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Development and Characterization of a Fixed-Dose Orodispersible Film of Telmisartan and Indapamide for Enhancing Dissolution

Dilara ÖRGÜL*, Aslı YİĞİT**, Yakup GÜLTEKİN***, Sema ARISOY****

Development and Characterization of a Fixed-Dose Orodispersible Film of Telmisartan and Indapamide for Enhancing Dissolution

SUMMARY

Fixed-dose combination (FDC) of antihypertensive drugs improves efficacy, safety and patient adherence over monotherapy. However, an FDC of telmisartan (TEL) and indapamide (IND), both poorly soluble BCS Class II drugs, has not been available yet. This study aimed to develop an orodispersible film (ODF) containing TEL and IND cyclodextrin (CD) inclusion complexes (ICs) to enhance solubility and improve patient adherence. In the first phase of the study, phase-solubility analysis was performed with β -CD, then the ICs were prepared via kneading and assessed for solubility enhancement and entrapment efficiency. In the second phase, ICs were incorporated into hydroxypropyl-methylcellulose (HPMC) based ODFs using solvent casting. The films were characterized for thickness, mass, and content uniformity, surface pH, water content, folding endurance, Young's modulus, FTIR, in vitro disintegration, and dissolution. AL-type phase-solubility plots confirmed 1:1 complexation and enhanced solubility by 1.7–4.1 fold, with entrapment efficiency >95%. ICs-loaded ODFs were thin (0.138 mm), uniform in mass (236.39 mg) and content (TEL 97.22%, IND 102.09%), nearly neutral pH, and low in water (2.68%). Mechanical testing showed adequate flexibility and stiffness (100 N/mm² modulus). Disintegration occurred in <35 s. Dissolution exceeded 90% for both drugs within 15 minutes in 6.8 phosphate buffer, whereas in 0.1N HCl, TEL showed complete release in 15 minutes and IND reached 97% after 20 minutes. In conclusion, TEL and IND ICs-loaded HPMC ODFs successfully overcame solubility limitations, offering an immediate-release formulation across the gastrointestinal pH range with favorable physicochemical properties and potential to improve patient adherence.

Keywords: Solubility Enhancement, Fixed-Dose Combinations, Orodispersible Film, Cyclodextrin, Telmisartan, Indapamide.

Çözünürlüğü Artırmaya Yönelik Telmisartan ve İndapamid İçeren Sabit Dozlu Bir Ağızda Dağılan Filmin Geliştirilmesi ve Karakterizasyonu

ÖZ

Antihipertansif ilaçların sabit-doza kombinasyonları (SDK), monoterapiye kıyasla tedavi etkinliğini, güvenliğini ve hasta uyumunu artırmaktadır. Ancak, her ikisi de düşük çözünürlüğe sahip BCS Sınıf II ilaçlar olan telmisartan (TEL) ve indapamid (IND)'i birlikte içeren bir SDK formülasyonu henüz mevcut değildir. Bu çalışma, TEL ve IND'nin siklodekstrin (SD) inklüzyon komplekslerini (İK) içeren bir ağızda dağılan film (ADF) formülasyonu geliştirerek çözünürlüğü artırmayı ve hasta uyumunu desteklemeyi amaçlamıştır. Çalışmanın ilk aşamasında, β -SD faz-çözünürlük analizi gerçekleştirilmiş, ardından İK'ler yoğunlaştırma yöntemiyle hazırlanmış ve çözünürlük artışı ile enkapsülasyon etkinliği açısından değerlendirilmiştir. İkinci aşamada, İK'leri hidroksipropil-metilselüloz (HPMC) bazlı ADF'lerle çözücü döküm yöntemiyle birleştirilmiştir. Hazırlanan filmler; kalınlık, kütle ve içerik tekdüzeliği, yüzey pH'sı, su içeriği, katlanma dayanıklılığı, Young modülü, FTIR, in vitro dağılma ve çözünme özellikleri bakımından karakterize edilmiştir. AL-tip faz-çözünürlük grafikleri, 1:1 kompleks oluşumunu doğrulamış ve çözünürlüğü 1,7–4,1 kat artırmış; enkapsülasyon etkinliği ise %95'in üzerinde bulunmuştur. İK yüklü ADF'ler ince yapılı (0,138 mm), kütle (236,39 mg) ve içerik bakımından tekdüze (TEL %97,22, IND %102,09), neredeyse nötr pH'lı ve düşük su içeriğine sahip (%2,68) bulunmuştur. Mekanik testler, yeterli esneklik ve rijitlik göstermiştir (100 N.mm⁻² modül). Filmler 35 saniyeden kısa sürede dağılmış, 6,8 fosfat tamponunda her iki ilaç da 15 dakika içinde %90'dan fazla çözünme göstermiştir. 0,1N HCl ortamında TEL 15 dakikada tamamen salınırken, IND 20 dakikada %97'ye ulaşmıştır. Sonuç olarak, TEL ve IND İK yüklü HPMC ADF'ler, çözünürlük sınırlamalarını aşarak gastrointestinal pH aralığında hızlı salım sağlayan, uygun fizikokimyasal özelliklere sahip ve hasta uyumunu artırma potansiyeli bulunan bir dozaj formu olarak başarılı şekilde geliştirilmiştir.

Anahtar Kelimeler: Çözünürlük Artırma, Sabit Doz Kombinasyonları, Ağızda Dağılan Film, Siklodekstrin, Telmisartan, İndapamid.

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* ORCID: 0000-0002-8947-4995, Department of Pharmaceutical Technology, Faculty of Pharmacy, Selcuk University, Konya, Turkey.

** ORCID: 0009-0005-7345-6070, Department of Pharmaceutical Technology, Faculty of Pharmacy, Selcuk University, Konya, Turkey.

*** ORCID: 0000-0001-6504-0757, Department of Pharmaceutical Technology, Faculty of Pharmacy, Selcuk University, Konya, Turkey.

**** ORCID: 0000-0003-2798-1884, Department of Pharmaceutical Technology, Faculty of Pharmacy, Selcuk University, Konya, Turkey.

* Corresponding Author; Dilara ÖRGÜL

E-mail: dilara.orgul@selcuk.edu.tr, dilaraorgul@gmail.com

INTRODUCTION

Hypertension is a chronic condition characterized by elevated arterial blood pressure, and it is a leading cause of cardiovascular disease. Today, hypertension has become one of the most prevalent global chronic diseases. Therefore, it represents a significant worldwide public health problem and a leading risk factor for mortality (Zhou et al., 2021). Currently, hypertension can be effectively controlled with low-cost pharmacological treatments. As recommended in the recent guidelines, most hypertension patients require more than one antihypertensive medication to achieve blood pressure control. However, this leads to a high medication burden and complex therapy, resulting in reduced medication adherence and uncontrolled blood pressure symptoms. Various initiatives implemented at the global, regional, and national levels increasingly recommend fixed-dose combinations (FDCs), which combine two or more active pharmaceutical ingredients (APIs) in a single dosage form, to enhance treatment efficacy, simplify regimens, and improve patient adherence (An et al., 2020). Accord-

ing to the European Medicines Agency (EMA) guideline for the clinical development of fixed-dose combinations (EMA/CHMP/158268/2017), compared to monotherapy, FDCs can provide a more rapid, potent therapeutic effect, reduce adverse effects, and improve compliance by simplifying drug administration (EMA, 2017). For these reasons, the development of FDC formulations is supported by a strong scientific and clinical rationale. One of the adopted strategies in clinical practice is the FDC of an angiotensin receptor blocker (ARB) with a thiazide or thiazide-like diuretic (Coca, Whelton, Camafort, López-López, & Yang, 2024). However, there are no existing dual FDC products containing an ARB, telmisartan (TEL), and a thiazide-like diuretic, indapamide (IND) (Figure 1). In a notable recent advancement, in 2025, the Food and Drug Administration (FDA) approved a fixed-dose triple-combination tablet comprising telmisartan, amlodipine, and indapamide (WIDAPLIKTM) for the management of hypertension, including as an initial therapeutic option (FDA, 2025).

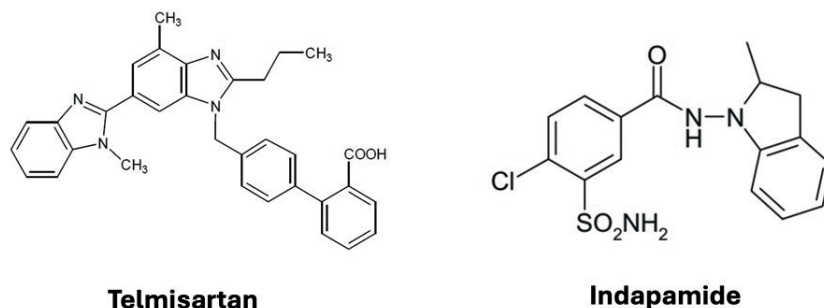


Figure 1. The chemical structures of telmisartan and indapamide.

TEL is a widely prescribed antihypertensive agent classified as a non-peptide angiotensin II receptor blocker. According to the biopharmaceutics classification system (BCS), it belongs to class II, displaying high membrane permeability but low aqueous solubility (Battershill & Scott, 2006). Therefore, various formulation strategies have been attempted to enhance its solubility and systemic exposure, including solid dispersions (Turek, Gach-Janczak, Różycka-Sokołowska,

Owsianik, & Bałczewski, 2025), nano-microparticles (Ha et al., 2024), self-emulsifying drug-delivery systems (Park et al., 2021), cocrystals (Dhibar et al., 2023), and cyclodextrin (CD) inclusion complexes (ICs) (Sharapova, Ol'khovich, & Blokhina, 2025). Likewise, although a thiazide-like diuretic, IND is well tolerated, clinically effective, and supported by a long history of therapeutic use. However its poor aqueous solubility remains a major impediment to optimal efficacy and

bioavailability. Because of its limited water solubility, IND is classified as BCS class II. Amorphous forms of IND have been developed as a potential approach to enhance its aqueous solubility; however, the inherent complexity of the amorphization process and the risk of residual solvents substantially limit their practical application (Cong et al., 2021; Wojnarowska et al., 2013). Taken together, there is a need to develop innovative strategies to enhance the dissolution of TEL and IND, thereby improving their oral bioavailability. Enhancing the solubility of BCS Class II drugs is a key strategy to overcome these limitations and improve oral bioavailability. Therefore, from past to present, a range of conventional methods such as super-critical fluid technology, cryogenic techniques, particle-size reduction, salt formation, and solid dispersion have been employed to enhance the solubility of these drugs, while newer approaches like vesicular systems, nanotechnological strategies, and cyclodextrin inclusion complexes have also been adopted in recent years (Kumari et al., 2023). Among these approaches, CD ICs have emerged as a prominent method in the pharmaceutical industry due to their versatility and safety in improving not only the solubility but also the stability and bioavailability of poorly water-soluble drugs. For example, in a recent study, Sharapova et al. showed that ICs of TEL with hydroxypropyl- β -cyclodextrin (HP- β -CD) and sulfobutylether- β -cyclodextrin (SBE- β -CD) improved its aqueous solubility by 23-fold and 7-fold, respectively, confirming the potential of cyclodextrins in enhancing poorly soluble drugs (Sharapova et al., 2025). However, studies on indapamide cyclodextrin systems remain scarce. To date, only a limited number of studies have described such complexes (Radi & Eissa, 2011).

Conventional oral dosage forms such as tablets and capsules have been widely used in the clinical management of diverse diseases because of their non-invasive nature, suitability for self-administration, long shelf life, and straightforward manufacturing processes. This condition also applies to FDC products used in hypertension management, the majority of which

are administered in tablet form (Lesutan, Andersen, & Lamprou, 2025). However, today, orodispersible films (ODFs) thin polymeric strips that disperse on the tongue within seconds have gained interest as an alternative to traditional tablets due to their attractive advantages. Due to their ability to rapidly disintegrate in saliva without the need for chewing, ODFs improve medication intake in both populations with dysphagia, such as geriatric patients, and the general population. Their rapid disintegration also facilitates drug dissolution and absorption, less first-pass metabolism, resulting in faster onset of action and potentially higher bioavailability (Morath, Sauer, Zaradzki, & Wagner, 2022). These attributes are especially valuable for BCS II drugs whose clinical performance is dissolution-limited (Islam et al., 2023).

Based on these considerations, the present study aimed to develop and characterise an ODF as a dual FDC containing telmisartan β -CD and indapamide β -CD ICs. Complexation with β -CD and incorporating into a single ODF formulation of both BCS Class II antihypertensives is intended to overcome the dissolution limitations that constrain their oral bioavailability while simultaneously enhancing treatment adherence for elderly hypertensive patients who experience difficulty swallowing conventional tablets.

MATERIALS AND METHODS

Materials

Indapamide was a kind gift from ILKO Pharmaceuticals, Turkey. Telmisartan was kindly provided by Nobel Pharmaceutical Industry, Turkey. β -CD was purchased from Wacker Chemie AG as pharmaceutical-grade products (Cavamax® W7 Pharma). HPMC (hydroxypropylmethyl cellulose) was supplied by Shin-Etsu Chemical. Glycerin was obtained from Sigma-Aldrich. All other chemicals were of analytical grade. Purified water was utilized during the study.

Methods

Construction of a Calibration Curve of Telmisartan and Indapamide

Spectrophotometric analysis of TEL and IND in

the formulations and *in vitro* dissolution samples was performed using a Shimadzu UV-Vis spectrophotometer (UV-1800). To determine the maximum absorption wavelengths (λ_{max}), stock solutions of each API were prepared at a concentration of 100 $\mu\text{g/mL}$ in two different solvents: 6.8 phosphate buffer and 0.1 N HCL. From these stock solutions, appropriate dilutions were made in volumetric flasks to obtain final concentrations of 10 $\mu\text{g/mL}$ for TEL and 6 $\mu\text{g/mL}$ for IND. These solutions were scanned in the UV range of 200–400 nm, and TEL exhibited a maximum absorbance at 295 nm, while IND showed maximum absorbance at 240 nm. At these λ_{max} values, calibration curves were constructed by preparing a series of dilutions ranging from 1 to 12 $\mu\text{g/mL}$ for IND and from 4 to 16 $\mu\text{g/mL}$ for TEL in both pH 6.8 phosphate buffer and 0.1 N HCL. The absorbance values of each dilution were spectrophotometrically measured at their respective λ_{max} . Based on the obtained absorbance and concentration data, two separate calibration curves were plotted for each API, and linear least-squares regression analysis was performed for all calibration curves.

Phase Solubility Studies

Phase solubility studies were carried out in accordance with the method of Higuchi and Connors to evaluate the effect of β -CD on the solubility of TEL and IND and assess ICs' molecular stoichiometry (Higuchi & Connors, 1965). In a pH 6.8 phosphate buffer, β -CD solutions were prepared at concentrations of 3, 6, 9, 12, 15, and 18 mM, and an excess amount of either TEL or IND was added to each solution. All β -CD concentrations were carried out in triplicate. The resulting suspensions were agitated in a thermostatted shaking water bath at 300 rpm and $37 \pm 2^\circ\text{C}$ for 24 h to reach equilibrium. After 24 h, samples were centrifuged at 10,000 rpm for 15 minutes, and the supernatants were filtered through 0.45 μm Polytetrafluoroethylene (PTFE) syringe filters. Drug concentrations in appropriately diluted filtrates were measured spectrophotometrically against corresponding β -CD blank solutions. Phase-solubility diagrams were con-

structed by plotting the measured TEL and IND concentrations (mM) versus β -CD concentration (mM). The intrinsic solubility (S_0) of TEL and IND in the absence of β -CD (0 mM) was obtained from the y-intercept of the linear portion of the plot. According to Higuchi and Connors' method, apparent stability constants (K_s) can be calculated from the slope and intercept (S_0) of phase solubility curves using Equation (1):

$$K_s = \frac{\text{Slope}}{S_0 \times (1 - \text{slope})} \quad (1)$$

Preparation of Inclusion Complexes and Physical Mixture

Inclusion complexes of the TEL and IND with β -CD were prepared using a modified kneading method, based on the 1:1 molar ratios previously determined from phase solubility studies (Khan et al., 2020). Briefly, the accurately weighed β -CD and API, in a 1:1 molar ratio, were triturated in a mortar. A water : ethanol (1:1 v/v) mixture was then slowly added dropwise to this mixture, with continuous stirring, until a homogeneous paste consistency was obtained. The resulting paste was subsequently dried at room temperature until a solid mass formed. Once dried, the mass was pulverized into a fine powder and then passed through an 80 μm sieve to ensure a uniform particle size distribution. The obtained ICs were then stored at room temperature in a desiccator and protected from light until further use. The physical mixtures were obtained by simple homogenization of host (β -CD) and guest (TEL or IND) powders, using a spatula for 5 minutes, in a 1:1 molar ratio. The samples were stored at room temperature in a desiccator and protected from light.

Characterization of Inclusion Complexes

Solubility Analysis

Solubility studies of ICs were conducted in both medium: purified water and pH 6.8 phosphate buffer, at room temperature, to evaluate the enhancement in solubility of TEL and IND through inclusion complexation. Excess amounts of TEL β -CD and IND β -CD inclusion complexes were added to 10 mL of

each medium, and the suspensions were applied to the same procedure used in the phase-solubility studies. After equilibration and filtration, the concentration of solubilized TEL and IND was determined spectrophotometrically ($n = 3$).

Determination of Entrapment Efficiency

To determine the amount of TEL and IND entrapped in the ICs, 30 mg of each IC was accurately weighed and dissolved in their respective optimal solvents, 0.1 M NaOH for TEL and ethanol for IND, using 10 mL volumetric flasks. Obtained solutions were agitated on a horizontal shaker for 4 hours to ensure complete dissolution. Afterward, each solution was filtered through a 0.45 μm PTFE syringe filter. Appropriate dilutions were made and the drug content analysed spectrophotometrically. All measurements were performed in triplicate for each IC sample. The entrapment efficiency (EE%) was calculated based on the measured TEL and IND concentrations using the following Equation (2) (Al-Heibshy, Başaran, Öztürk, & Demirel, 2020).

$$EE\ \% = \frac{\text{The measured amount of drug in ICs}}{\text{Initial Drug Content}} \times 100 \quad (2)$$

Preparation of Orodispersible Film

ODF formulations were developed using the solvent casting method, based on previously reported methods, with hydroxypropyl methylcellulose (HPMC) as the film-forming polymer and glycerin as the plasticizer (Yin et al., 2024). To determine polymer and plasticizer ratios, blank ODF formulations were initially prepared by testing various concentrations of HPMC and glycerin. The resulting blank ODFs were evaluated in terms of thickness, uniformity of mass, folding endurance, and disintegration time. Based on the results, the optimal concentrations were determined as 11.42 mg/cm² for HPMC and 5.71 mg/cm² for glycerin. After determining the polymer and plasticizer ratios, blank and TEL and IND β -CD ICs loaded ODFs were prepared as follows. An appropriate amount of HPMC was added to purified water in a beaker and allowed to swell overnight under gentle stirring. The required amount of glycerin

was then added dropwise to the swollen polymer solution with gentle stirring. In ICs loaded ODF, after the addition of glycerin, TEL β -CD ICs equivalent to a 40 mg/film dose of TEL and IND β -CD ICs equivalent to a 1.5 mg/film dose of IND were incorporated into the casting solution and stirred magnetically until complete dissolution. To remove air bubbles, the final casting solution was degassed by ultrasonication for 15 minutes. Then, the solution was cast onto petri plates and dried in an oven at 40°C for 48 hours to form thin films. Peeled films were cut into 2 $\times$ 3 cm pieces (6 cm² per film). The individual film pieces were wrapped in aluminum foil and stored in a desiccator until further use.

Characterization of Orodispersible Film

Thickness

Blank and ICs loaded ODF were cut into 3 $\times$ 2 cm (6 cm²) specimens for thickness determinations. Subsequently, ten film pieces were randomly selected, and each specimen was measured with a micrometer at five points: the four corners and the centre (Visser et al., 2017). The mean value and corresponding standard deviation were calculated for all measurements.

Uniformity of Mass

Uniformity of mass was assessed in accordance with the European Pharmacopoeia (9th edition, method 2.9.5), uniformity of mass for single-dose preparations. Twenty ICs-loaded ODFs were randomly selected and individually weighed using an analytical balance. The mean mass of the films was then calculated to evaluate uniformity.

Surface pH

To determine the surface pH, six blank and ICs-loaded ODFs were randomly selected. Since the pH of the dry films could not be measured directly, each film was first moistened with 2 mL of distilled water in a petri dish. The electrode of the pH meter was then placed in contact with the surface of the hydrated film at room temperature, and the pH value was recorded (Oudah & Al-Khedairy, 2025).

Water Content

Water content is a critical parameter in determining the hygroscopic nature of the film. In this study, the water content of three individual ODFs was measured using a moisture analyzer (OHAUS MB120) at 50 °C, and the measurement was continued until a constant film weight was obtained. Water content percent was calculated as per the following equation (3):

$$\text{Water content \%} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad (3)$$

Content Uniformity

Content uniformity analysis was performed to assess whether each film contained a consistent amount of API. Ten randomly selected TEL and IND ICs loaded ODFs were tested for uniformity of content for single-dose preparations in accordance with the European Pharmacopoeia 9th edition (method 2.9.6). For this purpose, each selected film was dissolved in phosphate buffer (pH 6.8), and appropriate dilutions were made. The drug content was then analyzed using UV spectrophotometry at 295 nm for TEL and 240 nm for IND.

Folding Endurance

For the folding-endurance test, three films from blank and IC-loaded ODFs were repeatedly folded 180° at the same point until breakage occurred. The number of folds required to break the film was recorded as the folding-endurance value (Khan et al., 2020).

Mechanical Properties – Young's Modulus

The mechanical properties of the blank and IC-loaded ODFs were evaluated using a Dynamic Mechanical Analyzer (DMA 8000, PerkinElmer). Based on the obtained stress-strain curves, Young's modulus values were calculated.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier-transform infrared (FTIR) spectroscopy was employed to perform physicochemical characterization of both the cyclodextrin inclusion complexes

and the orodispersible film formulations. Samples of pure telmisartan, indapamide, β -CD, the corresponding inclusion complexes, and physical mixtures of β -CD with each pure drug were analyzed. Additionally, for the ODF formulation, samples of HPMC, glycerin, blank and, ICs-loaded ODF formulations were also examined. All infrared spectra were recorded over the 4000–400 cm^{-1} range using a Bruker Vertex 70 IR spectrophotometer equipped with an attenuated transmittance reflectance.

Disintegration Time

The disintegration time of the blank and ICs-loaded ODFs was evaluated using two different methods: the petri dish and drop method (n:6). In the petri-dish method, 2 mL of purified water was added to a petri-dish, and the ODF was carefully placed onto the surface without agitation. The time required for the film to start disintegrating was recorded as the disintegration time. In the drop method, a single drop of purified water was applied onto the center of the ODF using a pipette. The time taken for the drop to form a visible hole in the film was measured (Pezik, Gulsun, Sahin, & Vural, 2021).

In Vitro Dissolution

In vitro dissolution studies of TEL- β -CD and IND- β -CD ICs loaded ODF were conducted based on the USP 42–NF 37 monographs of telmisartan and indapamide using a Pharma Test PTWS 120D dissolution tester (Germany). Two different dissolution media were selected to comply with the pharmacopeial requirements for both APIs: 0.1 N HCL and phosphate buffer at pH 6.8. Each test was performed in 900 mL of dissolution medium using the USP Apparatus II (paddle method) at 37 °C and 75 rpm for 45 minutes and repeated in triplicate. At predetermined time intervals, 5 mL aliquots were withdrawn from the medium and immediately replaced with an equal volume of fresh dissolution medium to maintain sink conditions. The collected samples were analyzed using UV spectrophotometry at 295 nm for TEL and 240 nm for IND.

RESULTS AND DISCUSSION

Construction of a Calibration Curve of Telmisartan and Indapamide

According to the literature, the maximum wavelengths for TEL and IND were determined to be 295 nm and 240 nm, respectively (Chavhan, Lawande, Salunke, Ghante, & Jagtap, 2013; Tarkase Kailash, Jadhav Manisha, Tajane Sachin, & Dongare Umesh, 2012). At these wavelengths, there is no interaction or overlap between TEL and IND. At these wavelengths, calibration curves were constructed over the concentration range of 1 to 12 $\mu\text{g/mL}$ for IND and 4 to 16 $\mu\text{g/mL}$ for TEL in 6.8 phosphate buffer and 0.1 N HCL (Figure 2). The calibration curves for TEL and

IND, generated in phosphate buffer (pH 6.8) and in 0.1 N HCl, exhibited excellent linearity with high correlation coefficients. Under buffered conditions, TEL showed a slope of 0.0483 with an intercept of 0.0131 ($R^2 = 0.9996$), while IND displayed a slope of 0.0575 with an intercept of 0.0176 ($R^2 = 0.9996$) (Figure 2a). In 0.1 N HCl, TEL's slope remained essentially unchanged at 0.0484 (intercept = 0.0157, $R^2 = 0.9996$), and IND's slope increased slightly to 0.0595 (intercept = 0.0023, $R^2 = 0.9991$) (Figure 2b). These findings confirm that both APIs comply with Beer's law over the tested concentration ranges, and the high linearity of the calibration curves underscores the suitability of the assay.

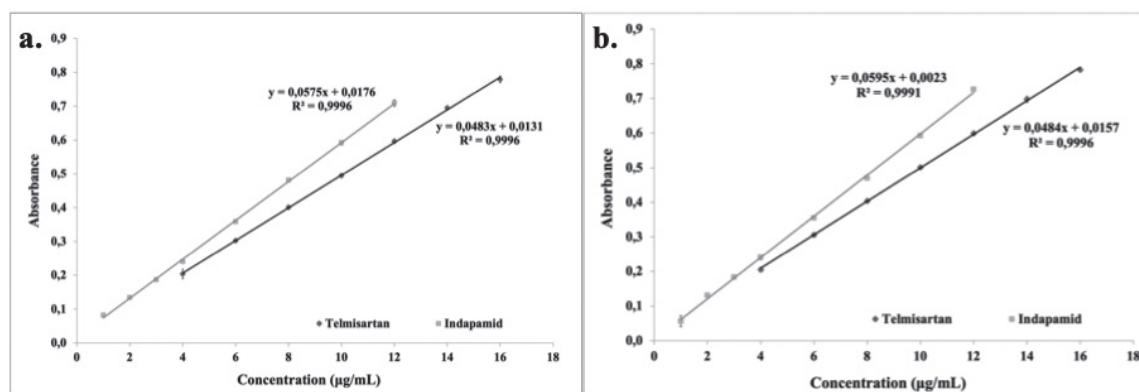


Figure 2. The calibration curve of TEL and IND in (a) phosphate buffer pH 6.8 and (b) 0.1 N HCl.

Phase Solubility Studies

Phase-solubility studies revealed the stoichiometric ratios, apparent stability constants, and complexation efficiencies of TEL and IND when complexed with β -CD. Figure 3 shows the phase-solubility diagrams of TEL and IND, plotted as solubilized drug concentration versus β -CD concentration. A similar phase-solubility profile was observed for both APIs, with the solubility of TEL and IND increasing linearly as the β -CD concentration increased from 0.0 to 18 mM. This increased solubility confirmed the existence of intermolecular interactions between host and guest molecules. High correlation coefficients, R^2

= 0.9761 for TEL and $R^2 = 0.9821$ for IND, of both plots confirmed strong linearity and exhibited AL-type phase-solubility behavior according to the Higuchi–Connors classification. As shown in Figure 3, the slopes of the TEL β -CD (0.0006) and IND β -CD (0.0216) curves are both less than unity, indicating the formation of 1:1 stoichiometric ICs with β -CD (Jambekar & Breen, 2016). Apparent stability constants (K_s) indicate the binding affinity between guest molecules and cyclodextrin. The calculated K_s values of 305.4 M^{-1} for TEL and 121.5 M^{-1} for IND fall within the typical 50–5000 M^{-1} range, indicating the formation of strong 1:1 ICs with β -CD, thereby resulting in enhanced aqueous solubility (Örgül, 2024).

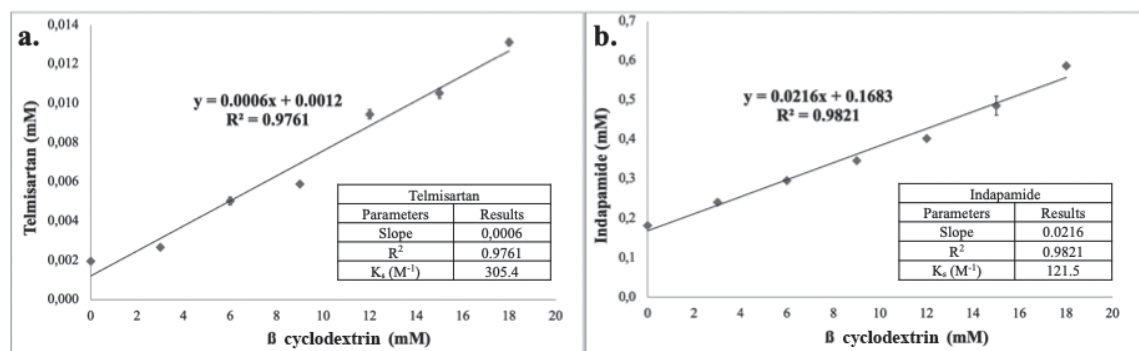


Figure 3. Phase-solubility diagrams of (a) telmisartan and (b) indapamide with β -cyclodextrin.

Characterization of Inclusion Complexes

Table 1 summarizes the solubility of TEL and IND both as free drugs and as β -CD ICs in two biorelevant media: purified water and pH 6.8 phosphate buffer. As expected for BCS Class II drugs, TEL and IND exhibited limited aqueous solubility; telmisartan dissolved to $0.79 \pm 0.1 \mu\text{g mL}^{-1}$ in purified water and $1.01 \pm 0.10 \mu\text{g mL}^{-1}$ in buffer, whereas indapamide reached $78.65 \pm 2.30 \mu\text{g mL}^{-1}$ and $63.53 \pm 2.10 \mu\text{g mL}^{-1}$, respectively. Complexation with β -CD markedly improved these values. The TEL β -CD ICs achieved solubilities of $2.52 \pm 0.7 \mu\text{g mL}^{-1}$ in water and $4.19 \pm 0.1 \mu\text{g mL}^{-1}$ in buffer, corresponding to 3.2 and 4.1-fold increases over the free drug. Although the relative enhancement for IND was more moderate, it remained significant. IND β -CD ICs yielded $136.87 \pm 5.2 \mu\text{g mL}^{-1}$ in purified water and $120.33 \pm 4.9 \mu\text{g mL}^{-1}$ in buffer, representing 1.74 and 1.89 fold increases, respectively.

These solubility increases corroborate the well-established mechanism whereby cyclodextrins form soluble inclusion complexes by encapsulating poorly water-soluble drug molecules within their hydrophobic cavities. However, the magnitude of solubility en-

hancement differed between the two APIs due to their distinct physicochemical properties. TEL is a highly lipophilic molecule with log P about 7, therefore it exhibits strong affinity for the hydrophobic cavity of β -cyclodextrin (β -CD), resulting in a higher stability constant (K_s) and, consequently, a more pronounced increase in solubility (Ruiz Picazo et al., 2018). By contrast, IND has a polar sulfonamide group that imparts a degree of intrinsic aqueous solubility and limits the relative impact of solubility enhancement by complexation (Wojnarowska et al., 2013). Additionally, both APIs exhibited distinct solubility values in purified water and pH 6.8 phosphate buffer, both as free drug and β -CD ICs. These variations arise from their well-known pH-dependent solubility profiles and align with values previously reported in the literature (Ruiz Picazo et al., 2018; Wojnarowska et al., 2013). Entrapment efficiency values were $98.88 \pm 0.7 \%$ for the TEL β -CD ICs and $95.67 \pm 0.3 \%$ for IND β -CD ICs. These high EE values confirm that the guest molecules were successfully accommodated within the β -CD cavity and, demonstrate the effectiveness of the kneading method in achieving near-complete drug entrapment.

Table 1. Entrapment efficiency and solubility results of inclusion complexes. Each value represents the mean standard deviation (n = 3).

APIs and Their β -CD ICs	Solubility ($\mu\text{g mL}^{-1}$)		Entrapment Efficiency %
	Purified Water	pH 6.8 Phosphate Buffer	
Telmisartan	0.79 $\pm$ 0.1	1.01 $\pm$ 0.1	-
Telmisartan β -CD ICs	2.52 $\pm$ 0.7	4.19 $\pm$ 0.1	98.88 $\pm$ 0.7 %
Indapamide	78.65 $\pm$ 2.3	63.53 $\pm$ 2.1	-
Indapamide β -CD ICs	136.87 $\pm$ 5.2	120.33 $\pm$ 4.9	95.67 $\pm$ 0.3 %

Characterization of Orodispersible Film

ODFs are thin, fast-dissolving drug delivery systems designed to improve ease of administration and distinguished from other solid dosage forms by their rapid disintegration properties. In the present study, an HPMC-based ODF containing FDCs of TEL and IND was successfully prepared using the solvent-casting method. HPMC was selected because it offers superior mechanical strength, dissolution performance, and drug-loading capacity compared with many alternative film formers. Therefore, along with HPC, HPMC is one of the two most commonly used film-forming polymers in ODF formulations (Rani et al., 2021; Takeuchi, Umemura, Tahara, & Takeuchi, 2018). Using HPMC as a film-forming polymer and glycerin as a plasticizer, the resulting TEL- β -CD and IND- β -CD ICs loaded ODF was smooth, flexible, and visually homogeneous, confirming the suitability of the formulation and manufacturing approach for orodispersible applications. The prepared ODF formulations were characterized in terms of thickness, uniformity of mass and content, surface pH, water content, folding endurance, Young's modulus, FTIR analysis, disintegration time, and *in vitro* dissolution (Table 2).

Thickness

Film thickness is a key parameter affecting the disintegration time and mechanical properties of the ODF. Similar to previously reported ODF thickness values, all films in this study exhibited thicknesses within the mean range of 0.138–0.140 mm (Vlad et al., 2023). The thickness of the ICs loaded ODFs (0.138 $\pm$ 0.009 mm) and the blank ODFs (0.140 $\pm$ 0.008 mm) was

found to be similar, indicating that the incorporation of ICs into the polymeric matrix did not significantly affect the film thickness. The solvent casting method produced films with uniform and reproducible thickness, suitable for orodispersible applications.

Uniformity of Mass

For the mass uniformity test, twenty ICs loaded ODFs were randomly selected and individually weighed using an analytical balance. The mean mass was found to be 236.39 $\pm$ 3.81 mg. All IC-loaded ODF formulations complied with pharmacopoeia criteria, indicating acceptable uniformity of mass.

Surface pH

The surface pH values of the developed blank and ICs-loaded ODF were found to be 7.04 $\pm$ 0.08 and 6.95 $\pm$ 0.05, respectively (Table 2). Since the pH of the buccal cavity ranges from 5.5 to 7.4, ODFs need to have a surface pH within this range to avoid mucosal irritation, as they rapidly dissolve upon placement in the oral cavity (Oudah & Al-Khedairy, 2025). The measured surface pH values indicate that the formulated films are compatible with the physiological pH of the oral environment and are therefore expected to ensure good patient compliance.

Water Content

Water content is a critical parameter in ODFs, as it affects film flexibility, mechanical strength, stability, and disintegration time. The percentage of water content was calculated 3.13 $\pm$ 0.25 % for blank ODFs and 2.68 $\pm$ 0.29 % for IC-loaded ODFs (Table 2). The residual water content of commercial films is typically below 10 %, with most falling under 5 % (Borges, Sil-

va, Coelho, & Simões, 2017). In this context, the obtained water content values are considered acceptable, and the low moisture levels observed suggest good stability of the prepared ODFs.

Uniformity of Content

Content-uniformity testing showed that telmisartan and indapamide were evenly dispersed in the films. Telmisartan yielded 38.89 ± 1.71 mg per unit (97.22 ± 4.28 %), while indapamide gave 1.53 ± 0.05 mg per unit (102.09 ± 3.33 %). All values fell within the limits specified by Ph. Eur. 9.0 (method 2.9.6), confirming that the formulation meets the pharmacopoeial requirement for content uniformity.

Folding Endurance

Folding endurance is an indicator of an ODF's resistance to repeated bending and is used to evaluate its suitability in terms of mechanical strength and flexibility. The folding endurance values for the blank and ICs-loaded ODFs were found to be 182.21 ± 5.09 and 164.33 ± 4.16 , respectively (Table 2). Although the incorporation of ICs slightly reduced the folding endurance compared to blank films, all ODFs exhibited high folding endurance values. These results suggest that the prepared films possess adequate flexibility and mechanical robustness, which are essential for storage and handling (Takeuchi, Ikeda, Tahara, & Takeuchi, 2020).

Table 2. Physicochemical and performance characteristics of blank and IC-loaded ODF.

ODFs	Parameters							
	Thickness	Uniformity of mass	Surface pH	Water content	Folding endurance	Content uniformity	Disintegration time	
							Petri Method	Drop Method
Blank ODF	0.140 ± 0.008 mm	-	7.04 ± 0.08	$3.13 \pm 0.25\%$	182.21 ± 5.09	-	29.7 ± 3.8	34.3 ± 2.1
ICs loaded ODF	0.138 ± 0.009 mm	236.39 ± 3.81 mg	6.95 ± 0.05	$2.68 \pm 0.29\%$	164.33 ± 4.16	97.22 ± 4.28 % (TEL) 102.09 ± 3.33 % (IND)	25.3 ± 3.1	32.4 ± 2.5

Mechanical Properties – Young's Modulus

The stress-strain curves obtained from DMA analysis exhibited a clear linear relationship with high correlation coefficients ($R^2 > 0.997$), indicating elastic behavior of both blank and ICs-loaded ODFs (Figure 4). The calculated Young's modulus of the blank ODFs was found to be 70 N/mm^2 , while the ICs-loaded ODFs showed a higher modulus of 100 N/mm^2 . This increase in stiffness can be attributed to the incorporation of ICs into the polymer matrix, which

may enhance intermolecular interactions and structural integrity. Similarly, in the literature, an increase in Young's modulus, also known as elastic modulus, has been reported with higher amounts of formulation components (Orgul, Eroglu, & Hekimoglu, 2017). Nevertheless, both values remained below the 550 N/mm^2 threshold reported for ODFs, confirming that the mechanical flexibility and performance of the films remain within acceptable limits for buccal application (Visser et al., 2015).

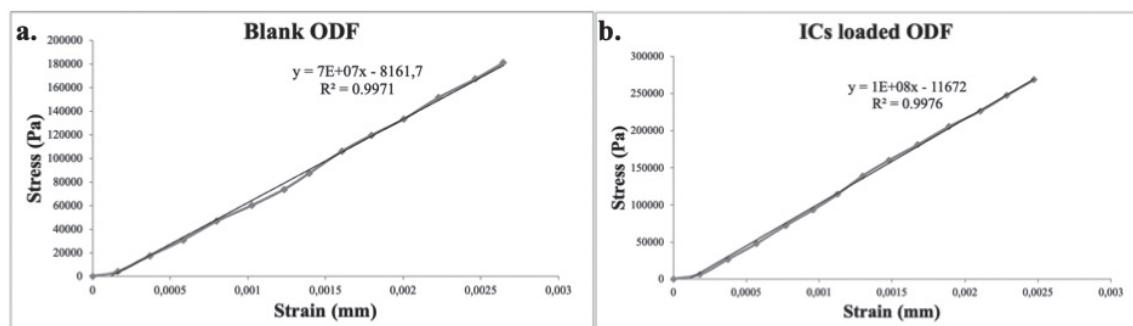


Figure 4. Stress–strain curves of (a) blank ODF and (b) ICs loaded ODF obtained from dynamic mechanical analysis.

FTIR Analysis

FTIR analysis was first used to evaluate the ICs between IND, TEL, and β -CD in the solid state, and the spectra of APIs, β -CD, physical mixture, and ICs were shown in Figure 5. Characteristic peaks of TEL were observed at 3500 cm^{-1} as a broad band attributed to O–H stretching of the carboxylic acid, 3061 cm^{-1} for aromatic C–H stretching, 2956 cm^{-1} for aliphatic C–H stretching, a sharp intense band at 1695 cm^{-1} corresponding to C=O stretching of the carboxylic acid, around 1600 cm^{-1} for aromatic C=C stretching, 1384 cm^{-1} arising from O–H bending/C–O stretching of the carboxylic acid and a diagnostic doublet at 757 and 741 cm^{-1} assigned to 1,2-disubstituted benzene ring vibrations in line with previously reported (Kaur et al., 2014) (Figure 5a). Further, the spectrum of IND exhibited characteristic peaks consistent with those reported in the literature (Gumieniczek et al., 2018). A strong band at 3296 and 3202 cm^{-1} corresponds to N–H stretching vibrations of the sulfonamide group. A weak absorption band around 2943 cm^{-1} was attributed to aliphatic C–H stretching. A sharp and intense peak at 1655 cm^{-1} was assigned to C=O stretching of the amide group. The peak at 1598 cm^{-1} is indicative of aromatic C–H stretching, while the bands at 1384 and 1168 cm^{-1} are characteristic of the S=O stretching vibrations of the sulfonamide group (Figure 5b). Similarly, the absorption bands in the FTIR spectrum of β -CD match those reported in the literature (Örgül, 2024). In the spectra of the physical mixture, characteristic peaks of both APIs (TEL or IND) and β -CD

were visible with minimal shifts and slightly reduced intensity due to overlapping bands of β -CD. The presence of characteristic peaks with only slight changes suggests the absence of significant chemical interactions between the API and β -CD in the physical mixture, supporting their mutual compatibility. Conversely, the FTIR spectra of the ICs exhibited noticeable shifts, broadening, and intensity reductions in the characteristic peaks of both APIs. These spectral modifications confirm that telmisartan and indapamide have been successfully incorporated into the β -CD cavity through non-covalent interactions such as hydrophobic, van der Waals, and hydrogen bonding, thereby forming stable ICs. In addition, in this study, possible interactions between the components of the ODF formulations were evaluated using FTIR spectra of HPMC, glycerin, blank ODF, and ICs-loaded ODF as depicted in Figure 6. HPMC spectrum exhibits distinct peaks such as a broad O–H stretching band around 3450 cm^{-1} , which is typical for cellulose derivatives, the C–H stretching peak at about 2940 cm^{-1} , and the strong band in the $1050\text{--}1100\text{ cm}^{-1}$ region corresponds to C–O–C ether stretches (Mahdavinia, Ettehadi, Amini, & Sabzi, 2015). The spectrum of glycerin is dominated by a broad and strong O–H stretching band around $3300\text{--}3200\text{ cm}^{-1}$, which is typical for polyols with extensive hydrogen bonding. The C–H stretching bands appear in the $2930\text{--}2870\text{ cm}^{-1}$ range, while the C–O stretching vibrations, indicative of alcohol groups, are clearly visible in the $1110\text{--}1020\text{ cm}^{-1}$ range (Wu, Feng, Hedoux, & Shalaev, 2022). These

spectral features are consistent for pure HPMC and glycerin, confirming their identity and chemical integrity. The blank ODF spectrum exhibits the characteristic profile of the HPMC–glycerin matrix, particularly in the fingerprint region. The prominent bands include a broad O–H stretching band at around 3300 cm^{-1} , aliphatic C–H stretches near 2930 cm^{-1} , a moisture-related H–O–H bending band at around 1640 cm^{-1} , and strong C–O–C ether peaks between 1100 and 1020 cm^{-1} . These results confirm that HPMC and glycerin are compatible, and the solvent casting method did not chemically alter the excipients. The ODF containing ICs largely preserves the characteristic HPMC and glycerin bands observed in the blank

ODF. Superimposed on this baseline are ICs peaks that emerge as broadened shoulders or markedly attenuated peaks for example, a low-intensity shoulder at about 1690 cm^{-1} attributable to the C=O stretch of the TEL β -CD IC, a diminished peak near 1650 cm^{-1} arising from the amide C=O of the IND β -CD IC, and a marked attenuation of the diagnostic aromatic doublet at 760–740 cm^{-1} (Figure 6). These ICs' specific bands, together with the unchanged HPMC and glycerin peaks, indicate that the inclusion complexes are molecularly dispersed within the film matrix without the formation of new covalent bonds, confirming the good physicochemical compatibility of all formulation components.

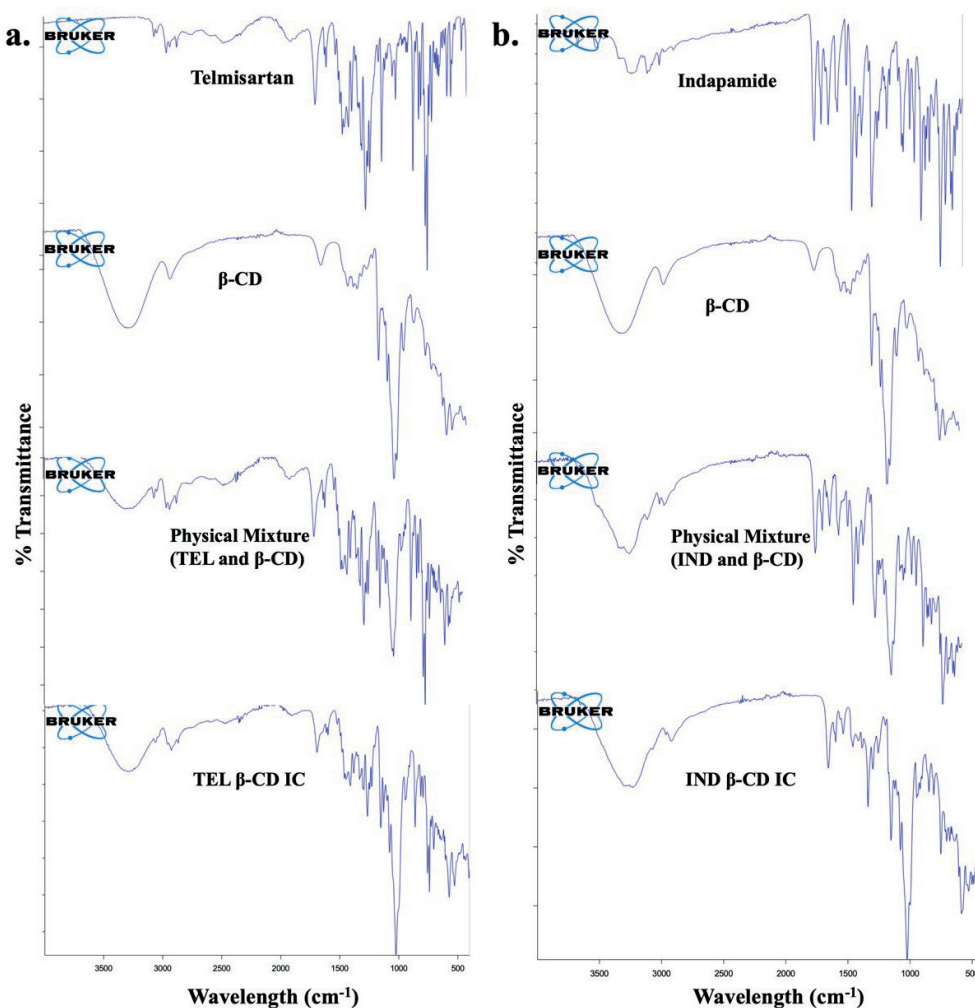


Figure 5. FTIR spectra of (a) TEL and (b) IND, β -CD, physical mixtures, and corresponding ICs.

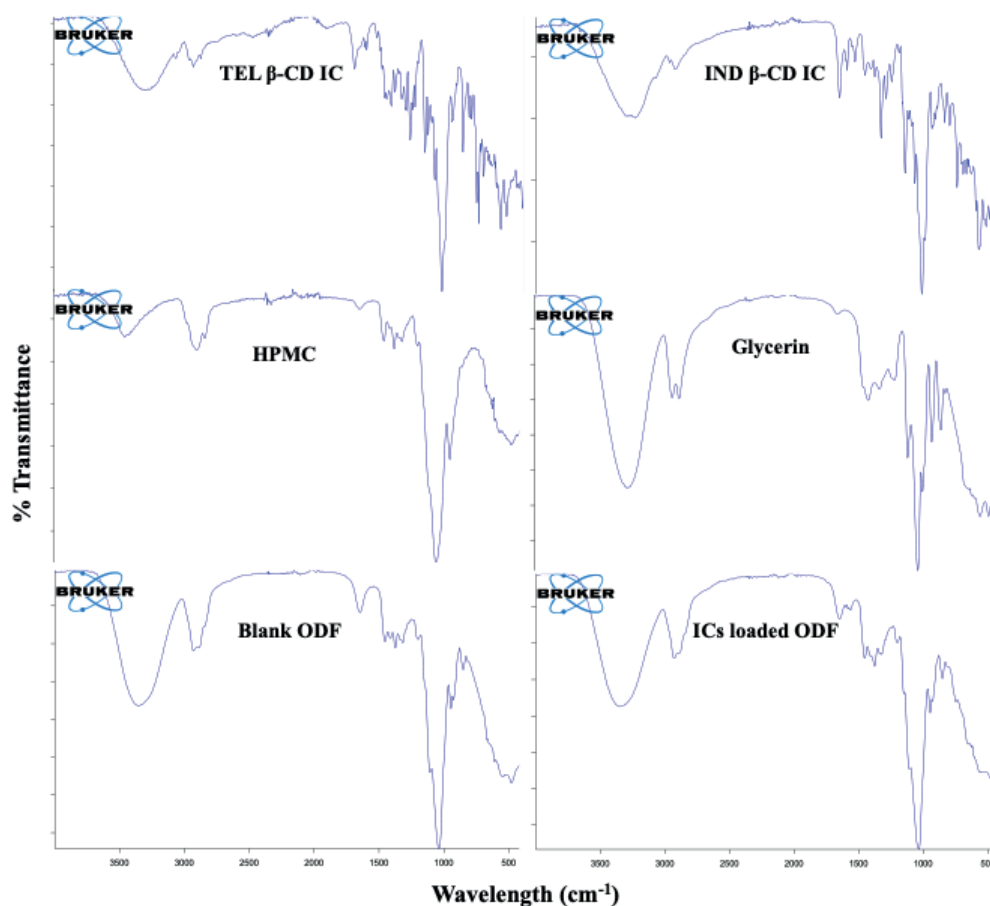


Figure 6. FTIR spectra of individual components (TEL β -CD IC, IND β -CD IC, HPMC, and glycerin), blank ODF, and ICs-loaded ODF formulation.

Disintegration Time

Disintegration time is a critical quality attribute for ODFs, specifically designed to dissolve rapidly in the oral cavity. As shown in Table 2, the disintegration times of the developed ODF formulations ranged between 25.3 ± 3.1 and 34.3 ± 2.1 seconds. Although the drop method resulted in slightly longer disintegration times compared to the petri dish method, all values remained below 35 seconds, with no statistically significant difference observed between blank and ICs-loaded ODFs for either method. According to the European and United States Pharmacopoeia (USP), the maximum allowable disintegration time for orodispersible dosage forms is 180 seconds. With the prepared ODF formulations, disintegration times

were achieved well below this limit. Factors such as the film-forming polymer, film thickness, and production method are known to influence disintegration behavior. In this study, the use of HPMC, a hydrophilic polymer, facilitated faster water penetration into the film matrix, contributing to shorter disintegration times. These findings are in agreement with the literature, confirming that HPMC-based ODFs can achieve rapid disintegration suitable to enable oral administration (Zaki et al., 2023).

In Vitro Dissolution

The release profiles of TEL and IND from TEL β -CD and IND β -CD ICs loaded ODF in 0.1 N HCl and phosphate buffer pH 6.8 are given in Figure 7. As shown in Figure 7a, TEL exhibited a rap-

id dissolution profile in 0.1 N HCl, achieving nearly 100% drug release within 15 minutes, whereas IND showed a slightly slower yet efficient release, reaching approximately 97% within 20 minutes. In pH 6.8 phosphate buffer (Figure 7b), both APIs demonstrated fast-release profiles, with more than 90% of the drug released within the first 15 minutes and complete release observed by 30 minutes. Although TEL is highly soluble in acidic or basic conditions, it has limited solubility under neutral pH (Ruiz Picazo et al., 2018). However, the formation of β -CD IC successfully overcame this pH-dependent solubility limitation,

enabling immediate drug release (>90% in 15 minutes) even under neutral conditions. Similarly, IND which is a weak base with pH-dependent solubility (Wojnarowska et al., 2013), showed a similar rapid dissolution across both media due to complexation with β -CD. These findings confirm that the β -CD ICs significantly enhanced the solubility and dissolution rates of both poorly soluble drugs. In addition to the formation of ICs, the enhanced dissolution profiles were also attributed to the high hydrophilicity of the HPMC-based ODF matrix, increased surface area, and resulting rapid disintegration of the films.

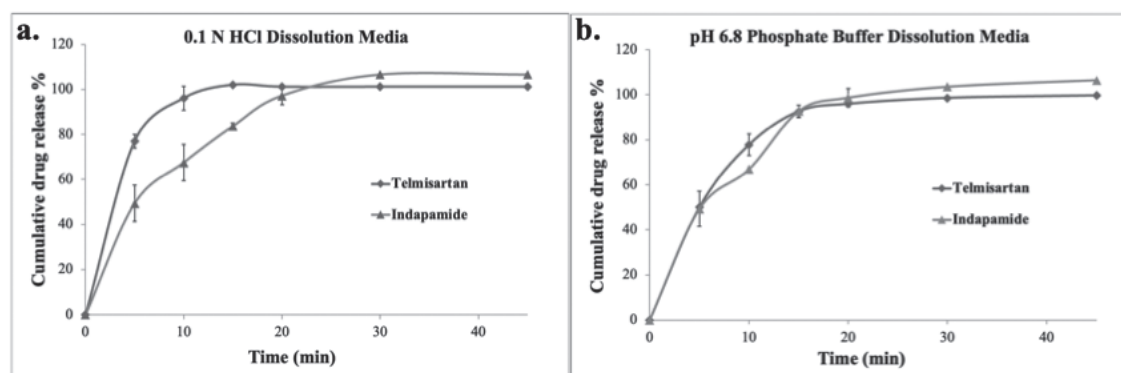


Figure 7. *In vitro* cumulative drug release profiles of Telmisartan and Indapamide from their β -CD ICs loaded ODFs in (a) 0.1 N HCl and (b) pH 6.8 phosphate buffer ($n = 3$, mean $\pm$ SD).

Conclusion

In this study, an FDC ODF formulation containing TEL and IND β -ICs was successfully manufactured by solvent casting using HPMC and glycerin. β -CD complexation increased the solubility of both APIs, with greater than 90% encapsulation efficiency, confirming effective host–guest interaction. Incorporating these ICs into an HPMC-based ODF produced proper, flexible, and thin films with uniform mass and content that disintegrated in less than 35 seconds and achieved almost complete release of both APIs within 20 minutes in both acidic (0.1 N HCl) and neutral (pH 6.8) media. Furthermore, compatibility of components was confirmed through physicochemical analysis, and all critical quality attributes of the ODFs met pharmacopeial limits, demonstrating the robustness of the process. Overall, the developed ODF in this

study effectively overcame the solubility limitations of TEL and IND, providing an immediate-release FDC dosage form that improves patient adherence and is suitable for *in vivo* evaluation.

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AUTHOR CONTRIBUTION STATEMENT

The conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing – original draft, writing – review & editing, project administration (DO), the investigation, literature review, writing – original draft, data curation, formal analysis, project administration (AY), the conceptualization, methodology, formal analysis, data cu-

ration, project administration (YG), the formal analysis, data curation, writing – review & editing (SA).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Synthesis of Novel Benzofuran-Based Thiazole Hybrids and Investigation of Their Antioxidant and Anticholinesterase Activities

Betül KAYA^{*,}, Zahra MARYAM^{**}, Nour El-Huda DAOUD^{***}, Emine Rana BAĞCI^{****},^{*****}, Abdüllatif KARAKAYA^{*****},^{*****}, Tuğba ERÇETİN^{*****}, Ulviye Acar ÇEVİK^{*****}

Synthesis of Novel Benzofuran-Based Thiazole Hybrids and Investigation of Their Antioxidant and Anticholinesterase Activities

Benzofuran Temelli Yeni Tiyazol Hibritlerinin Sentezi ve Antioksidan ve Antikolinesteraz Aktivitelerinin İncelenmesi

SUMMARY

In this study, a novel series of benzofuran derivatives (2a-2k) was synthesized, and the structures of the obtained compounds were elucidated by ¹H NMR, ¹³C NMR, and HRMS spectra. The compounds were investigated for their anticholinesterase and antioxidant potentials. The ferrous ion chelating activity of the compound 2g at 50 μ M concentration was 68.25%, surpassing those of rutin and BHT (13.21% and 2.57%, respectively). Besides, according to the DPPH radical scavenging activity assay, compound 2g exhibited the most potent antioxidant activity with an IC₅₀ value of $30.14 \pm 0.005 \mu$ M, when compared to standard gallic acid (IC₅₀ $29.48 \pm 0.014 \mu$ M). Furthermore, compounds 2d (10.33%) and 2i (20.17%) showed the highest inhibitory activity against AChE and BChE, respectively. Compound 2i exhibits important interactions with the active site of BChE, according to the *in silico* molecular docking study. Also, the *in silico* ADMET study revealed the desirable pharmacokinetic properties of compound 2i. Therefore, compounds 2g and 2i could be deemed as promising candidates for designing dual functional antioxidant and butyrylcholinesterase inhibitors in the management of Alzheimer's disease.

Keywords: Benzofuran, Alzheimer's disease, anticholinesterase, DPPH assay, ferrous ion-chelating activity.

ÖZ

Bu çalışmada, bir seri yeni benzofuran türevi bileşik (2a-2k) sentezlenmiş ve elde edilen bileşiklerin yapıları ¹H NMR, ¹³C NMR ve HRMS spektrumları ile aydınlatılmıştır. Bileşikler antikolinesteraz ve antioksidan potansiyelleri açısından incelenmiştir. 50 μ M konsantrasyonda, 2g bileşiğinin (%68,25), demir iyonu şelatlama aktivitesinin rutin ve BHT'den (sırasıyla %13,21 ve %2,57) daha yüksek olduğu bulunmuştur. Ayrıca, DPPH radikal süpürücü aktivite deneyine göre, 2g bileşiği, referans gallik asitle (IC₅₀ = $29,48 \pm 0,014 \mu$ M) karşılaştırıldığında, $30,14 \pm 0,005 \mu$ M'lik bir IC₅₀ değeriyle en güçlü antioksidan aktiviteyi göstermiştir. Bunun dışında, 2d (%10,33) ve 2i (%20,17) bileşikleri, sırasıyla AChE ve BChE'ye karşı en yüksek inhibitör aktiviteyi göstermiştir. Bileşik 2i, *in silico* moleküler yerleştirme çalışmasına göre BChE'nin aktif bölgesiyle önemli etkileşimler sergilemiştir. Ayrıca, *in silico* ADMET çalışması, bileşik 2i'nin istenen farmakokinetik özelliklerini ortaya koymuştur. Bu nedenle, 2g ve 2i bileşikleri, Alzheimer hastalığının yönetiminde çift fonksiyonlu antioksidan ve bütirilcholinesteraz inhibitörlerinin tasarlanması için umut verici adaylar olarak öne çıkmıştır.

Anahtar Kelimeler: Benzofuran, Alzheimer hastalığı, antikolinesteraz, DPPH testi, demir iyon şelatlama aktivitesi.

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* ORCID: 0000-0002-1713-9485 Vocational School of Health Services, Pharmacy Services, Bilecik Şeyh Edebali University 11000, Bilecik, Turkey

** ORCID: 0000-0003-4233-727X Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, Eskişehir 26470, Turkey.

*** ORCID: 0000-0003-1332-9609 Department of Pharmaceutical Chemistry, Graduate Education Institute, Anadolu University, Eskişehir 26470, Turkey.

**** ORCID: 0009-0004-7452-0532 Department of Pharmaceutical Chemistry, Graduate Education Institute, Anadolu University, Eskişehir 26470, Turkey.

***** ORCID: 0009-0004-7452-0532 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar 03030, Turkey

***** ORCID: 0009-0003-9619-6705 Department of Pharmaceutical Chemistry, Graduate Education Institute, Anadolu University, Eskişehir 26470, Turkey.

***** ORCID: 0009-0003-9619-6705 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Zonguldak Bulent Ecevit University, 67600 Zonguldak, Turkey

***** ORCID: 0000-0001-7774-7266 Department of Pharmacognosy, Faculty of Pharmacy, Eastern Mediterranean University, Via Mersin 10, TR-99628 Famagusta, North Cyprus, Turkey

***** ORCID: 0000-0003-1879-1034 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, Eskişehir 26470, Turkey.

* Corresponding Author; Betül KAYA

E-mail: betul.kaya@bilecik.edu.tr

INTRODUCTION

Alzheimer's disease (AD) is a chronic condition characterized by the irreversible degeneration of neurons in the central nervous system (CNS), resulting in progressive impairment of memory, reduced ability to speak and understand language, and other cognitive impairments that eventually lead to death (Goyal et al., 2017). There are an estimated 50 million cases of AD worldwide, according to the World Alzheimer's Disease Report, and this figure is expected to increase rapidly to 152 million cases by 2050 (Zong et al., 2023). Neurofibrillary tangles (NFT) and extracellular β -amyloid plaques ($A\beta$) are the primary pathological features of AD (Li et al., 2018). The decreased levels of cholinergic neurotransmitter, namely acetylcholine (ACh) in neural synapses, and metal dyshomeostasis linked to accelerated oxidative processes also potentiate the risk of AD. However, the exact pathogenesis of AD remains elusive, and comprehending the process of AD has been deemed essential for developing effective anti-AD drugs

(Pravin and Jozwiak, 2022; Sepehri et al., 2022).

Based on the cholinergic theory, anticholinesterase inhibitors offer a solid foundation for treating AD by preserving cholinergic functionality. Anticholinesterase inhibitors perform their action by inhibiting two isoforms of human cholinesterase enzymes: acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE). AChE is the primary enzyme for the disintegration of 80% of acetylcholine present in the body and is mostly found in the CNS (Marucci et al., 2021). Thus, the suppression of AChE and BChE enzymes maintains ACh level by decreasing its breakdown rate, improving short-term memory and other Alzheimer's symptoms at the cognitive level. Anticholinesterase inhibitors are considered first-line therapy for mild-to-moderate symptoms of AD. To date, four medications are approved by the FDA to treat AD symptoms, namely donepezil, memantine, rivastigmine, and galantamine (Figure 1), which are acetylcholinesterase inhibitors except memantine (Goyal et al., 2018).

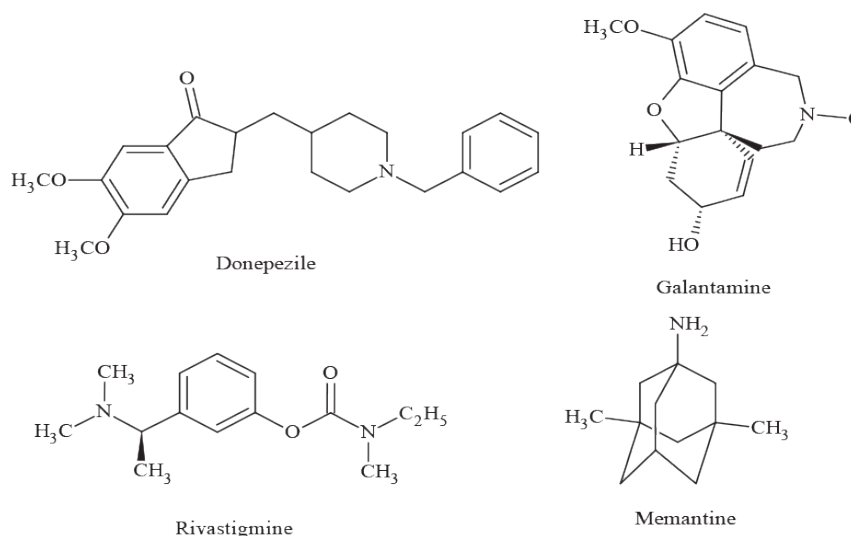


Figure 1. FDA-approved drugs used in the treatment of AD.

In the field of medicinal chemistry, heterocyclic scaffolds such as benzofuran (Irfan et al., 2023b), furan (Loğoğlu et al., 2010), pyrazole (Karrouch et al., 2018), oxadiazole (Irfan et al., 2023a), and thiazole (Ripain and Ngah, 2021) have garnered considerable

attention because of their remarkable biological applications. Benzofuran, in particular, has drawn great attention and is considered a valuable class and a key structural motif in the field of drug discovery. Based on their structure, benzofuran-containing

molecules have a variety of pharmacological actions, including anti-hyperlipidemic, anti-inflammatory, analgesic, antiviral, antibacterial, anticancer, and anti-Alzheimer's properties (Arce-Ramos et al., 2023; Dawood 2019; Patel et al., 2024; Sahu et al., 2024). Thus, the benzofuran chemical core has been the subject of numerous synthetic investigations. Recently, it has been revealed that benzofuran-containing heterocyclic scaffolds (Figure 2), including hydroxypyridinone-containing benzofuran (compound 7d), coumarin-linked benzofuran (compound 9j), tacrine-derived benzofuran (compound 15), and benzofuran-based benzyl pyridinium (compound 5e) are powerful AChE inhibitors (Baharloo et al., 2015; Hiremathad et al.,

2018a; Hiremathad et al., 2018b; Fancellu et al., 2020). Besides, the benzofuran scaffold may be considered as a bioisosteric alternative to the indanone ring present in donepezil and galantamine, and benzofuran derivatives, therefore, have been explored as anti-Alzheimer's disease agents due to the structural variety while maintaining the possibility of interaction with the cholinesterase active site (Fancellu et al., 2020; Gebeş-Alperen et al., 2025). Moreover, thiazole scaffolds have appeared as prominent pharmacophores in medicinal chemistry for their numerous outstanding therapeutic efficacies, including anti-acetylcholinesterase efficacy (Haroon et al., 2021; Jehangir et al., 2024; Saglik et al., 2020; Xu et al., 2020).

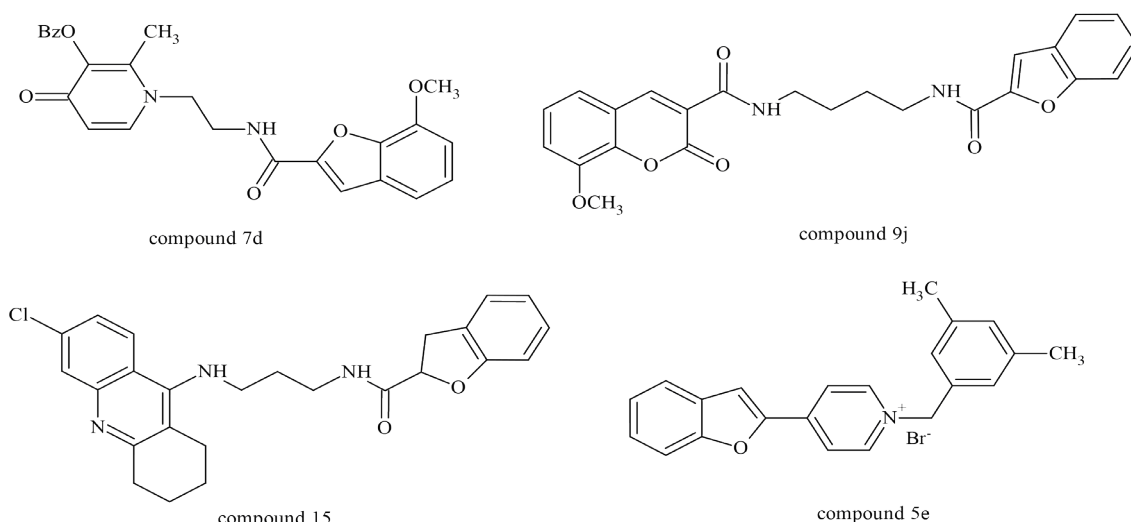


Figure 2. Some examples of the benzofuran-based compounds that have been reported to have AChE inhibitory activity

In this study, a novel series of benzofuran derivatives including a thiazole ring was synthesized, characterized through NMR and HRMS techniques, and tested for their AChE/BChE inhibitory activities and antioxidant effects.

RESULTS AND DISCUSSION

Chemistry

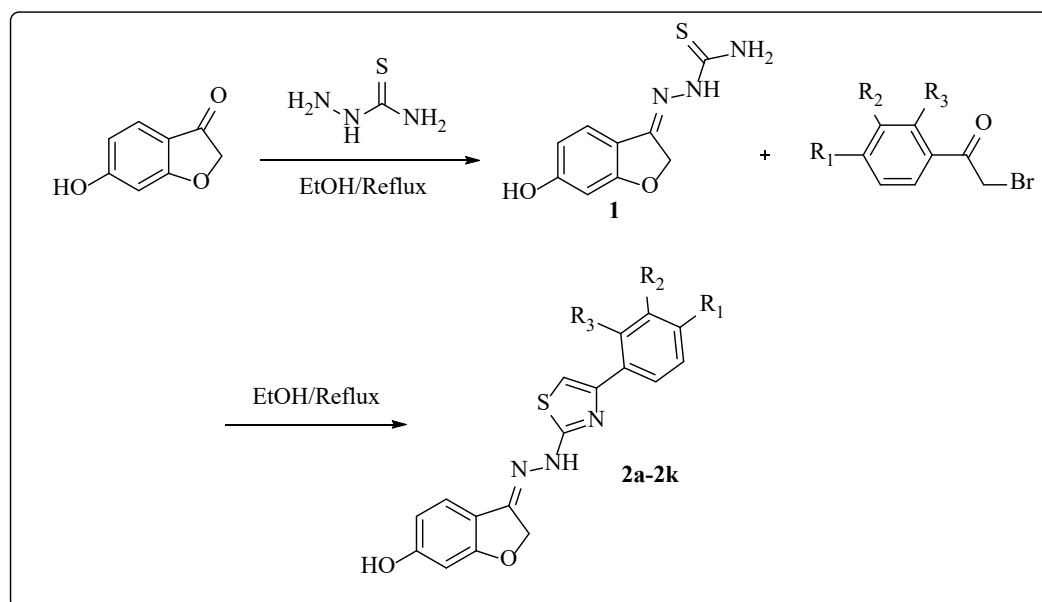
In this study, new benzofuran derivatives **2a-2k** were yielded as outlined in Scheme 1. The target compounds were obtained via a two-step synthetic

procedure. Firstly, 6-hydroxybenzofuran-3(2H)-one was reacted with thiosemicarbazone under reflux in ethanol to synthesize the corresponding thiosemicarbazone (**1**). Secondly, the reaction of compound **1** with appropriate 2-bromoacetophenone derivatives in ethanol afforded the title compounds **2a-2k**. The structures of synthesized compounds were elucidated through NMR and HRMS techniques.

In the ¹H NMR spectra of the compounds, -CH₂ protons belonging to the benzofuran ring were

observed at between 4.68-4.71 ppm as singlets. In the ^{13}C NMR spectra of compounds, the peak due to $-\text{CH}_2$ carbons was obtained at between 56.24 and 56.52 ppm. The signals of all other aliphatic and

aromatic protons and carbons were detected in their expected regions. HRMS spectra corresponded with the proposed structures for all compounds.



Comp.	R ₁	R ₂	R ₃	Comp.	R ₁	R ₂	R ₃
2a	-NO ₂	-H	-H	2g	-Cl	-Cl	-H
2b	-OCH ₃	-H	-H	2h	-F	-H	-F
2c	-CN	-H	-H	2i	-Cl	-H	-H
2d	-F	-H	-H	2j	-H	-H	-H
2e	-Br	-H	-H	2k	-H	-NO ₂	-H
2f	-CH ₃	-H	-H				

Scheme 1. The synthetic route and substituents of the title compounds **2a-2k**. Reagents and conditions: first step: thiosemicarbazide, EtOH, reflux, 3h; second step: appropriate 2-bromoacetophenone derivative, EtOH, reflux, 4h.

AChE and BChE inhibitory activity

The inhibitory activity of compounds **2a-2k** was measured against AChE and BChE using Galantamine hydrobromide as a reference, and the results were depicted in Table 1. Compounds **2c** and **2d** were detected as the most potent compounds, displaying 10.33 and 10.13% inhibition against AChE, respectively. The compounds represented a better inhibitory profile against BChE than AChE. Compounds **2i** and

2h were found to be the most effective agents against BChE, with the inhibition percentage of 20.17% and 16.67%, respectively. This differential behavior can be explained by better binding of disubstituted groups with BChE. Based on these results, it can be suggested that *para*-cyano and *para*-fluoro groups are favorable for AChE inhibition, while *para*-chloro and *ortho,para*-difluoro substituents are suitable for BChE inhibitory activity.

Table 1. The inhibitory effects (%) of the compounds **2a-2k** against cholinesterases at 50 μ M reaction concentrations.

Compound	AChE	BChE
2a	NA	NA
2b	NA	NA
2c	10.13 $\pm$ 0.002	8.37 $\pm$ 0.003
2d	10.33 $\pm$ 0.008	4.94 $\pm$ 0.007
2e	NA	NA
2f	1.43 $\pm$ 0.001	5.67 $\pm$ 0.002
2g	NA	2.14 $\pm$ 0.001
2h	NA	16.67 $\pm$ 0.009
2i	2.05 $\pm$ 0.001	20.17 $\pm$ 0.011
2j	6.81 $\pm$ 0.004	2.78 $\pm$ 0.001
2k	3.76 $\pm$ 0.001	7.64 $\pm$ 0.003
Gal HBr 50 μ M	97.89 $\pm$ 0.01	62.48 $\pm$ 0.01

*NA: not-active

Antioxidant Activity

The antioxidant potential of synthesized compounds **2a-2k** was measured using DPPH free radical-scavenging and ferrous ion chelating activities (Table 2). In the DPPH test, compounds **2b**, **2g**, and **2k** showed promising antioxidant activities with IC₅₀ values of 44.17 $\pm$ 0.012 μ M, 48.11 $\pm$ 0.014 μ M, 30.14 $\pm$ 0.005 μ M, and 44.28 $\pm$ 0.009 μ M, respectively. In particular, compound **2g**, with *meta*, *para*-dichloro substituent on the phenyl, ring exerted approximately equal antioxidant activity to the reference drug gallic acid. It can be suggested that *para*-methoxy (**2b**),

para-fluoro (**2d**), *meta*, *para*-dichloro (**2g**), and *meta*-nitro (**2k**) groups have a positive contribution to the DPPH radical-scavenging activity. According to the ferrous ion chelating potential of the compounds **2a-2k**, compounds **2c**, **2d**, **2i**, and **2j** were found to have better chelating capacity (5.34%, 3.99%, 4.70% and 5.08%, respectively, at 50 μ M) than BHT (2.57% at 50 μ M). It can be claimed that *para*-cyano (**2c**), *para*-fluoro (**2d**), and *para*-chloro (**2i**) substituents on the phenyl ring and also no substitution on the phenyl ring, have a positive contribution to the ferric ion chelating activity.

Table 2. The effects of the synthesized compounds **2a-2k** at 50 μM and references on ferric ion chelating and DPPH free radical-scavenging (inhibition % $\pm$ S.E.M).

Compound	DPPH	ION CHELATING
2a	6.68 ± 0.009	NA
2b	58.09 ± 0.006	2.08 ± 0.008
2c	38.98 ± 0.031	5.34 ± 0.012
2d	53.14 ± 0.042	3.99 ± 0.005
2e	37.47 ± 0.012	1.78 ± 0.003
2f	46.56 ± 0.005	1.79 ± 0.001
2g	68.25 ± 0.023	1.66 ± 0.019
2h	46.46 ± 0.008	1.38 ± 0.008
2i	46.41 ± 0.002	4.70 ± 0.012
2j	5.68 ± 0.003	5.08 ± 0.009
2k	57.50 ± 0.001	2.41 ± 0.006
RUTIN 50 μM	-	13.21 ± 0.007
RUTIN 100 μM	-	28.14 ± 0.011
BHT 50 μM	-	2.57 ± 0.004
BHT 100 μM	-	7.06 ± 0.009
Gallic Acid	70.29 ± 0.005	

*NA: not-active

Table 3. DPPH free radical-scavenging activity of the synthesized compounds **2a-2k** and gallic acid (IC_{50})

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
2a	$> 60 \mu\text{M}$	2g	30.14 ± 0.005
2b	44.17 ± 0.012	2h	53.29 ± 0.011
2c	$> 60 \mu\text{M}$	2i	53.31 ± 0.015
2d	48.11 ± 0.014	2j	$> 60 \mu\text{M}$
2e	$> 60 \mu\text{M}$	2k	44.28 ± 0.009
2f	53.21 ± 0.008	Gallic Acid	29.48 ± 0.014

Molecular Docking Study and ADMET

This molecular docking illustrates how compound **2i** binds to the active site of BChE, providing detailed insights into its binding interactions and potential efficacy. During the preparation step, compound **2i**,

a proton was transferred between the nitrogen atom of hydrazine and the thiazole ring due to the selected pH range (5-9) and different pKa values for each of them; N (hydrazine) 1.17 and N (thiazole) 9.63 (see Figure 3).

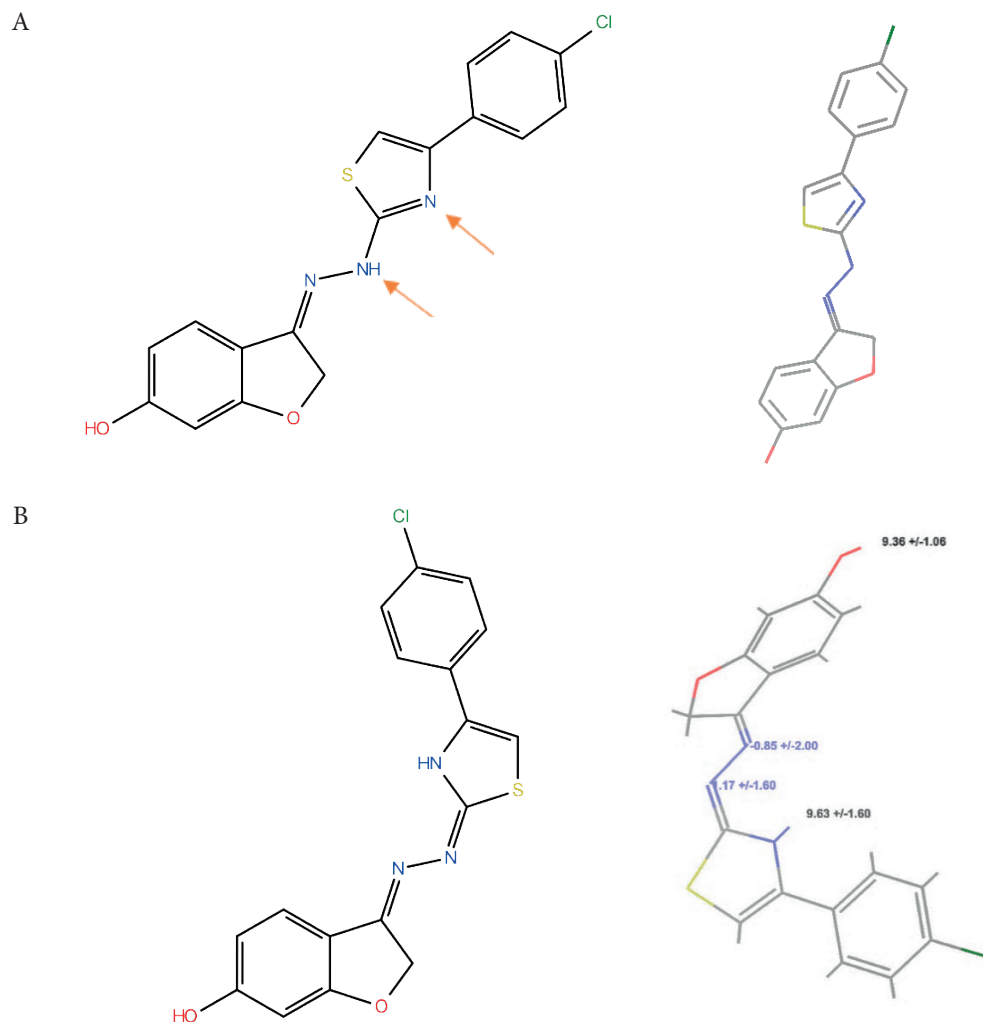


Figure 3. Show compound **2i**, (A) before preparation, (B) after preparation step for docking, and the predicted pKa for each atom in compound **2i**, which justifies the different generated forms for the compound before and after preparation on the pH range (5-9).

The docking results are presented in Table 4. The docking scores (glide gscore) for both SP and XP docking modes are -7.392 and -7.279 Kcal/mol, respectively. Protonated and deprotonated forms.

However, the docking gscore for the co-crystallized ligand (Tacrine) was lower in the XP mode (-6.301 Kcal/mol), which indicates that our compound **2i** binds more effectively to the BChE.

Table 4. The computational results from docking compound **2i** in BChE enzyme (4BDS) (Kcal/mol); different docking scores with the glide property.

Docking Mode	Standard precision mode (SP)	Extra precision mode (XP)
Docking score	-7.392	-7.279
Glide ligand efficiency	-0.308	-0.303
Glide gscore	-7.392	-7.279
Glide energy	-46.888	-41.624

The binding correspondence of BChE with compound **2i** (Figure 4) is instituted by two bond types: the hydrogen bond, which is the stronger, and the Pi-Pi stacking bond, which is the weaker. The hydrogen bond is suggested to be a crucial aid in stabilizing the binding of our compound with the BChE binding pocket. The stronger bond type was found between the phenolic OH group of our

compound (**2i**) with Tyr 128 and Glh197 residues from the active site (two H-bonds). The weaker one formed between the hydrophobic parts of the benzene rings and the thiazole ring from our compound with Trp82, Tyr332, and Phe329 residues from the active site; formed five Pi-Pi stacking bonds. The calculated glide gscore is -7.279 Kcal/mol.

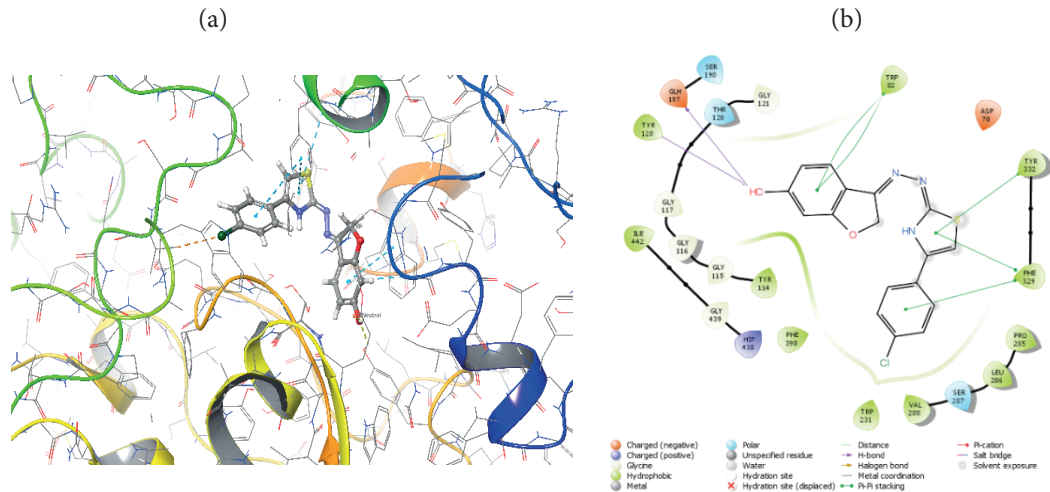


Figure 4. (a) 3D (b) 2D binding interactions between BChE with compound **2i** from XP mode, demonstrating hydrogen bonds with Glh197 and Tyr 128. Five pi-pi stacking with Trp82, Tyr332, and Phe329. The glide gscore is -7.279 Kcal/mol (PDB ID 4BDS).

After we evaluated the interaction of compound **2i** with BChE, we need to check its toxicity and biological effects to figure out its potential use as a medicine. For that, we applied a computational ADMET study, which helped to predict how our lead compound affects human metabolism. The result of it is summarized in Table 5, which lists the computed

ADMET parameters for our compound **2i**. According to the data, our compound may qualify as a drug because the values for Rule 5 (Lipinski, 2004) and 3 (Lipinski et al., 2012) are less than 3. Moreover, it predicts good oral absorption and high permeability in MDCK and Caco2 cells. While it also shows a few side effects, good safety, and drug-like properties.

Table 5. Predicted ADMET characteristics of the compound **2i**.

Title	2i	Reference Range
Molecular weight (MW)	357.814	130–725
dipole	4.892	1.0–12.5
SASA	602.208	300–1000
FOSA	53.934	0–750
FISA	80.032	7–330
PISA	344.502	0–450
WPSA	123.741	0–175
Volume (Å ³)	1024.594	500–2000
Hydrogen bond donor (HBD)	2	0–6
Hydrogen bond acceptor (HBA)	3.5	2.0–20.0
Glob (Sphere = 1)	0.8161812	0.75–0.95
QPlogPC16	11.99	4.0–18.0
QPlogP _o t	17.476	8.0–35.0
QPlogP _w	9.814	4.0–45.0
Predicted octanol/water partition coefficient (QPlogP _o /w)	4.361	–2.0–6.5
QPlogS	–5.866	–6.5–0.5
CIQPlogS	–6.109	–6.5–0.5
Predicted IC ₅₀ value for blockage of HERG K ⁺ channels (QPlog HERG)	–6.149	Concern if it is below –5
Predicted Caco2 cell permeability (QPP Caco) (nm/sec)	1725.676	Less than 25 is poor More than 500 is great
Predicted brain/blood partition coefficient (QPlogBB)	–0.115	–3.0–1.2
Predicted MDCK cell permeability (QPPMDCK) (nm/sec)	4249.394	Less than 25 is poor More than 500 is great
Prediction of binding to human serum albumin (QPlogK _h a)	0.516	–1.5–1.5
Oral Absorption (for Human)	3	-
Human Oral Absorption as %	100	Less than 25% is poor More than 80% is high
PSA	64.89	7–200
Rule 5	0	In max of 5
Rule 3	1	In max of 3
Jm	0.016	-

MATERIAL AND METHOD

Chemistry

All starting materials and chemicals used in the synthetic route were supplied by commercial suppliers. Melting points were determined using the MP90 digital melting point apparatus (Mettler Toledo, OH, USA) and were uncorrected. ¹H-NMR

and ¹³C-NMR spectra were recorded through a NMR spectrometer (Bruker, Billerica, MA, USA) in DMSO-*d*₆. HRMS spectra were recorded on a LCMS-IT-TOF system (Shimadzu, Kyoto, Japan). Thin Layer Chromatography (TLC) using Silica Gel 60 F254 TLC plates (Merck KGaA, Darmstadt, Germany) was performed to monitor the progress of the chemical reactions.

Synthesis of 2-(6-hydroxybenzofuran-3(2H)-ylidene)methyl-hydrazine-1-carbothioamide (1) (CAS Number: 1535209-86-9): Ethanol (100 mL) was used to dissolve thiosemicarbazide (2.89 g, 0.032 mol) and 6-hydroxybenzofuran-3(2H)-one (4.8 g, 0.032 mol). The mixture was refluxed for three hours. After completion of the reaction, the reaction mixture was chilled in an ice bath, and the precipitated product was filtered and crystallized from ethanol (Laczkowski et al., 2013).

Synthesis of target compounds (2a-2k): The appropriate 2-bromoacetophenone derivative (2.5 mmol) and 2-(6-hydroxybenzofuran-3(2H)-ylidene)methyl-hydrazine-1-carbothioamide (**1**) (2.5 mmol) were dissolved in ethanol (50 mL). The mixture was refluxed for four hours. After TLC screening, the mixture was cooled in an ice bath, and the precipitated product was filtered and crystallized from ethanol.

3-(2-(4-(4-Nitrophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2a): Yield: 78 %, M.P.= 155.4 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 4.70 (2H, s, CH₂), 6.51 (1H, s, Aromatic CH), 6.58 (1H, d, J= 8.44 Hz, Aromatic CH), 7.31 (1H, br.s, Aromatic CH), 7.45 (1H, d, J= 8.96 Hz, Aromatic CH), 8.02 (2H, d, J= 7.88 Hz, Aromatic CH), 8.27 (2H, d, J= 8.00 Hz, Aromatic CH). ¹³C-NMR (100 MHz, DMSO-d₆): δ= 56.48, 112.23, 113.40, 124.27, 124.54, 125.49, 126.94, 130.04, 139.18, 145.00, 150.08, 155.80, 167.18, 176.05, 197.25. HRMS (m/z): [M+H]⁺ calcd for C₁₇H₁₂N₄O₄S: 369.0652; found: 369.0651.

3-(2-(4-(4-Methoxyphenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2b): Yield: 76 %, M.P.= 112.9 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 3.82 (3H, s, -OCH₃), 4.69 (2H, s, CH₂), 6.53 (1H, s, Aromatic CH), 6.59 (1H, d, J= 8.48 Hz, Aromatic CH), 7.01-7.03 (2H, m, Aromatic CH), 7.44 (1H, d, J= 8.28 Hz, Aromatic CH), 7.67 (1H, d, J= 7.92 Hz, Aromatic CH), 7.91 (2H, d, J= 7.60 Hz, Aromatic CH). ¹³C-NMR (100 MHz, DMSO-d₆): δ= 55.78, 56.52, 98.08, 100.94, 112.58, 114.15, 115.07, 125.42, 127.63, 130.68, 160.60, 163.74, 167.16, 170.76, 176.03,

197.18. HRMS (m/z): [M+H]⁺ calcd for C₁₈H₁₅N₃O₃S: 354.0907; found: 354.0901.

3-(2-(4-(4-Cyanophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2c): Yield: 69 %, M.P.= 129.4 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 4.70 (2H, s, CH₂), 6.51 (1H, s, Aromatic CH), 6.58 (1H, d, J= 8.52 Hz, Aromatic CH), 7.45 (2H, d, J= 8.36 Hz, Aromatic CH), 7.92-7.94 (2H, m, Aromatic CH), 8.01-8.04 (2H, m, Aromatic CH). ¹³C-NMR (100 MHz, DMSO-d₆): δ= 56.48, 98.76, 112.24, 113.39, 125.48, 126.58, 126.77, 129.23, 132.64, 132.84, 133.22, 145.27, 158.66, 167.19, 176.04, 197.26. HRMS (m/z): [M+H]⁺ calcd for C₁₈H₁₂N₄O₂S: 349.0754; found: 349.0746.

3-(2-(4-(4-Fluorophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2d): Yield: 77 %, M.P.= 91.2 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 4.70 (2H, s, CH₂), 6.52 (1H, s, Aromatic CH), 6.58 (1H, d, J= 8.24 Hz, Aromatic CH), 7.45 (2H, d, J= 8.44 Hz, Aromatic CH), 7.80-7.82 (2H, m, Aromatic CH), 8.04-8.05 (2H, m, Aromatic CH). ¹³C-NMR (100 MHz, DMSO-d₆): δ= 56.48, 98.76, 112.24, 113.40, 116.01, 116.35 (d, J= 23.8 Hz), 125.49, 128.61, 131.67, 132.28 (d, J= 9.4 Hz), 141.86, 150.26, 167.19, 176.05, 197.27. HRMS (m/z): [M+H]⁺ calcd for C₁₇H₁₂N₃O₂FS: 342.0707; found: 342.0708.

3-(2-(4-(4-Bromophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2e): Yield: 75 %, M.P.= 100.5 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 4.68 (2H, s, CH₂), 6.36 (1H, s, Aromatic CH), 6.49-6.57 (2H, m, Aromatic CH), 7.43 (1H, s, Aromatic CH), 7.63-7.88 (4H, m, Aromatic CH). ¹³C-NMR (100 MHz, DMSO-d₆): δ= 56.24, 104.08, 112.49, 113.41, 120.71, 122.74, 123.66, 125.32, 130.68, 132.34, 141.39, 148.78, 168.08, 176.21, 197.45. HRMS (m/z): [M+H]⁺ calcd for C₁₇H₁₂N₃O₂SBr: 401.9906; found: 401.9913.

3-(2-(4-(4-Methylphenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2f): Yield: 79 %, M.P.= 98.7 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ: 2.32 (3H, s, CH₃), 4.71 (2H, s, CH₂),

6.38 (1H, s, Aromatic CH), 6.59 (2H, d, J = 8.28 Hz, Aromatic CH), 7.46 (1H, d, J = 8.20 Hz, Aromatic CH), 7.45-7.47 (1H, m, Aromatic CH), 7.63 (2H, br.s, Aromatic CH), 7.85 (2H, br.s, Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 21.30, 56.48, 102.33, 112.25, 113.40, 125.48, 126.14, 128.73, 129.66, 130.03, 143.98, 150.63, 167.20, 170.67, 176.05, 197.26. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_2\text{S}$: 338.0958; found: 338.0968.

3-(2-(4-(3,4-Dichlorophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2g): Yield: 70 %, M.P. = 116.6 °C. ^1H -NMR (400 MHz, DMSO- d_6): δ : 4.70 (2H, s, CH_2), 6.51 (1H, s, Aromatic CH), 6.58 (1H, d, J = 8.44 Hz, Aromatic CH), 7.45 (2H, d, J = 8.04 Hz, Aromatic CH), 7.90 (1H, d, J = 8.32 Hz, Aromatic CH), 8.03 (1H, s, Aromatic CH), 8.11-8.13 (1H, m, Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 56.48, 98.76, 102.86, 112.23, 113.41, 125.49, 126.20, 127.82, 128.65, 130.56, 131.35, 131.51, 131.81, 148.78, 167.18, 176.05, 197.25. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{11}\text{N}_3\text{O}_2\text{SCl}_2$: 392.0022; found: 392.0027.

3-(2-(4-(2,4-Diflorophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2h): Yield: 73 %, M.P. = 117.8 °C. ^1H -NMR (400 MHz, DMSO- d_6): δ : 4.69 (2H, s, CH_2), 6.56 (1H, s, Aromatic CH), 6.61 (1H, d, J = 8.24 Hz, Aromatic CH), 7.12 (1H, s, Aromatic CH), 7.24 (1H, s, Aromatic CH), 7.37 (1H, s, Aromatic CH), 7.44 (1H, d, J = 8.36 Hz, Aromatic CH), 7.50 (1H, s, Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 56.47, 98.77, 105.54, 112.31, 113.30, 125.40, 130.84 and 130.96 (dd, J_1 = 3.7 Hz, J_2 = 11.6 Hz), 132.17, 148.16, 162.25, 165.34, 167.37, 170.03, 176.04, 197.25. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{11}\text{N}_3\text{O}_2\text{F}_2\text{S}$: 360.0613; found: 360.0612.

3-(2-(4-(4-Chlorophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2i): Yield: 77 %, M.P. = 123.9 °C. ^1H -NMR (400 MHz, DMSO- d_6): δ : 4.70 (2H, s, CH_2), 6.51 (1H, s, Aromatic CH), 6.58 (1H, d, J = 8.28 Hz, Aromatic

CH), 7.44-7.47 (2H, m, Aromatic CH), 7.55-7.60 (3H, m, Aromatic CH), 7.76 (1H, d, J = 7.08 Hz, Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 98.75, 112.23, 113.39, 125.47, 128.04, 128.80, 129.23, 129.46, 129.57, 130.53, 133.17, 167.19, 176.05, 197.26. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{12}\text{N}_3\text{O}_2\text{SCl}$: 358.0412; found: 358.0419.

3-(2-(4-Phenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2j): Yield: 78 %, M.P. = 129.8 °C. ^1H -NMR (400 MHz, DMSO- d_6): δ : 4.71 (2H, s, CH_2), 6.43 (1H, m, Aromatic CH), 6.53-6.59 (2H, m, Aromatic CH), 7.10 (2H, s, Aromatic CH), 7.25 (3H, s, Aromatic CH), 7.92 (1H, s, Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 56.49, 98.78, 112.25, 113.40, 125.46, 126.27, 127.46, 128.58, 128.82, 129.12, 129.48, 131.92, 167.20, 176.05, 197.23. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$: 324.0801; found: 324.0809.

3-(2-(4-(3-Nitrophenyl)thiazol-2-yl)hydrazineylidene)-2,3-dihydrobenzofuran-6-ol (2k): Yield: 76 %, M.P. = 146.4 °C. ^1H -NMR (400 MHz, DMSO- d_6): δ : 4.70 (2H, s, CH_2), 6.51 (1H, s, Aromatic CH), 6.58 (1H, d, J = 8.48 Hz, Aromatic CH), 7.45 (2H, d, J = 7.80 Hz, Aromatic CH), 7.71-7.73 (2H, m, Aromatic CH), 8.20 (2H, br.s., Aromatic CH). ^{13}C -NMR (100 MHz, DMSO- d_6): δ = 56.48, 98.75, 112.23, 113.40, 120.45, 122.72, 123.25, 125.48, 130.56, 130.72, 132.01, 132.34, 148.53, 148.73, 167.18, 176.04, 197.24. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}_4\text{S}$: 369.0652; found: 369.0658.

Determination of AChE and BChE Inhibitory Activities

The ChE inhibitory activity of the synthesized compounds was determined using an Ellman-modified spectrophotometric technique, as described in our previous studies (Karakaya et al., 2023a; Kaya et al., 2024; Karakaya et al., 2024). Donepezil hydrochloride (Sigma-Aldrich, USA) was employed as the reference drug.

Determination of Antioxidant Activity

Ferrous ion-chelating effect

The ferrous ion-chelating effect of all compounds and references was evaluated through the method reported by Chua et al., as described in our previous studies (Karakaya et al., 2023b; Karakaya et al., 2024; Kaya et al., 2024). BHT and rutin were employed as standard compounds.

DPPH Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the compounds was determined via Blois's UV technique, as described in our previous studies (Karakaya et al., 2023b; Karakaya et al., 2024; Kaya et al., 2024). Gallic acid was used as the standard compound.

Molecular Docking Study

To design effective AChE inhibitors and or BChE, ligands must target both the peripheral anionic site (PAS) and catalytic anionic site (CAS), and maximize inhibition (Ghanei-Nasab et al., 2016; Kuzu et al., 2024). Potent AChE inhibitors often include aromatic groups to bind PAS and basic-centered groups to interact with CAS; for instance, donepezil demonstrates high inhibition by binding both sites (Ferreira et al., 2021).

The AChE active site, measuring approximately 20 Å deep and 5 Å wide, includes essential residues like Tyr70, Asp72, Tyr121, Trp279, and Tyr334 for structural integrity. The deeper CAS contains His440, Glu327, Ser200, and Trp84. Similarly, BChE has a comparable active site structure, with a catalytic triad of Ser198, His438, and Glu325, as well as CAS residues Glu197, Trp231, and Phe329, and PAS residues Asp70 and Tyr332, forming a wider binding pocket (Kuzu et al., 2024).

Successful inhibition relies on ligand interactions with these residues to effectively block cholinesterase activity (Kuzu et al., 2024). Molecular docking, a rapid, cost-effective modeling technique, evaluates these atomic interactions between small molecules and receptors, supporting drug discovery efforts (Agarwal and Mehrotra, 2016; Meng et al., 2011).

In our study, we conducted docking analyses on BChE (PDB ID: 4BDS) (Kryger et al., 1999; Nachon et al., 2013) using Maestro v12.8.117 from Schrödinger Suite 2021-2 (Schrödinger, 2021a). These specific crystal structures, selected for their biological relevance (Kuzu et al., 2024), were analyzed across a pH range of 5–9, aligning with AChE's active pH range of 7.5–8.0 (Tran-Minh et al., 1999). We developed our methodology based on Kuzu group (Kuzu et al., 2024) and Kaya group (Kaya et al., 2025) methods in protein selection (precision and relevance) and software choice. We conducted this *in-silico* analysis to validate our *in-vitro* findings regarding the compound **2i**, which is identified as the most effective BChE inhibitor among others. Our objective is to illustrate its docking behavior within the active site of BChE, providing detailed insights into its binding interactions and potential efficacy.

Preparation of the compound 2i

We sketched compound **2i** in Maestro and prepared for docking with the LigPrep tool (Schrödinger, 2021b). Through that, it created all stereoisomers and tautomers for the most stable conformers (32 in max) with its ionization state at neutral pH (7.0 ± 2.0); here we applied Epik to calculate charge for ionized atoms.

Protein structure preparation

The protein structures (4BDS) were prepared for docking using the Schrödinger Impeded Protein Preparation tool (Schrödinger, 2021c). Through that, it inserted hydrogen and created zero bond ordering for metals, cap terminal, and generated ionic state at neutral pH (7.0 ± 2.0) with Epik. Then we eliminated the co-crystal reagents except for the model ligand (Tacrine), and we restored the ionization states for the protein's polar residues to their original state. In addition, all water molecules were removed that literature proved not included in the complex formation (Ghanei-Nasab et al., 2016; Kuzu et al., 2024). Finally, we used the PROPKA procedure to produce hydrogens at pH 7. We minimized all proteins using the OPLS4 force field to decrease

steric clash between side chains, with a convergence of heavy atoms to an RMSD of 3 Å according to the method reported by Kuzu et al (Kuzu et al., 2024).

Grid generation and docking protocol

Using the incorporated Schrödinger panel to create a grid (Schrödinger, 2021d) as a mandatory pre-step for docking. We created a grid box around the urease enzyme's active site, including the co-crystal ligands. It is 20°A in each dimension from the ligand's center, with 0.25 as the cutoff radius scaling and 1.0 as a factor for the van der Waals.

We then docked our compound twice using another incorporated panel for docking using GLIDE, first using standard precision (SP) mode and then using extra precision (XP) mode (Schrödinger, 2021e). Through that, we applied default settings and selected the final docked pose of each compound based on the highest Glide score.

For validation, the co-crystallized ligand (Tacrine) was re-located in the active site of BChE by applying the same docking modes. RMSD value equals 0.4171 and SP docking score equals -7.681.

ADMET

We used an incorporated Qik-prop (Schrödinger, 2021f) tool in Schrödinger to calculate and predict ADMET and physicochemical properties of our compound, including distribution, absorption, excretion, metabolism, and toxicity.

CONCLUSION

Regarding the significant role of the benzofuran moiety in AD management drugs, a novel series of benzofuran derivatives was successfully synthesized and characterized in this study. Compounds were tested for their cholinesterase inhibitory activity and antioxidant potential. The results revealed that compounds exhibited better BChE inhibition than AChE. Compound **2i** showed the highest inhibitory activity against BChE. Moreover, the computational docking study showed that our lead compound **2i** interacts significantly with the active site of BChE. Additionally, ADMET profiling using QikProp suggests that our

lead (**2i**) possesses favorable pharmacokinetic properties. Considering antioxidant activity, compound **2g** showed promising DPPH scavenging and ferrous ion chelating activity. Thus, in the future, compounds **2i** and **2g** can serve as lead compounds for the development of new dual-targeted anti-AD agents that can serve as butyrylcholinesterase inhibitors and antioxidants.

Supporting Information Summary

In the supporting information, the researcher can view the full characterization data of compounds.

AUTHOR CONTRIBUTION STATEMENT

BK and UAÇ conceived and designed the study, BK, ZM, UAÇ, ERB and AK performed the synthesized compounds, BK and UAÇ performed the analysis of the compounds. TE performed anti-acetylcholinesterase (AChE), anti-butyrylcholinesterase (BChE) and antioxidant activities. NEHD performed the docking and ADMET studies. All authors reviewed and edited the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Antifungal and Anti-Biofilm Activities of Cosmetic Ingredients Against Clinical Dermatophyte Isolates

Cemre ÖZKANCA*, Sibel DÖŞLER**

Antifungal and Anti-Biofilm Activities of Cosmetic Ingredients Against Clinical Dermatophyte Isolates

SUMMARY

The skin, with its low pH and sweat glands, acts as a physical barrier against microbial colonization. Dermatophytes, however, can develop resistance through biofilm formation and changes in membrane permeability. Cosmetic products applied to the skin may also contribute to this stress. This study evaluated the antifungal and antibiofilm effects of commonly used cosmetic raw materials against clinical dermatophyte isolates. Eleven isolates were tested: five *Microsporum canis*, four *Trichophyton rubrum*, and one each of *T. mentagrophytes* and *T. tonsurans*. Biofilm formation was assessed by crystal violet staining, antifungal activities by microbroth dilution, and antibiofilm activities against mature biofilms. Among the isolates, four *M. canis* were strong biofilm producers, one *M. canis* and two *T. rubrum* moderate, while others were weak. Minimum inhibitory concentrations ranged from 7.8–250 µL/mL for tea tree oil, 4–625 µL/mL for vitamin A, and 4–500 µL/mL for octocrylene, glycerin, or panthenol. Benzophenone-4 showed moderate activity (256–5000 µg/mL), whereas the UV filter DHHB was selectively active against *M. canis* (8–256 µg/mL). Vitamin A, glycerin, and octocrylene were most effective against *M. canis*, while panthenol and tea tree oil were more active against *T. rubrum*. Cosmetic ingredients reduced mature biofilms up to 3-log₁₀ cfu/mL, with tea tree oil, vitamin A, panthenol, Centella asiatica extract, and benzophenone-4 showing strong antibiofilm effects. These results indicate that cosmetic components possess significant antifungal and antibiofilm properties, offering a promising adjunct or alternative strategy for treating dermatophyte infections. Further research is needed to validate their clinical efficacy and safety.

Keywords: Cosmetic ingredient, dermatophyte, anti-biofilm, antifungal activity.

Kozmetik İçeriklerin Klinik Dermatofit İzolatlarına Karşı Antifungal ve Anti-Biyofilm Aktivitelerinin İncelenmesi

ÖZ

Deri, düşük pH değeri ve ter bezleri ile mikroorganizmaların kolonizasyonuna engel olmak üzere fiziksel bir bariyer sağlamaktadır. Dermatofitler, biyofilm oluşturma yetenekleri ve hücre zarı geçirgenliği değişiklikleri gibi çevresel stres faktörleri nedeniyle antifungal direnç geliştirebilmektedir. Cilde uygulanan kozmetik ürünler de bu strese katkıda bulunma potansiyelindedir. Bu çalışmada, yaygın kullanılan kozmetik hammaddelerin, klinik dermatofit suşlarına karşı antifungal ve anti-biyofilm etkinliğinin değerlendirilmesi amaçlanmıştır. Beş *Microsporum canis*, dört *Trichophyton rubrum*, birer *Trichophyton mentagrophytes* ve *Trichophyton tonsurans* olmak üzere toplam 11 suşun biyofilm oluşturma kapasiteleri kristal viyole boyama yöntemiyle belirlenmiştir. Antifungal aktiviteler mikrodilüsyon tekniği ile belirlenirken, etkili maddelerin antibiyofilm aktiviteleri olgun biyofilmlere karşı test edilmiştir. 11 suş arasında dört *M. canis* kuvvetli, bir *M. canis* ile iki *T. rubrum* orta ve diğer izolatlar zayıf biyofilm oluşturma özelliği göstermiştir. Çay ağacı yağı, A vitamini ve oktokrilen, gliserin ya da panthenol için minimum inhibitör konsantrasyonları sırasıyla 7,8-250, 4-625 ve 4-500 µL/mL arasında bulunmuştur. Benzofenon-4, 256-5000 µg/mL ile orta düzeyde aktifken UV filtresi DHHB, *M. canis*e karşı 8-256 µg/mL ile seçici olarak etkili bulunmuştur. Özellikle vitamin A, gliserin ve oktokrilen *M. canis*e karşı, panthenol veya çay ağacı yağı ise *T. rubrum*'a karşı en aktif maddeler olarak bulunmuştur. Kozmetik hammaddeler, olgun biyofilmleri 3-log₁₀ cfu/mL'ye kadar inhibe etmiş olup, bu sonuçlara göre özellikle çay ağacı yağı, vitamin A, panthenol, Centella asiatica ekstresi ve benzofenon-4'ün güçlü antibiyofilm aktivitelerine sahip olduğu belirlenmiştir. Kozmetik bileşenler önemli antifungal ve antibiyofilm özellikler göstererek dermatofit enfeksiyonlarının tedavisinde umut verici bir alternatif yaklaşım sunmaktadır. Bu maddelerin klinik etkinlik ve güvenliğini doğrulamak için daha fazla araştırma yapılması gerekmektedir.

Anahtar Kelimeler: Kozmetik ürünler, dermatofit, anti-biyofilm, antifungal aktivite.

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* ORCID: 0000-0002-0342-2060, Hamidiye Vocational School of Health Services, The University of Health Sciences, Istanbul, Türkiye.

** ORCID: 0000-0001-5223-4755, Department of Pharmaceutical Microbiology, Istanbul University, Faculty of Pharmacy, Istanbul, Türkiye

INTRODUCTION

Dermatophytes are a group of organisms responsible for infections in keratin-rich tissues, including the skin, hair, and nails. Under normal circumstances, the skin does not provide a suitable environment for microorganisms to thrive due to its low pH and the presence of sweat glands (Blaak & Staib, 2018). However, under certain conditions, some infections can occur due to fungi in keratin-rich environments, colonizing the stratum corneum of the skin, hair shafts, and nails (Weitzman & Summerbell, 1995). These infections, which are commonly referred to as dermatophytosis, ringworm, or tinea, are prevalent worldwide and pose significant health concerns. Dermatophytes belong to three main genera: *Trichophyton*, *Microsporum*, and *Epidermophyton*, and their infections manifest in various forms depending on the site of infection (Weitzman & Summerbell, 1995).

Since fungal cells are eukaryotic, like mammalian cells, generally a substance that is toxic to fungi can also be toxic to humans, and the development of new antifungal agents poses a serious problem (Nivoix et al., 2020). Besides the limited number of antifungal drugs, antifungal resistance is increasing worldwide and poses a serious problem for the treatment of clinically encountered infections. In clinical studies, a high rate of resistance is especially observed against the azole group, which is one of the main antifungal groups in the treatment of dermatophytes (Oliveira Carvalho et al., 2013). There are different mechanisms for fungal resistance, such as mixing the drug targets, increasing cell permeability, forming a biofilm, and stress triggering by the improper use of antifungal agents (Khurana et al., 2019; Revie et al., 2018). In the midst of the difficulties encountered in managing dermatophyte infections, biofilms -organized groups of fungal cells surrounded by a self-generated extracellular matrix that sticks to surfaces- are gaining more focus (Donlan, 2002). The ability of dermatophytes to form biofilms is crucial for their

survival and contributes to their resilience against antifungal therapies. *Trichophyton rubrum* and *Microsporum canis* are among the most frequently reported species to form biofilms, which protect fungal cells from antifungal agents and immune responses. These biofilms contribute to chronic infections and complicate treatment strategies (Costa-Orlandi et al., 2014; Markantonatou et al., 2023).

In dermatophyte infections, biofilms can develop on the skin, nails, and other areas, creating a protective environment for the fungi, which leads to several consequences such as heightened resistance, ongoing infections, and evasion of the immune system (Markantonatou et al., 2023). Consequently, the existence of biofilms calls for the formulation of innovative treatment approaches capable of effectively penetrating and disrupting these structures. Conventional antifungal therapies might be less effective against infections associated with biofilms. As a result, recent research is concentrating on discovering and developing new anti-fungal agents and/or combination therapies specifically targeting the biofilms.

While much of the research is dedicated to finding new treatments for resistant fungi, it's equally vital to examine how existing cosmetics influence biofilm formation. Cosmetics, which are applied to the skin's surface, are readily accessible as over-the-counter products. There are many types of cosmetics on the market that have moisturizing, protective, and caring effects (Halla et al., 2018), and create an environmental stress to fungi. Therefore, the addition of active cosmetic components like essential oils into topical products can present a viable approach for controlling dermatophyte infections. Their potential natural antifungal characteristics, along with their capacity to break down biofilms, render them valuable options for creating innovative therapies for dermatophytosis.

Recent studies highlight the importance of exploring alternative approaches, such as integrating antifungal agents into cosmetic formulations, to

address dermatophyte biofilms. These formulations may act as adjunct therapies, particularly for superficial infections, offering both antifungal activity and biofilm disruption (Halla et al., 2018). It has been established that several natural active compounds, especially essential oils, used in cosmetic formulations demonstrate encouraging antifungal effects against dermatophytes (Dall'oglio et al., 2015). The antifungal properties of these substances are linked to their capability to disrupt the fungal cell membrane and interfere with the production of vital cellular components. Additionally, UV filters such as benzophenone derivatives are recognized for their antimicrobial properties, possibly resulting from the generation of reactive oxygen species (ROS) when exposed to UV light. Some research indicates antifungal activity against organisms like *Candida albicans*, implying that these compounds may also hinder the growth of dermatophytes and the formation of biofilms (Hanson et al., 2006; Wang et al., 2013). This study aims to explore the antifungal and antibiofilm efficacy of certain raw cosmetic materials as a potential alternative or complementary strategy for treating dermatophyte infections.

MATERIALS AND METHODS

Microorganisms: A total of 11 clinical fungal isolates consisting of five *M. canis*, four *T. rubrum*, one *Trichophyton tonsurans*, and one *Trichophyton mentagrophytes*, obtained from specimens submitted to the Mycology Laboratories of Prof. Dr. Süleyman Yalçın City Hospital between January-March 2022. Identifications of all isolates were made by MALDI-TOF analysis. *T. rubrum* ATCC 28188 was used as a standard strain.

Tested substances: Natural products such as tea tree oil (TTO), *Hypericum perforatum* L. oil, *Centella asiatica* extract; UV filters in sunscreens such as benzophenone-4, octocrylene, ethylhexyl methoxycinnamate, diethylamino hydroxybenzoyl hexyl benzoate (DHHB); and glycerin, panthenol, collagen, hyaluronic acid, zinc oxide, vitamin A (VITA), and also standard antifungal itraconazole were

kindly provided by their respective manufacturers. Solid substances (e.g., benzophenone-4, *C. asiatica* extract, and VITA) were accurately weighed using a precision balance and dissolved in solvents suitable for their solubility properties following the manufacturer's recommendations. The solvents other than water were ethanol for *C. asiatica* extract, and DMSO for DHHB, ethylhexyl methoxycinnamate, *Hypericum perforatum* oil, octocrylene, TTO, and zinc oxide. All solutions were freshly prepared daily, and the concentrations were expressed as µg/mL for solid substances and µL/mL for liquids, consistent with standard practices in antifungal testing. To assess the potential antifungal effects of the solvents, solvent controls were also included in the experiments.

Media: Roswell Park Memorial Institute (RPMI)-tt1640 medium buffered with morpholine propane sulfonic acid (MOPS, Sigma-Aldrich), and adjusted to pH 7.0, was used for minimum inhibitory concentration (MIC) determinations and biofilm formations. Sabouraud Dextrose Agar (SDA) was used as a solid medium for mold growth and colony counts. Resazurin dye (0.1 mg/mL) was used for examining the growth of microorganisms.

Minimum inhibitory concentrations (MICs): For determining the antifungal activities of cosmetic substances, the Minimum Inhibitory Concentrations (MICs) against 11 fungal isolates were determined by the microbroth dilution technique according to the Clinical and Laboratory Standards Institute (CLSI) document M38, with minor modifications (CLSI, 2017). Two-fold serial antifungal dilutions in RPMI-1640 medium ranging from 5000 to 5 µg/mL for solid substances and 500 to 0.4 µL/mL for liquids were tested in a 96-well, U-shaped microplates. Each well was inoculated with fungi suspension diluted to give a final concentration of 5×10^3 cfu/mL, the trays were covered and placed in plastic bags to prevent evaporation and incubated at 30°C for 3-7 days. At the end of the incubation, 25 µL of resazurin dye was added to the wells, and after 1-3 h incubation, purple-colored wells were evaluated as growth

negative and pink colored wells as positive. The lowest concentration that inhibited the visible growth was determined as the MIC value. *T. rubrum* ATCC 28188 and itraconazole diluted from 64 to 0.06 µg/mL were used as a standard strain and standard antifungal, respectively.

Biofilm formation: A total of 11 fungal isolates were cultured on SDA medium, after incubation at 30°C for 7-14 days, 5 mL physiologically buffered saline (PBS) solution was added to the colonies, and molds were collected. The resulting suspension was added to 24-well flat-bottom cell-culture microplates to give a final concentration of 1×10^6 cfu/500 µL in RPMI-1640 medium and incubated at 30°C for 7 days. After the incubation, the medium was aspirated, and the wells were washed three times with 500 µL PBS to remove unattached fungi. To ensure the fixation of biofilms, 500 µL of 99% methanol was added to each well for 15 minutes, then methanol was aspirated, and plates were left to dry. 500 µL of 0.1% crystal violet dissolved in water was added to the wells, left for 5 minutes to stain, then the excess stain was aspirated, the wells were washed with tap water, and air dried. For re-solubility of the stain, 500 µL 95% ethanol was added, and the plates were incubated on an orbital shaker for 30 min. Then, the optical density was measured at 595 nm (BioTek EON ELISA reader). RPMI-1640 medium was used as a negative control, and *T. rubrum* ATCC 28188 standard strain was used as a positive control (Costa-Orlandi et al., 2014).

Determination of biofilm inhibition: The antibiofilm activities of cosmetic substances were tested against mature dermatophyte biofilms (Costa-Orlandi et al., 2014; Dos Santos & Dias-Souza, 2017) for the first time, the characteristics of *Trichophyton rubrum* and *T. mentagrophytes* biofilms. Biofilm formation was analyzed by light microscopy, scanning electron microscopy (SEM). According to the MIC results, 200 µL solutions of each active cosmetic ingredients were prepared in RPMI-1640 medium at a concentration of 10,000 µg/mL for solid substances and 1000 µL/mL for liquids and were added to the mature biofilms (described as above) in a 96-well, flat-

bottom tissue culture microtiter plates. After 7 days incubation at 30°C, the plates were washed twice with 200 µL PBS and placed in an ultrasonic water bath for 15 minutes to disrupt the biofilms. Then, the plates were stirred on a microplate vortex for 10 minutes, and 20 µL samples from each well, directly or after subsequent 1/10 dilutions, were plated on SDA. After the 3-7 days of incubation at 30°C, the colonies were counted. RPMI-1640 medium was used as a negative control, and *T. rubrum* ATCC 28188 standard strain was used as a positive control.

Statistical analysis: A bootstrap analysis was employed to estimate the mean $\log_{10}$ cfu/mL values and 95% confidence intervals (CI) for each cosmetic ingredient's antimicrobial effect against dermatophytes. This resampling strategy was chosen to address the impediments related to small test sizes, guaranteeing the vigor and unwavering quality of the factual results. A total of 1000 bootstrap emphases were performed for each group, and the confidence intervals were calculated at a 95% certainty level (Efron & Tibshirani, 1994). All experiments were performed duplicate, and in cases of any inconsistent results, a third replicate was conducted to confirm the findings.

RESULTS

Minimum inhibitory concentrations (MICs): According to the microbroth dilution test results, which were summarized in Table 1, the MIC ranges of cosmetic substances against fungal isolates were 4–250 µL/mL for TTO and glycerine, 4–62.5 µL/mL for VITA, followed by 4–500 µL/mL for octocrylene and panthenol. Benzophenone-4 showed moderate MIC activity with 256–5000 µg/mL, and the UV-filter DHHB was selectively active against *M. canis* with 8–256 µg/mL. Itraconazole was used as a standard antifungal for the standardization of the study, and its MIC values against *T. rubrum* ATCC 28188 standard strain were obtained within the quality control limits of CLSI throughout the study. Also, the solvent control results confirmed that the solvents at the highest concentrations, which used in the antifungal activity assays, exhibited no antifungal effect.

Table 1. Minimum inhibitor concentrations of cosmetic ingredients against dermatophytes ($\mu\text{L/mL}/\mu\text{g/mL}$)

	<i>M. canis</i> (n:5)	<i>T. rubrum</i> (n:4)	<i>T. mentagrophytes</i> (n:1)	<i>T. tonsurans</i> (n:1)
BEN	256-2500	1250-2500	625	5000
CEA	1250-5000	1250-2500	2500	5000
COL	-	-	-	-
DHNB	8-256	1250-2500	2500	5000
EMC	-	-	-	-
GLI	4-250	31-156	125	15.6
HPO	-	-	-	-
HYA	-	-	-	-
OCT	4-62.5	15-500	125	15.6
PAN	4-250	31-156	500	15.6
TTO	4-250	62.5-156	62.5	7.8
VITA	4-62.5	15-500	250	4
ZNC	-	-	-	-
ITR	0.125-1	0.125-0.5	0.03	1

BEN: Benzophenone-4, **CEA:** *Centella asiatica* extract, **COL:** Collagen, **DHNB:** Diethylamino hydroxybenzoyl hexyl benzoate, **EMC:** Ethylhexyl methoxycinnamate, **GLI:** Glycerine, **HPO:** *Hypericum perforatum* L. Oil, **HYA:** Hyaluronic acid, **OCT:** Octocrylene, **PAN:** Panthenol, **TTO:** Tea Tree Oil, **VITA:** Vitamin A, **ZNC:** Zinc oxide, “-”: No activity”

Biofilm formation: As shown in Table 2, among the total 11 dermatophyte isolates, four *M. canis* were strong biofilm formers, one *M. canis* and two *T. rubrum* isolates were moderate, and the others were weak biofilm formers.

Table 2. Biofilm-forming capacities of the isolates

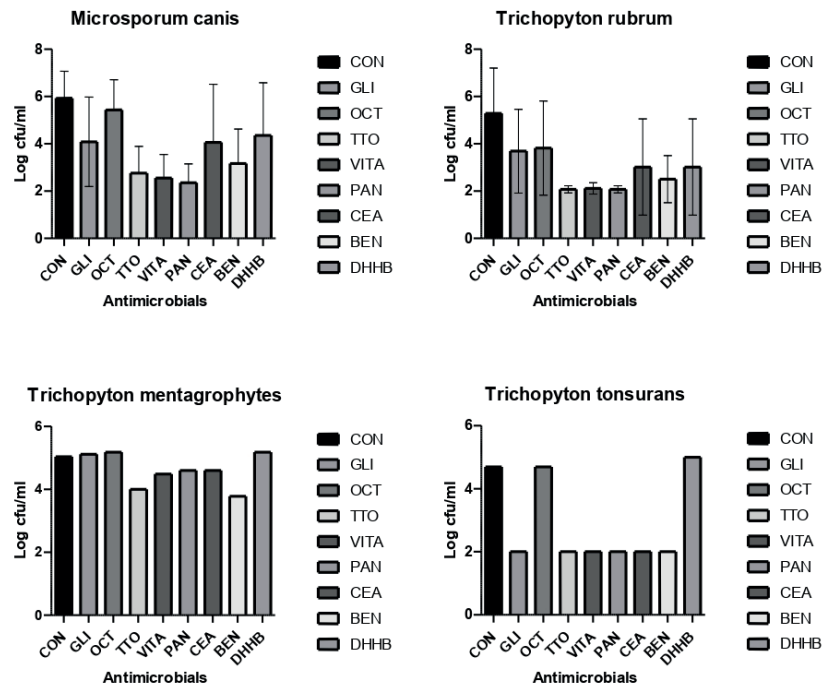
Dermatophyte isolates	Number of biofilm-forming isolates		
	Strong	Moderate	Weak
<i>Microsporum canis</i> (n:5)	4	1	-
<i>Trichophyton mentagrophytes</i> (n:1)	-	-	1
<i>Trichophyton rubrum</i> (n:4)	-	2	2
<i>Trichophyton tonsurans</i> (n:1)	-	-	1

Determination of antibiofilm activities: Against the mature biofilms of 11 dermatophyte isolates, the antibiofilm activities of cosmetic ingredients were summarized in Table 3. According to these results, as shown in Figure 1, cosmetic substances inhibited the mature biofilms up to 3-log_{10} cfu/mL. Especially TTO, VITA, panthenol, *C. asiatica* extract, and benzophenone-4 have strong antibiofilm activities.

Table 3. Antibiofilm activities of cosmetic substances

Cosmetic ingredients	Number of isolates showing the following log ₁₀ CFU/mL decrease at the 24 th hour											
	<i>M. canis</i> (n:5)			<i>T. mentagrophytes</i> (n:1)			<i>T. rubrum</i> (n:4)			<i>T. tonsurans</i> (n:1)		
	≥1	≥2	≥3	≥1	≥2	≥3	≥1	≥2	≥3	≥1	≥2	≥3
BEN	0	3	2	1	0	0	0	0	3	0	0	1
CEA	1	1	2	0	0	0	0	0	2	0	0	1
DHNB	1	1	1	0	0	0	0	0	2	0	0	0
GLI	1	1	2	0	0	0	2	0	1	0	0	1
OCT	2	1	0	0	0	0	1	0	1	0	0	0
PAN	0	1	4	0	0	0	0	0	3	0	0	1
TTO	0	2	3	1	0	0	0	0	3	0	0	1
VITA	0	2	3	0	0	0	0	0	3	0	0	1

BEN: Benzophenone-4, CEA: *Centella asiatica*, DHNB: Diethylamino hydroxybenzoyl hexyl benzoate, GLI: Glycerine, OCT: Octocrylene, PAN: Panthenol, TTO: Tea Tree Oil, VITA: Vitamin A.

**Figure 1.** Effects of cosmetic ingredients against the mature biofilms of dermatophytes.

CON: Control, GLI: Glycerine, OCT: Octocrylene, TTO: Tea Tree Oil, VITA: Vitamin A, PAN: Panthenol, CEA: *Centella asiatica*, BEN: Benzophenone-4, DHNB: diethylamino hydroxybenzoyl hexyl benzoate. The graph was created using the GraphPad Prism 7.0 program.

The results were expressed as the number of live fungi in biofilms as log₁₀ cfu/mL. Each bar is representative of the mean values of two independent tests, and error bars indicate the standard deviations

between isolates. Control bars indicate the viable cells in mature biofilms without any antimicrobial treatment.

Statistical analysis: Bootstrap analysis was chosen to address the limitations associated with small sample sizes and to enhance the robustness of the results; this method offers significant advantages over traditional parametric methods as it does not rely on parametric assumptions and provides reliable confidence intervals regardless of the data distribution characteristics. Among the tested compounds, TTO and VITA consistently demonstrated the lowest mean $\log_{10}$ cfu/mL values across all dermatophyte species, indicating strong antifungal and antibiofilm activities. For *M. canis*, TTO and VITA had mean $\log$ cfu/mL values of 2.0 (95% CI: 2.0–2.0). Similarly, TTO and VITA showed superior performance against *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans*, with mean $\log$ cfu/mL values of 2.5 (95% CI: 2.5–2.5) and 3.0 (95% CI: 3.0–3.0), respectively.

In contrast, compounds such as benzophenone-4, DHHB, and *C. asiatica* extract exhibited higher mean $\log_{10}$ cfu/mL values, ranging from 5.5 to 7.0, suggesting limited antifungal efficacy. The narrow confidence intervals across all tested groups highlight the robustness of the results, despite the small sample size.

DISCUSSION

Dermatophyte infections are common fungal infections that are not life-threatening but decrease the quality of life because of their persistent behaviors, and they become particularly challenging to treat due to the formation of biofilms. These biofilms confer increased resistance to antifungal agents and contribute to the persistence and recurrence of infections. In clinics, generally oral or topical antifungals are used alone or in combination for the treatment of dermatophytosis, but studies show that the biofilm-forming makes it complicated, especially in nail infections (Fanning & Mitchell, 2012). For example, Danielli et al. demonstrated the biofilm-forming properties of *M. canis* isolates (Danielli et al., 2017). In another study, Costa-Orlandi et al. reported that *T. rubrum* isolates have more biofilm-forming

capacities than *T. mentagrophytes* (Costa-Orlandi et al., 2014). In this study, when we perform the biofilm formation assay for 11 clinical dermatophyte isolates, as shown in Table 2, especially *M. canis* isolates (4/5) were strong biofilm formers, while half of the *T. rubrum* isolates (2/4) formed moderate biofilms, and all others formed weak biofilms. These results are similar to other studies.

While research on antifungal agents that disrupt biofilms shows promise for treating infections, this study focused on investigating the antifungal and antibiofilm effects of cosmetic raw materials against clinical dermatophyte isolates. For this purpose, *H. perforatum* oil, TTO, *C. asiatica* extract, collagen, hyaluronic acid, VITA, and different UV filter' antifungal activities were examined against dermatophyte isolates. As shown in Table 1, while some raw materials have no antifungal activity, especially VITA, glycerin and octocrylene were active against *M. canis*, and panthenol and TTO against *T. rubrum* isolates, according to the MIC values. Also, TTO and benzophenone-4 were the most active agents against *T. mentagrophytes*, and VITA for *T. tonsurans*. It's thought that the differences might be a result of the fungal species differences and their clinical antifungal resistance properties.

Among the studied cosmetic ingredients, the substances that have antifungal activity against dermatophytes were used for the antibiofilm assays. Because of the biofilms' extracellular matrix provides protection for dermatophytes under undesirable conditions, antifungals and immune mechanisms (Pereira, 2021), the frequently used cosmetic raw materials might be an alternative treatment agent alone or in combination with antifungals. In this study, although the antibiofilm activities of these raw materials against dermatophyte biofilms vary depending on the substances and strain, especially TTO, VITA, panthenol, and benzophenone-4 can cause a $\geq 2 \log_{10}$ decrease from the survival cell counts, except for *T. mentagrophytes*. Besides them, while the glycerin and *C. asiatica* extracts were active against

biofilms of especially *T. tonsurans*, followed by the *T. rubrum* and *M. canis*, the DHHB had a limited effect against *T. rubrum* or *M. canis*, and octocrylene was generally not effective, except for *T. rubrum*.

TTO is one of the well-known natural antiseptic derived from *Melaleuca alternifolia* which is frequently used in various cosmetic products such as shampoos, massage oils, nail and hand creams, or detergents, and especially used for the treatment of vaginal candidiasis and dermatophytes (Hammer et al., 2002; Larson & Jacob, 2012). TTO contains several active components of monoterpenes and alcohols, such as terpinen-4-ol, which disrupt the fungal cell membrane, leading to increased permeability and leakage of cellular contents, which compromises the structural integrity of the fungal cell, ultimately causing cell death. Also, TTO components can cause oxidative stress in fungal cells by generating ROS, which can damage essential cellular components such as lipids, proteins, and DNA, and further compromise cell viability (Abd El-Salam et al., 2023; Fitriani & Surini, 2022). Additionally, the proliferation of fungal pathogens is effectively curtailed by TTO by disrupting the key enzymatic functions and cellular processes (Upadhyay et al., 2023). For example, Roana et al. showed that TTO has antimicrobial activity against *T. rubrum* isolates either alone or in combination with some antifungals (Roana et al., 2021). Similarly, we showed that TTO has antifungal activities against all studied dermatophytes, and it has strong antibiofilm activities on mature biofilms of *M. canis*, *T. rubrum*, and *T. tonsurans*.

VITAs are a group of unsaturated nutritional organic compounds, including retinol, retinal, and several provitamins, responsible for the biological activities (Beckenbach et al., 2015). Although VITA is included in many preparations that are used for the skin, especially for the treatment of acne as a face cream (Leyden et al., 2017). Besides its cosmetic effects, some studies showed that, it has an antifungal activity against a broad-spectrum of human opportunistic pathogens, for example Campione et al.

showed that retinoid, a product of VITA, is effective in the treatment of nail infections, especially caused by *T. rubrum* and *T. mentagrophytes* (Campione et al., 2015). But the mechanism underlying the inhibition of growth and proliferation of certain fungal species by retinoids remains unclear (Pistoia et al., 2022). In this study, VITA showed strong antifungal and antibiofilm activities against *M. canis*, *T. tonsurans*, and *T. rubrum*.

Panthenol is an active substance that is frequently used in dermatology for its moisturizing, emollient, and barrier properties, which protect the skin, and has some therapeutic properties (Pavlačková et al., 2019). Although panthenol has not been directly studied for its antifungal activity, its role in skin health can indirectly support antifungal treatments. For example, Chohnan et al. found that panthenol effectively inhibits the growth of staphylococci, which can complicate the fungal infections by damaging the skin barrier (Chohnan et al., 2014). Also, some studies have shown that panthenol increases the effectiveness of antiseptics such as chlorhexidine gluconate (Kramer et al., 2016). However, under normal conditions, panthenol is required for the growth of pathogenic microorganisms; in our study, it showed $>2 \log_{10}$ decrease in all dermatophyte biofilms, except for *T. mentagrophytes*. This might be due to the competition for interacting with another vitamin B pathway used by fungi (Saliba et al., 2005).

UV filters such as benzophenone-4, octocrylene, and DHHB are organic chemicals developed to protect the skin from the harmful UV rays of the sun by absorbing those rays and reducing the effects of direct sun exposure (Giokas et al., 2007). Because of their UV ray filtering properties, UV filters are widely used in sunscreens as a barrier that protects skin from the sun, and skin cancer (Silvia Díaz-Cruz et al., 2008). However, the antifungal activity of benzophenone-4 has not been extensively studied; similar benzophenone derivative bicyclic imides were found to have significant antimicrobial activities, including antifungal effects (Fadel & Al-Azzawi,

2022). Octocrylene is also an organic compound used in sunscreens and cosmetics. It is a viscous, oily liquid that is clear and colorless, and it can convert to benzophenone-4 through the retro-aldol condensation. Octocrylene can penetrate the skin as a photosensitizer, resulting in an increased production of free radicals under illumination (Hanson et al., 2006). While the antifungal properties of octocrylene is unclear due to limited direct evidence, in a study some poly (phenylene ethynylene)-based cationic conjugated polyelectrolytes (CPEs) and oligophenylene ethynylenes (OPEs) compounds, which have structural similarities with UV filters such as octocrylene, showed significant antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans*, particularly when exposed to UV radiation (Wang et al., 2013).

To the best of our knowledge, there are no studies about all UV filter' antimicrobial or antifungal properties. In this study, it was found that UV filters benzophenone-4, octocrylene, and DHHB showed good antifungal properties against the studied dermatophytes, especially octocrylene, which has the lowest MIC values, except for *T. mentagrophytes*. According to antibiofilm assays, while benzophenone-4 decreases the survival of cells in biofilms by more than $\geq 2 \text{ Log}_{10}$, except for *T. mentagrophytes*, octocrylene and DHHB could only decrease the *M. canis* and *T. rubrum* biofilms by 1-2 Log_{10} .

Glycerin is a substance that is frequently used in many medical products as a therapeutic or in personal care/cosmetic products due to its fragrance, hair moisturizer feature, humectant, oral hygiene product, and skin protective properties (Becker et al., 2019). Especially its hygroscopic action causes it to extract water from the bacteria and fungi, hence preventing them from proliferation (Linser, 2002). It is also known that some substances obtained from glycerin have antibacterial and antifungal properties (Len et al., 1996). *C. asiatica* L., also known as *Gotu Kola*, is a plant used especially in South Asia,

showing antidiabetic, antioxidant, and wound healing properties (Chandrika & Prasad Kumarab, 2015), and its extracts are effective antimicrobial agents against fungi such as *Aspergillus niger* and *C. albicans* (Wong & Ramli, 2021). In this study, similar to other studies, glycerin and *C. asiatica* extract showed strong antifungal activity on a weight basis, against all studied dermatophytes, but they could only decrease the *T. tonsurans* biofilms by more than $\geq 2 \text{ Log}_{10}$. Against other biofilms, they have limited antibiofilm activities except for *T. mentagrophytes*.

The practical applicability of our findings may be impacted by the differences in the concentrations of cosmetic ingredients assessed in our antibiofilm assays compared with the usual levels in cosmetic formulations. For example, TTO and Panthenol were tested at 1000 $\mu\text{L/mL}$, in the 0.1% to 2% (1000 to 20,000 $\mu\text{g/mL}$) and 0.1% to 5% (1000 to 50,000 $\mu\text{g/mL}$) ranges, respectively, which are commonly used in skin care products for their antifungal properties (Carson et al., 2006; Ebner et al., 2002; Enshaieh et al., 2007). Considering the densities of TTO and Panthenol, it is thought that although it is more advantageous for TTO, these effective values remain within the usage limits for both substances. Similarly, glycerin is typically incorporated at 2% to 10% (20,000 to 100,000 $\mu\text{g/mL}$) as a humectant, and was assessed at 1000 $\mu\text{L/mL}$, within the safety limits (Fluhr et al., 2008). Unfortunately, VITA was also tested at 1000 $\mu\text{L/mL}$, exceeding the safer 0.01% to 0.1% (100 to 1000 $\mu\text{g/mL}$) range used in anti-aging and acne treatments (Mukherjee et al., 2006), which may limit its practical utility despite its potent antibiofilm activity. Benzophenone-4 tested at 10,000 $\mu\text{g/mL}$, align reasonably with their typical cosmetic ranges of 0.1% to 5% (1000 to 50,000 $\mu\text{g/mL}$) (Santos et al., 2012), supporting the relevance of their antibiofilm effects in formulations. Lastly, octocrylene and DHHB, tested at 10,000 $\mu\text{g/mL}$, are used in sunscreens at 1% to 10% (10,000 to 100,000 $\mu\text{g/mL}$) (Santos et al., 2012), hinting that their limited activity might improve at higher concentrations. These findings underscore

the importance of balancing efficacy and safety when adapting our results for cosmetic applications, with future studies needed to test concentrations more reflective of industry standards.

The results obtained through bootstrap analysis confirm the significant antifungal potential of TTO and VITA against dermatophyte biofilms. These findings align with previous studies reporting the potent antifungal properties of TTO and VITA. The low log cfu/mL values and narrow CIs suggest that these compounds could be promising candidates for developing innovative antifungal formulations. However, compounds like benzophenone-4 and *C. asiatica* extract demonstrated limited efficacy, emphasizing the need for further optimization or combination strategies to enhance their antifungal effects. Bootstrap analysis also validated the reliability of the findings, providing robust statistical support despite the inherent limitations of small sample size experiments.

The findings of this study suggested that incorporating cosmetic ingredients like TTO, VITA, and panthenol into topical formulations could provide a supplementary approach to managing dermatophyte infections. These products may serve as standalone options for mild infections or as adjunctive treatments alongside conventional antifungals to target biofilm-associated resistance. Clinical trials are essential to establish optimal concentrations, assess safety profiles, and confirm their efficacy in real-world applications (Campione et al., 2015; Halla et al., 2018). However, because the antibiofilm assays employed very high concentrations (1000 µL/mL or 10.000 µg/mL), direct translation of these levels into topical formulations could pose practical challenges—both in terms of skin tolerance (irritation, sensitization) and formulation stability (viscosity changes, emulsion breakdown).

As an overall result of the study, the antifungal and antibiofilm properties of TTO, VITA, panthenol, and benzophenone-4 were extremely high, followed by

the *C. asiatica* extract and glycerin against *M. canis*, *T. rubrum*, and *T. tonsurans* isolates. On the other hand, *T. mentagrophytes* was recorded as the most resistant isolate in this study. According to these results, we think that the substances used in cosmetic products could be effective in the treatment of persistent fungal infections and fungal biofilms on the skin. However, further research and clinical trials are essential to validate efficacy in clinical settings, evaluate the long-term safety to optimize their use, and establish standardized guidelines for their application in dermatology. Understanding the antifungal effects of cosmetic active ingredients can lead to innovative solutions for treating dermatophyte infections. Such studies can bridge the gap between cosmetic science and medical mycology, providing new avenues for both preventive and therapeutic applications.

This study is limited by its *in vitro* design, which may not fully replicate the complex environment of clinical infections. Also, one of the limitations of this study is the relatively small number of clinical isolates tested (n=11). These isolates were collected from specimens submitted to the Mycology Laboratories of Prof. Dr. Süleyman Yalçın City Hospital between January and March 2022. The primary reason for the limited number of isolates was the reduced patient admissions during this period, attributed to the ongoing COVID-19 pandemic, which significantly impacted healthcare service utilization (Moynihan et al., 2021). Consequently, the generalizability of the findings may be constrained, and further studies with larger and more diverse isolate sets are needed to validate the antifungal and anti-biofilm activities of the tested cosmetic ingredients. Further research is required to evaluate the long-term safety and efficacy of them through controlled *in vivo* and clinical trials.

AUTHOR CONTRIBUTION STATEMENT

SD and CÖ jointly developed the hypothesis for the study. CÖ conducted the experimental work. The manuscript was prepared collaboratively by both

authors, with shared responsibility for reviewing and finalizing the text.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Binding Behavior of Anticancer Agent Trametinib Across Different Mammalian Serum Albumins: A Comparative Perspective

Cem ERKMEN^{*,**}

Binding Behavior of Anticancer Agent Trametinib Across Different Mammalian Serum Albumins: A Comparative Perspective

Antikanser Ajan Trametinib'in Farklı Memeli Serum Albüminlerine Bağlanma Davranışı: Karşılaştırmalı Bir Bakış

SUMMARY

Understanding the binding interactions between drugs and serum albumins is crucial for evaluating pharmacokinetics, bioavailability, and species-specific therapeutic responses. In this study, the binding behavior of the anticancer agent trametinib (TMB) using serum albumins derived from four distinct mammals, namely human (HSA), bovine (BSA), goat (GSA), and sheep (SSA)—was investigated using steady-state fluorescence spectroscopy. TMB caused a concentration-dependent quenching of intrinsic albumin fluorescence, indicating complex formation. Analysis of Stern–Volmer and double logarithmic plots revealed that the quenching mechanism was predominantly static in nature, as supported by high bimolecular quenching rate constants ($k_q \sim 10^{12} \text{ M}^{-1} \text{ s}^{-1}$), which exceeded the diffusion-controlled limit. Binding parameters, including the association constant (K_d) and binding stoichiometry (n), were calculated, with all albumins exhibiting a 1:1 binding ratio. Among the studied proteins, BSA displayed the strongest affinity for TMB, followed by GSA, HSA, and SSA. Thermodynamic evaluation via Gibbs free energy change (ΔG°) confirmed the spontaneity of the interaction, with ΔG° values ranging from -25.2 ± 0.10 to $-28.9 \pm 0.10 \text{ kJ mol}^{-1}$. These results not only provide molecular-level insight into TMB–albumin interactions but also support the rational selection of animal models in preclinical pharmacological studies based on their albumin binding profiles.

Keywords: Trametinib, Serum albumin, Fluorescence spectroscopy, Protein–ligand interaction, Pharmacokinetics.

ÖZ

İlaçların serum albüminleriyle olan bağlanma etkileşimlerinin anlaşılması, farmakokinetik, biyoyararlanım ve türlere özgü terapötik yanıtların değerlendirilmesi açısından büyük önem taşımaktadır. Bu çalışmada, antikanser ajan trametinib'in (TMB) insan (HSA), sığır (BSA), keçi (GSA) ve koyun (SSA) olmak üzere dört farklı memeli türüne ait serum albüminleriyle olan bağlanma davranışı, kararlı durum floresans spektroskopisi kullanılarak araştırılmıştır. TMB, albüminlerin içsel floresansında konsantrasyona bağlı olarak bir sönmülemeye neden olmuş ve bu durum kompleks oluşumunu işaret etmiştir. Stern–Volmer ve çift logaritmik grafiklerin analizi, sönmüleme mekanizmasının ağırlıklı olarak statik karakterde olduğunu göstermiştir; bu durum, difüzyon kontrollü sınırın üzerinde olan yüksek biyomoleküler sönmüleme hız sabitleriyle ($k_q \sim 10^{12} \text{ M}^{-1} \text{ s}^{-1}$) desteklenmiştir. Bağlanma parametreleri, yani bağlanma sabiti (K_d) ve stokiyoometri (n) hesaplanmış; tüm albüminlerin 1:1 bağlanma oranı sergilediği bulunmuştur. İncelenen proteinler arasında BSA, TMB'ye en yüksek afinitiyi göstermiştir; bunu sırasıyla GSA, HSA ve SSA takip etmiştir. Gibbs serbest enerji değişimi (ΔG°) üzerinden yapılan termodinamik değerlendirme, bağlanma etkileşimlerinin kendiliğinden gerçekleştiğini doğrulamış ve ΔG° değerlerinin -25.2 ± 0.10 ile $-28.9 \pm 0.10 \text{ kJ mol}^{-1}$ arasında değiştiği gösterilmiştir. Bu sonuçlar, TMB–albümin etkileşimlerine moleküler düzeyde ışık tutmakla kalmayıp, prelinik farmakolojik çalışmalarda albümin bağlanma profillerine dayalı olarak uygun hayvan modelinin rasyonel seçimini de desteklemektedir.

Anahtar Kelimeler: Trametinib, Serum albümin, Floresans spektroskopisi, Protein–ligand etkileşimi, Farmakokinetik.

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* ORCID: 0000-0001-5944-3912, Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul Aydın University, Istanbul, Türkiye.
** ORCID: 0000-0001-5944-3912, Application and Research Center for Advanced Studies, Istanbul Aydın University, Istanbul, Türkiye.

INTRODUCTION

Trametinib (TMB; Figure 1) is a targeted anticancer agent belonging to the class of mitogen-activated protein kinase kinase (MEK) inhibitors. TMB has gained significant importance in oncology, particularly for the treatment of cancers with B-Raf proto-oncogene (BRAF), serine/threonine kinase V600E (Valine

→ Glutamic acid) or V600K (Valine → Lysine) mutations, most notably metastatic melanoma. Approved by the FDA in 2013, TMB is often used as monotherapy or in combination with BRAF inhibitors such as dabrafenib to enhance therapeutic efficacy and overcome resistance mechanisms (Corcoran et al., 2015; Infante et al., 2013; Odogwu et al., 2018).

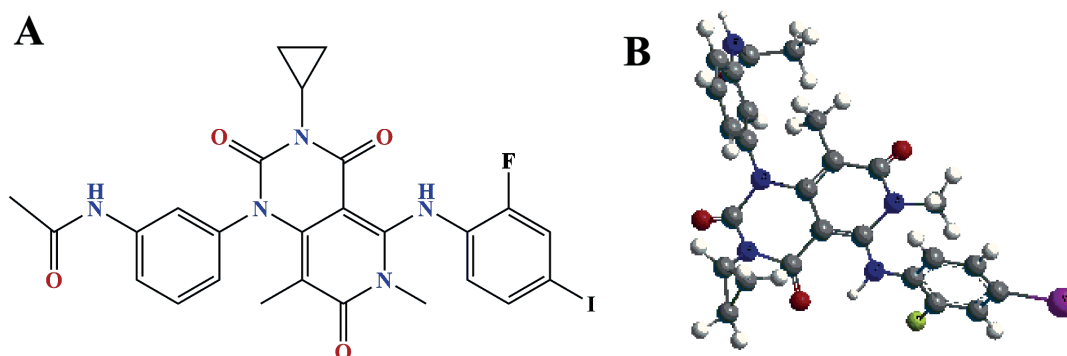


Figure 1. 2D (A) and 3D (B) chemical structures of TMB.

Pharmacologically, TMB selectively inhibits MEK1 and MEK2, critical kinases in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway. This pathway regulates cellular proliferation, differentiation, and survival. By targeting MEK, TMB effectively reduces the downstream activation of ERK, thereby inhibiting tumor cell proliferation and promoting apoptosis (Blumenschein et al., 2015; Caunt et al., 2015). TMB is administered orally, with a bioavailability of approximately 72% and a relatively long half-life of 3–4 days, allowing once-daily dosing. It is metabolized primarily via deacetylation and is not extensively processed by cytochrome P450 enzymes, which reduces the likelihood of certain drug–drug interactions (Lugowska et al., 2015; Marani et al., 2023; Tan et al., 2023).

Clinically, TMB has demonstrated improved progression-free and overall survival in patients with advanced melanoma. However, its pharmacokinetic profile, including distribution and bioavailability, may be influenced by binding interactions with serum albumins, which play a crucial role in drug transport and bioavailability (Voon et al., 2022). Understand-

ing TMB's binding affinity to serum albumins can provide valuable insights into its therapeutic efficacy, toxicity, and potential drug–drug interactions, highlighting the importance of albumin-binding studies in the context of personalized medicine and targeted therapy. Therefore, examining drug–albumin binding characteristics is essential in preclinical studies. However, interspecies variability in albumin structure and binding behavior may lead to divergent pharmacological outcomes between animals and humans. Consequently, the selection of appropriate animal models that closely mimic human serum albumin binding patterns is crucial for accurately predicting drug behavior and optimizing translational relevance in preclinical research (Feroz et al., 2014).

Elucidating drug–protein interactions requires highly sensitive and specific analytical techniques, and fluorescence spectroscopy has emerged as one of the most powerful tools in this context. Its exceptional sensitivity, rapid response, and non-destructive nature make it particularly well-suited for monitoring subtle molecular changes associated with ligand binding. In the case of serum albumins, this technique

allows precise detection of alterations in intrinsic fluorescence, primarily originating from tryptophan residues, which are highly responsive to microenvironmental shifts upon drug interaction. Beyond its sensitivity, fluorescence spectroscopy also provides valuable quantitative data, such as binding constants and quenching parameters, enabling detailed characterization of drug–protein interactions at the molecular level (Erkmen & Kabir, 2024; Lakowicz, 2006).

In this study, the binding interactions between the anticancer agent TMB and serum albumins from four different mammalian species—human, bovine, goat, and sheep—were examined for the first time in a comparative framework. By examining the binding affinities and interaction profiles across these distinct albumins, this work provides valuable insights into species-specific pharmacological behaviors. Therefore, the results generated through this investigation may contribute to the rational selection or design of animal models that closely mimic the biological conditions regarding drug–protein interactions, thereby supporting more predictive preclinical evaluations in drug development.

MATERIALS AND METHODS

Chemicals

Fatty acid-free albumins sourced from human (HSA), bovine (BSA), goat (GSA), and sheep (SSA) origins, together with the anticancer agent trametinib, were procured from Sigma-Aldrich and utilized directly without undergoing any additional purification steps. All other chemicals and solvents applied throughout the study were of analytical grade and employed as received.

Preparation of solutions

The proteins were initially dissolved in a 60 mM phosphate buffer solution (PBS, pH 7.4) to prepare stock solutions. The concentrations of HSA, BSA, and SSA were determined spectrophotometrically at 280 nm using their respective molar extinction coefficients: 35,700 M⁻¹ cm⁻¹ (HSA), 43,827 M⁻¹ cm⁻¹ (BSA), and 42,925 M⁻¹ cm⁻¹ (SSA) (Khan, Chaturve-

di & Khan, 2013). For GSA, which lacked established extinction coefficients, protein concentrations were estimated using the colorimetric method described by Lowry et al. (Lowry et al., 1951).

TMB stock solution was prepared by dissolving an appropriate amount of the compound in 1 mL of dimethyl sulfoxide (DMSO), followed by dilution with 60 mM PBS (pH 7.4) to a final volume of 10 mL. The prepared solution was stored in the dark at 4°C until use to maintain stability. All absorbance measurements were carried out using a Shimadzu model UV-2600 double-beam spectrophotometer with 1 cm path length quartz cuvettes.

Analytical procedures for the TMB–albumin interaction

The interaction of TMB with serum albumins derived from multiple mammalian species was examined through fluorescence quenching-based titration, following a previously established protocol (Duman et al., 2023; Tayyab et al., 2019). Varying concentrations of TMB (1.5–15 µM) were incrementally added to a constant concentration (3 µM) of albumin in a final volume of 3 mL, prepared using 60 mM PBS (pH 7.4). The mixtures were incubated at 25 °C for 1 hour to allow equilibrium binding. Fluorescence spectra were recorded using a JASCO FP-8300 spectrofluorometer equipped with a 1 cm path length quartz cuvette. The excitation wavelength was set at 280 nm, and emission was scanned in the range of 300–420 nm.

Fluorescence data processing and binding analysis

To account for inner filter interference, fluorescence measurements were corrected in accordance with the equation proposed by Lakowicz (Lakowicz, 2006):

$$F_{cor} = F_{obs} 10^{(A_{ex} + A_{em})/2} \quad (1)$$

In this equation, F_{cor} corresponds to the fluorescence value corrected for inner filter effects, whereas F_{obs} is the observed intensity. The absorbance at excitation (A_{ex}) and emission (A_{em}) wavelengths is incorporated to compensate for potential ligand-induced

spectral distortions.

Subsequent analysis of the adjusted fluorescence values was carried out through the Stern–Volmer approach, enabling calculation of the K_{SV} constant and clarification of the quenching type involved:

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (2)$$

Here, the fluorescence signals recorded in the absence (F_0) and presence (F) of TMB, the quencher, are denoted as such; $[Q]$ indicates the concentration of this quencher; k_q is the bimolecular quenching rate constant, and τ_0 is the average lifetime of the fluorophore in the absence of the TMB, assumed to be 10^{-8} s for serum albumins (Lakowicz, 2006).

To determine the binding parameters, the modified Stern–Volmer equation was applied to calculate the association constant (K_a) and the number of binding sites (n):

$$\log(F_0 - F) / (F_0 - F) = n \log K_a - n \log [(1 - (F_0 - F) / [P_T]) / (F_0 - F)] \quad (3)$$

Where $[L_T]$ and $[P_T]$ denote the total concentrations of the ligand and protein, respectively.

Finally, the standard Gibbs free energy change (ΔG^0) for the interaction was calculated using the following thermodynamic relationship:

$$\Delta G^0 = -RT \ln K_a \quad (4)$$

Where R is the universal gas constant ($8.3145 \text{ J mol}^{-1} \text{ K}^{-1}$) and T is the absolute temperature in Kelvin.

RESULTS AND DISCUSSION

Fluorescence quenching of serum albumins triggered by TMB

Among various analytical techniques, fluorescence spectroscopy is frequently employed to investigate the binding interactions between macromole-

cules and their ligands, offering valuable insight into the underlying mechanisms. In proteins, particularly serum albumins, intrinsic fluorescence is notably sensitive to structural and environmental changes triggered by the binding of low-molecular-weight compounds. When a small molecule, such as drugs, pesticides, and ions, interacts with these proteins, it often results in a measurable decrease in fluorescence intensity, indicative of quenching phenomena and conformational alterations (Lakowicz, 2006).

Firstly, to evaluate and correct for possible inner filter effects, the UV–Vis absorption spectra of TMB in the presence of different serum albumins were recorded (Figure 2). Figures 2A–D show the spectra for HSA, BSA, GSA, and SSA, respectively, with protein concentration fixed at $3 \mu\text{M}$ and TMB concentration gradually increased from 0 to $15 \mu\text{M}$. In all cases, the absorbance intensity increased progressively with rising TMB concentration, particularly in the range of 260–300 nm, which corresponds to the characteristic absorption bands of TMB. This increase reflects the additive contribution of TMB absorption to the intrinsic absorption of the albumins. Importantly, the absorbance values at both excitation (280 nm) and emission (~ 340 nm) wavelengths were significantly influenced by TMB concentration, confirming the necessity of applying inner filter effect corrections in the fluorescence analyses. Accordingly, all fluorescence intensities were corrected following Lakowicz's method using the measured absorbance values at excitation and emission wavelengths, ensuring accurate determination of quenching and binding parameters (Lakowicz, 2006).

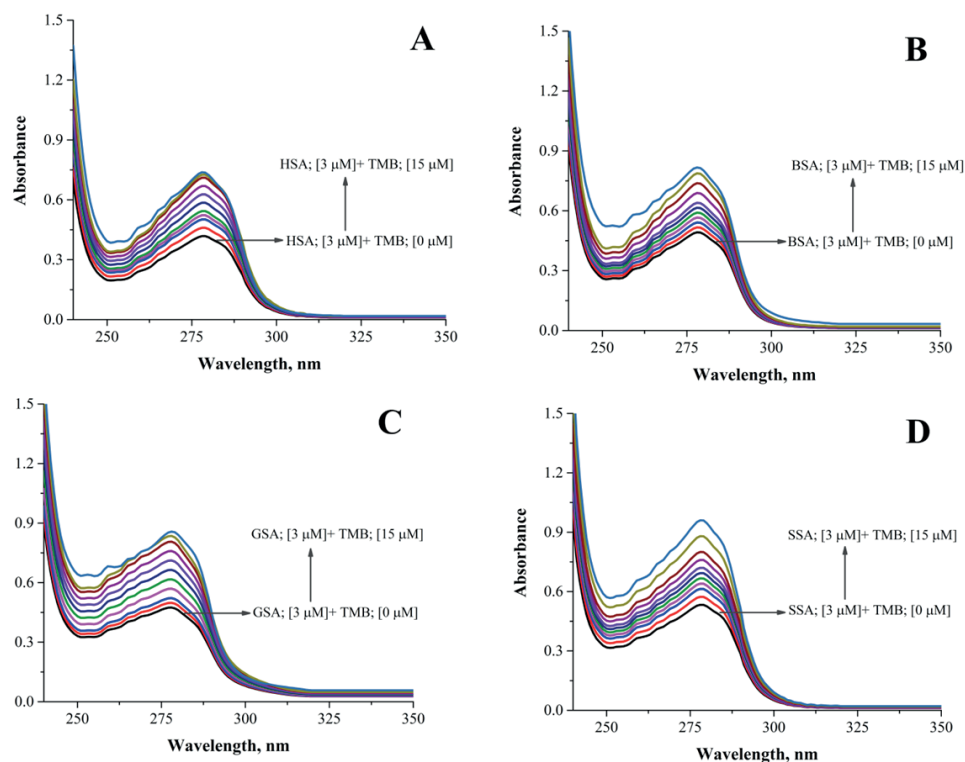


Figure 2. UV-Vis absorption spectra for various serum albumins obtained at increasing TMB levels. Figures correspond to: (A) HSA, (B) BSA, (C) GSA, and (D) SSA.

Figure 3 presents the fluorescence emission profiles of four different mammalian serum albumins (each at a concentration of $3\ \mu\text{M}$): HSA (Figure 3A), BSA (Figure 3B), GSA (Figure 3C), and SSA (Figure 3D). These spectra were obtained both in the absence and the presence of progressively increasing concentrations of TMB, ranging from 1.5 to $15\ \mu\text{M}$. Table 1 summarizes the native fluorescence characteristics of different albumins, including their emission intensities and peak wavelengths. As illustrated, significant differences were observed in the fluorescence intensities of the studied proteins, following the trend: $\text{SSA} > \text{GSA} > \text{BSA} > \text{HSA}$. In contrast, the maximum fluorescence emissions for these proteins were confined to a relatively narrow range between 339 and $342\ \text{nm}$. These findings are consistent with previously reported data regarding the fluorescence behavior of different serum albumins (Boon Kim, Abdul Kadir & Tayyab, 2008; Feroz et al., 2014; Ishtikhar et al., 2018). The intrinsic fluorescence of proteins is predominantly

attributed to their aromatic residues, especially tryptophan and tyrosine, as reported in prior studies (Lakowicz, 2006). Therefore, the observed variations in emission spectra among these albumins are likely influenced by both the quantity and spatial arrangement of tryptophan and/or tyrosine residues within the protein's tertiary structure. Notably, in class B albumins, tryptophan residues are the predominant contributors to the overall fluorescence signal (Lakowicz, 2006). Table 1 provides information regarding the number and sequence positions of tryptophan residues in the primary structures of the examined albumins. Notably, the position of Trp residues appears to be highly conserved across albumins from different species. As illustrated, albumins containing two tryptophan residues—such as BSA, SSA, and GSA—exhibited both stronger fluorescence signals and greater maximum emission wavelengths than those possessing a single tryptophan residue—such as HSA.

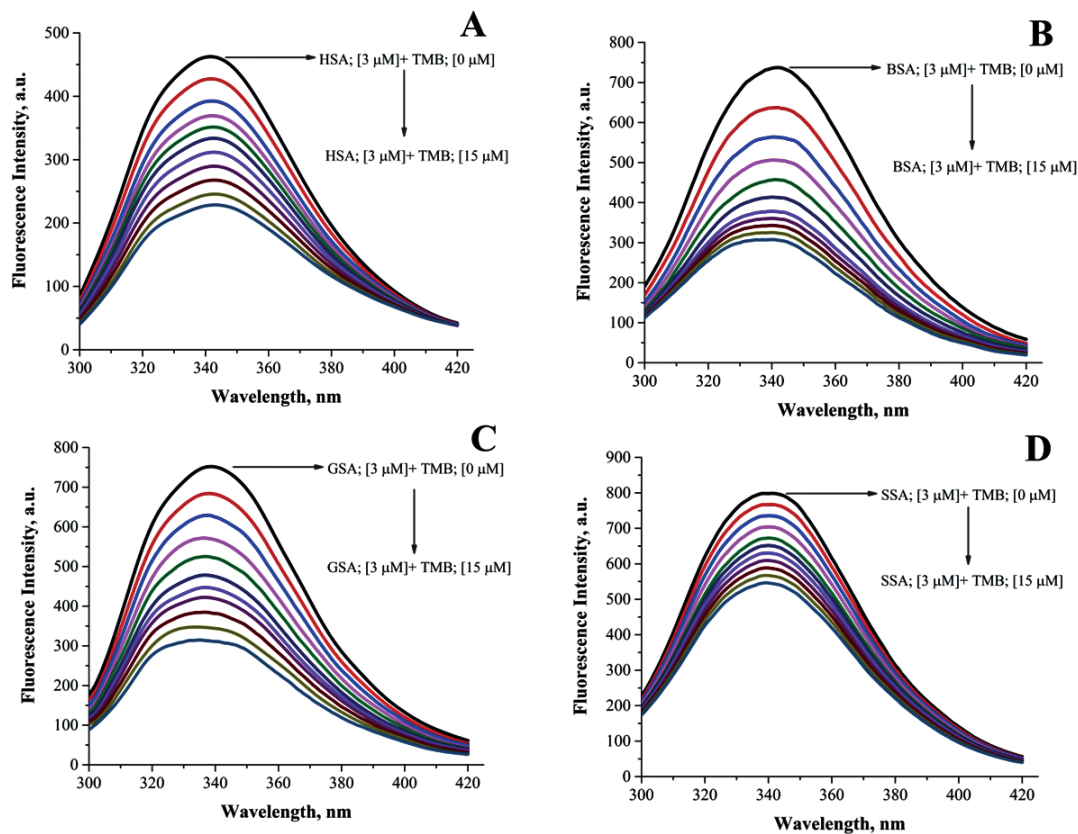


Figure 3. Fluorescence emission profiles for various serum albumins obtained at increasing TMB levels. Figures correspond to: (A) HSA, (B) BSA, (C) GSA, and (D) SSA.

Table 1. Analysis of fluorescence responses of mammalian albumins in both the presence and absence of TMB.

Albumin	Intensity (a.u.)	Emission maximum (nm)	Trp residu ^{a*}	% Quenching
BSA	737.03	341	Trp-134, Trp-213	59
GSA	751.96	339	Trp-134, Trp-213	55
HSA	462.53	342	Trp-214	48
SSA	798.57	341	Trp-134, Trp-213	32

*a**: Obtained from the Univesal Protein Resource (Uniprot) database at www.uniprot.org

Titration of albumin solutions with increasing concentrations of TMB resulted in a marked reduction in the intrinsic fluorescence of all albumin types examined. As shown in Figure 4A, this quenching effect intensified progressively with higher TMB levels. Nevertheless, the extent of fluorescence suppression varied among the different albumins. At a TMB-to-protein molar ratio of 5:1, BSA, GSA, and HSA exhibited the most significant decreases in fluorescence—59%, 55%, and 48% respectively—whereas the remaining

albumin, SSA, showed approximately 32% quenching. The observed decline in fluorescence intensity upon TMB addition strongly supports the occurrence of a ligand–protein interaction (Ladokhin, 2000).

Fluorescence-based investigation of the interaction mechanism between TMB and serum albumins

The reduction of protein fluorescence intensity caused by a ligand (quencher) can occur via different mechanisms, primarily classified as collisional (dynamic), static, or a combination of both (mixed)

quenching (Lakowicz, 2006). Collisional quenching involves transient interactions where the quencher collides with the fluorophore while it is excited, leading to non-radiative energy dissipation. In contrast, static quenching arises from the formation of a non-fluorescent ground-state complex between the ligand and the protein, which prevents the fluorophore from being excited (Braslavsky, 2007). The mixed quenching mechanism involves contributions from both static complex formation and collisional encounters, often characterized by more complex fluorescence decay behavior.

To elucidate the binding mechanism between TMB and serum albumins, fluorescence titration results were analyzed using the Stern–Volmer formalism (Eq. 2). The resulting Stern–Volmer curves for the various TMB–albumin systems are presented in Figure 4B, and the corresponding K_{SV} are summarized in Table 2. Since the slope of the Stern–Volmer plot corresponds to the K_{SV} , it reflects the magnitude of fluorescence quenching. The relatively high K_{SV} values determined for HSA, BSA, and GSA are consistent with the significant decrease in fluorescence values observed for these albumins at a TMB-to-albumin molar ratio of 5:1. Moreover, using the K_{SV} values obtained from the Stern–Volmer analysis (Eq. 2), the bimolecular quenching rate constants (k_q) for the various TMB–albumin systems were calculated and determined to be approximately $10^{12} \text{ M}^{-1} \text{ s}^{-1}$. In general, collisional (dynamic) quenching is characterized by k_q values around $10^{10} \text{ M}^{-1} \text{ s}^{-1}$, which corresponds to the diffusion-controlled limit in aqueous media. When the calculated k_q values exceed this value, it typically suggests the involvement of a static quenching mechanism. Therefore, given that the calculated k_q values for the TMB–albumin systems were on the order of $10^{12} \text{ M}^{-1} \text{ s}^{-1}$ —well above the diffusion-controlled limit—it can be inferred that the quenching mechanism is predominantly static in nature, suggesting complex formation between TMB and the albumin proteins. The occurrence of static quenching in the TMB–HSA system had previously been validated through

temperature-dependent studies, where an inverse relationship between quenching efficiency and temperature was observed (Suo et al., 2018). Our findings are consistent with these observations, as the calculated quenching parameters and the high bimolecular quenching constants further support a static quenching mechanism in the TMB–albumin interactions.

Binding parameters, including the K_a and the number of binding sites (n), for the TMB–albumins interaction were derived from fluorescence quenching results by Eq. 3. The applied analytical approach offers an advantage over traditional fluorescence-based methods, as it does not require prior assumptions about the concentrations of free or bound ligand species (Sahoo, Hennig & Nau, 2006). Figure 4C presents the linearized double-logarithmic curves corresponding to the different TMB–albumin complexes, from which the K_a and n were determined. The corresponding numerical results are summarized in Table 2. Considering the determined K_a values, the albumins examined in this study can be grouped into two categories: GSA, BSA, and HSA exhibited comparatively stronger binding affinities for TMB, while the remaining protein, SSA, demonstrated weaker interaction. Among them, BSA displayed the highest affinity toward TMB, whereas SSA showed the lowest binding strength.

The comparative analysis revealed clear differences in the binding affinity of TMB toward the four albumins, following the order BSA>GSA>HSA>SSA. These variations can be attributed to structural and sequence-specific factors of the proteins. For instance, BSA and GSA possess two tryptophan residues (Trp-134 and Trp-213), strategically located within hydrophobic pockets that often correspond to drug-binding regions. This structural feature may create a more favorable microenvironment for stabilizing TMB through hydrophobic and van der Waals interactions, resulting in its higher binding constants. In contrast, HSA contains only a single tryptophan residue (Trp-214), which may partially limit the extent of fluorescence quenching and decrease the over-

all binding strength. The lowest affinity observed for SSA suggests that subtle conformational or surface accessibility differences, despite also containing two tryptophan residues, may reduce the stability of the TMB–protein complex. Notably, the binding stoichiometry (n) for all TMB–albumin systems was approximately 1.0, indicating that a single TMB molecule interacts with each albumin molecule in a 1:1 binding ratio. Overall, from a pharmacological standpoint, these protein-binding differences are highly relevant. Stronger binding to BSA and GSA suggests that TMB may exhibit altered free drug fractions and distribution profiles in bovine and goat models compared to humans. While BSA has long been used as a surrogate for HSA in binding studies due to its availability and structural similarity, our results indicate that its higher binding affinity may lead to an underestimation of the pharmacologically active free fraction of TMB. Conversely, the relatively weaker binding in SSA indicates that sheep models may not adequately mimic human binding behavior. Therefore, GSA, which demonstrated an intermediate affinity closer to HSA, could represent a more suitable preclinical

model for predicting TMB's pharmacokinetic behavior in humans.

The binding process was further evaluated through the evaluation of the ΔG^0 using Equation 4. The obtained ΔG^0 values ranged from -25.2 ± 0.10 to -28.86 ± 0.10 kJ mol⁻¹ (Table 2), indicating that the interaction between TMB and serum albumins is thermodynamically favorable and occurs spontaneously under physiological conditions. The magnitude of these negative ΔG^0 values reflects moderate to strong non-covalent binding forces, typically associated with hydrogen bonding, hydrophobic interactions, and van der Waals forces. These interactions are consistent with those commonly observed in small-molecule–protein complexes. Furthermore, the similarity in ΔG^0 values across different albumins suggests that TMB binds comparably, possibly targeting structurally conserved domains such as Sudlow's sites I or II. These findings are also in agreement with previous reports (Suo et al., 2018) on TMB–HSA interaction, where spontaneous binding with similar ΔG^0 ranges has been reported, supporting the role of serum albumins as efficient transporters of anticancer agents.

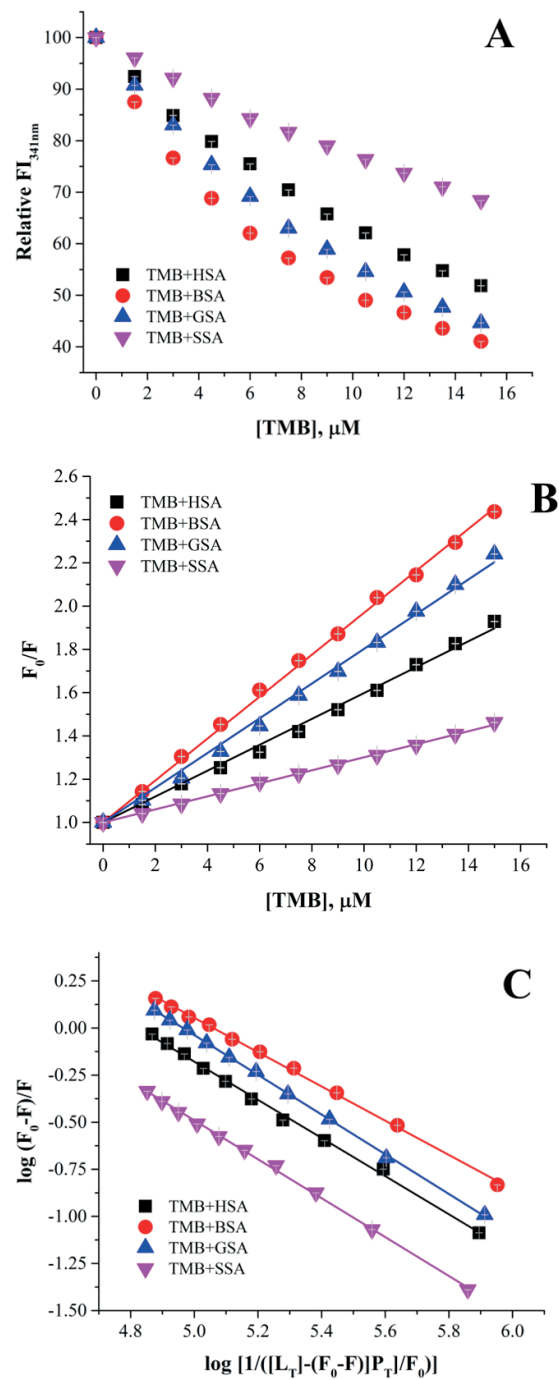


Figure 4. (A) Relative decrease in fluorescence intensity of various serum albumins upon TMB interaction. Analysis of fluorescence quenching titration data: (B) Stern–Volmer plots and (C) double logarithmic plots illustrating the binding interaction between TMB and various serum albumins ($n = 3$).

Table 2. Binding properties between TMB and various serum albumins, as obtained in PBS 7.4 at 298 K (n = 3).

Systems	$K_{sv} \times 10^4 (M^{-1})$	$K_a \times 10^4 (M^{-1})$	n	R^2	$\Delta G^0 (kJ mol^{-1})$
TMB-BSA	9.47 ± 0.09	11.46 ± 0.09	0.92	0.998	-28.86 ± 0.10
TMB-GSA	8.49 ± 0.08	9.22 ± 0.09	1.05	0.997	-28.32 ± 0.10
TMB-HSA	6.26 ± 0.06	6.67 ± 0.07	1.01	0.999	-27.52 ± 0.10
TMB-SSA	3.06 ± 0.05	3.35 ± 0.06	1.03	0.999	-25.82 ± 0.10

CONCLUSION

This study provides the first comparative analysis of TMB interactions with serum albumins derived from four different mammalian species—HSA, BSA, GSA, and SSA—using steady-state fluorescence spectroscopy. The fluorescence quenching observed upon titration of TMB into albumin solutions indicates the formation of non-fluorescent complexes, suggesting significant binding interactions. Stern–Volmer analysis and calculated bimolecular quenching constants revealed that the quenching mechanism is predominantly static, consistent with complex formation in the ground state. All albumins demonstrated a 1:1 binding stoichiometry with TMB, indicating that one drug molecule associates with one albumin molecule.

Among the species studied, BSA exhibited the highest binding affinity, followed by GSA, HSA, and SSA. Thermodynamic evaluation through ΔG^0 values confirmed that the interactions are spontaneous and thermodynamically favorable, with binding likely driven by non-covalent interactions such as hydrogen bonding and hydrophobic forces. The comparable ΔG^0 values across species also imply a structurally conserved binding environment.

These findings enhance our understanding of how trametinib interacts with albumins from different species and underscore the relevance of protein-binding studies in drug development. Importantly, they support the careful selection of preclinical animal models based on albumin binding similarity to humans, thereby improving the translational accuracy of pharmacokinetic predictions and therapeutic outcomes.

AUTHOR CONTRIBUTION STATEMENT

The author was responsible for developing the hypothesis, conducting literature research, performing experiments, and preparing and reviewing the manuscript (CE).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Hypericum perforatum Oil-Loaded Niosomal Gel for Vaginal Delivery

Emine Dilek ÖZYILMAZ*

Hypericum perforatum Oil-Loaded Niosomal Gel for Vaginal Delivery

Vajinal İçin Hypericum perforatum Yağ Yüklü Niosomal Jel

SUMMARY

The objective of this study is to formulate and assess a niosomal gel formulation with Hypericum perforatum oil for potential vaginal administration. Different Span®60:Tween®80 ratios (1:1, 1:2, and 2:1) were used to prepare the niosomes using the thin-film hydration method, and the physicochemical properties of the prepared niosomes were assessed by particle size, polydispersity index (PDI), and zeta potential. The F2 formulation was selected based on optimal particle size and stability conditions. Morphological analysis with SEM confirmed spherical and homogeneously dispersed vesicles. The selected niosomal formulation was incorporated into a 2% HPMC gel base to prepare a vaginal gel product. The developed gel had an adequate viscosity ($17,000 \pm 0.520$ cP), a physiologically relevant pH value (5.5 ± 0.2), and an effective entrapment of Hypericum oil ($71.6 \pm 0.4\%$). In vitro drug release studies across a cellulose membrane showed sustained release of the niosomal gel compared to the non-niosomal product. Stability studies conducted over 3 months under accelerated stability conditions revealed negligible changes in physicochemical properties. Overall, the niosomal gel system efficiently promoted the retention and controlled release of Hypericum perforatum oil, justifying its potential as a promising carrier for vaginal drug delivery purposes.

Keywords: Hypericum perforatum oil, niosomes, HPMC vaginal gel, stability.

ÖZ

Bu çalışmanın amacı, potansiyel vajinal uygulama için Hypericum perforatum yağı içeren bir niozomal jel formülasyonu formüle etmek ve değerlendirmektir. İnce film hidrasyon yöntemi kullanılarak niozomlar hazırlamak için farklı Span®60:Tween®80 oranları (1:1, 1:2 ve 2:1) kullanılmış ve hazırlanan niozomların fizikokimyasal özellikleri partikül boyutu, polidispersite indeksi (PDI) ve zeta potansiyeli ile değerlendirilmiştir. F2 formülasyonu, optimum partikül boyutu ve stabilite koşulları temelinde seçilmiştir. SEM ile yapılan morfolojik analiz, küresel ve homojen olarak dağılmış vezikülleri doğrulamıştır. Seçilen niozomal formülasyon, vajinal jel hazırlamak için %2'lik bir HPMC jel bazına eklenmiştir. Geliştirilen jel, yeterli bir viskoziteye (17.000 ± 0.520 cP), fizyolojik olarak anlamlı bir pH değerine ($5,5 \pm 0,2$) ve Hypericum yağının etkili bir şekilde tutulmasına ($71,6 \pm 0,4$) sahipti. Seliüloz membran üzerinden yapılan in vitro etkin madde salım çalışmaları, niozomal olmayan ürüne kıyasla niozomal jelin sürekli salınımını göstermiştir. Hızlandırılmış stabilite koşulları altında 3 ay boyunca yürütülen stabilite çalışmaları, fizikokimyasal özelliklerde ihmal edilebilir düzeyde değişiklikler olduğunu ortaya koymuştur. Genel olarak, niozomal jel sistemi, Hypericum perforatum yağının tutulmasını ve kontrollü salımını etkili bir şekilde desteklemiş ve vajinal ilaç iletimi için umut verici bir taşıyıcı olma potansiyelini kanıtlamıştır.

Anahtar Kelimeler: Hypericum perforatum yağı, niozomlar, HPMC vajinal jel, stabilite.

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* ORCID: 0000-0002-1791-3055, Eastern Mediterranean University, Faculty of Pharmacy, Department of Pharmaceutical Technology, North Cyprus via Mersin 10, Famagusta, Mersin 10, Türkiye.

* Corresponding Author; Asst. Prof. Dr. Emine Dilek ÖZYILMAZ
E-mail: emine.ozyilmaz@emu.edu.tr

INTRODUCTION

The vaginal route presents a promising option for delivering drugs locally, thanks to its expansive surface area, abundant blood supply, and the ability to avoid the liver's first-pass metabolism. However, the unique characteristics of the vaginal environment, such as its fluctuating pH, various enzymes, and natural self-cleaning process can often limit how long drugs stay effective and how well they work. This is why creating mucoadhesive, biocompatible, and stable delivery systems is crucial to tackle these challenges and improve treatment outcomes (Karmazyn & Zulfakar, 2021; Osmalek et al., 2021).

Hypericum perforatum oil, better known as St. John's Wort oil, is a well-known herbal remedy celebrated for its anti-inflammatory, antioxidant, and wound-healing benefits. Its traditional applications for skin and mucosal issues are increasingly backed by scientific research. However, the tendency to be oily and sensitive to oxidation poses significant hurdles in formulation, especially when aiming for mucosal tissues like the vaginal lining. These challenges call for a delivery method that not only stabilizes the oil but also ensures it stays in place longer and releases its benefits in a controlled manner (Ishak & Handy, 2023; Pereira et al., 2024).

Niosomes vesicles made from non-ionic surfactants have come to the forefront as effective carriers for both water-soluble and oil-soluble compounds. Their resemblance to biological membranes, straightforward preparation, and solid physical stability make them appealing for delivering drugs through mucosal routes. When designed correctly, niosomes can encapsulate lipophilic substances like *Hypericum* oil, shielding them from breakdown and allowing for controlled release in the mucosal setting. Adding these vesicles into a gel base boosts their ability to stick, ex-

tends the time they stay in contact, and enhances how well patients accept the treatment (Abdelkader et al., 2020; Vashist et al., 2020).

This study is all about creating and characterizing a new niosomal gel formulation that includes *Hypericum perforatum* oil, specifically designed for vaginal use. The formulation process utilizes the thin-film hydration method, incorporating a Span/Tween-based surfactant system and sodium citrate buffer as the hydration medium to enhance electrostatic stabilization. Comprehensive physicochemical evaluations such as particle size, zeta potential, pH, and viscosity control were performed to determine how suitable this system is for vaginal delivery. The aim of the study is to develop a stable, biocompatible, and effective topical formulation that can deliver *Hypericum* oil right where it's needed while tackling the physiological challenges associated with the vaginal route.

MATERIALS AND METHODS

Hypericum perforatum extract was purchased from Merck KGaA (Darmstadt, Germany). Span®60, Tween®80, cholesterol, ethanol, sodium citrate, glycerin, and hydroxypropyl methylcellulose (HPMC) were obtained from Interlab (Sigma-Aldrich, Türkiye). All solvents were of analytical grade and used without further purification. Throughout the study, freshly distilled water was utilized in all preparations.

Preparation of niosome formulations containing *Hypericum perforatum* oil

Formulations were prepared using different ratios of Span®60 and Tween®80 (1:1, 1:2, and 2:1), and their physicochemical properties were subsequently evaluated, with particular emphasis on particle size and zeta potential to determine stability and dispersion characteristics (Shehata et al., 2021). Table 1 presents the components and their respective amounts used in the preparation of the niosomal formulations.

Table 1. Formulations of niosomes

Ingredients	Quantity		
	F ₁	F ₂	F ₃
<i>Hypericum perforatum</i> extract	50 mg	50 mg	50 mg
Span®60	200 mg	150 mg	100 mg
Tween®80	100 mg	150 mg	200 mg
Cholesterol	100 mg	100 mg	100 mg
Ethanol	2 mL	2 mL	2 mL
0.1 M sodium citrate solution	100 mL	100 mL	100 mL

The predetermined amounts of surfactants (Span®60 and Tween®80), cholesterol, and *Hypericum perforatum* oil were dissolved in ethanol. The solvent was then evaporated using a rotary evaporator at 60 °C under reduced pressure, forming a thin lipid film on the inner surface of the flask. The resulting dry film was hydrated with 10 mL of 0.1 M sodium citrate buffer by magnetic stirring for 30 minutes. To reduce the particle size of the obtained niosomal formulation, the dispersion was sonicated at 40°C for 10 min, applying approximately 25 kHz power, and then subjected to magnetic stirring for another 5 min. This process was repeated twice to ensure homogeneity and optimal size reduction (Chasanah et al., 2021; Rezaei et al., 2024; Yeo, 2022).

Characterization studies on the niosomal formulations

A comprehensive characterization was carried out on niosomal formulations prepared with varying Span®60 to Tween®80 ratios, including assessments of particle size, surface charge (zeta potential), encapsulation efficiency (EE%), pH, and morphological characteristics. The most suitable Span®60 to Tween®80 ratio was determined according to the characterization results, and the corresponding niosomal formulation was optimized accordingly (Obeid et al., 2022; Temprom et al., 2022; Witika et al., 2022).

Determination of particle size, polydispersity index (PDI), and zeta potential

Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, UK) to determine the mean particle di-

ameter, polydispersity index (PDI), and zeta potential of the developed niosomal formulations. Each formulation was analyzed three times at 25 °C to obtain accurate and consistent data. PDI values reflected the size distribution profile, whereas zeta potential was indicative of the electrostatic stability of the vesicles.

Evaluation of encapsulation efficiency (EE%)

The encapsulation efficiency of the niosomal formulations was determined by centrifuging 1 mL of the niosomal formulation at 15,000 rpm for 45 minutes. The supernatant containing free drug was analyzed using the validated UV spectrophotometric method at 268 nm (Alahmad et al., 2022; Błońska-Sikora et al., 2025). The EE% was calculated using the formula:

EE% was calculated using the formula:

$$EE\% = [(Total\ drug - Free\ drug) / Total\ drug] \times 100$$

Equation 1

Evaluation of pH

The pH of niosome formulations was determined using a calibrated digital pH meter (Ohaus, USA) at ambient temperature (25±1°C). Each formulation was tested in triplicate, and the average value was recorded. pH measurements were evaluated for compatibility with physiological vaginal pH (~4.5), as maintaining this range is important to minimize mucosal irritation and ensure patient compliance during intravaginal administration (Gvozdevan & Staynova, 2025).

Morphological characteristics

The structural features of the niosomal systems were characterized by Scanning Electron Microscopy (SEM) (Thermo Fisher Scientific, USA). A dilut-

ed aliquot of the niosomal dispersion was deposited onto an aluminum stub that had been pre-prepared with double-sided conductive carbon tape. The film was allowed to air-dry under vacuum at ambient temperature to minimize any alteration of the vesicular architecture. After drying, the specimens received a sputtered gold overlay generated by a vacuum coater to improve electrical conductivity during imaging. Imaging was performed at an accelerating voltage of 15 kV, and high-magnification micrographs were recorded to evaluate the morphology, dimension distribution, and surface topology of the niosomes.

Preparation of non-niosomal *Hypericum perforatum* oil - HPMC gel

HPMC (2% w/v) was gradually dispersed in 30 mL of warm water with gentle stirring and allowed to swell for 10–15 min. After cooling to room temperature, *Hypericum perforatum* oil was added and gently mixed using a magnetic stirrer.

Preparation of niosomal vaginal gel

The required amount of HPMC (2% w/v) was gradually dispersed in 30 mL of warm water with gentle stirring and allowed to swell for 10–15 minutes. After cooling to room temperature, the optimized niosome formulation was added and mixed slowly using a magnetic stirrer (Limpongsa et al., 2023).

Evaluation of the structural and functional properties of HPMC vaginal gel

The HPMC vaginal gel products were characterized in comprehensive characterization studies to evaluate their physicochemical and functional attributes. Visual inspection was done to inspect color, transparency, and homogeneity (Bondre et al., 2023; Zhao et al., 2022). Room temperature pH was determined to ensure compatibility with the natural vaginal pH. Viscosity with a viscometer (Brookfield, USA) was determined to evaluate flow behavior and the convenience of application. Uniformity of drug content was determined spectrophotometrically following appropriate extraction procedures.

To determine the content of *Hypericum perfor-*

atum oil in the gel formulation, precisely 1 g of the gel was accurately weighed and dissolved in 10 mL of ethanol by vortexing for 5 minutes, followed by sonication in an ultrasonic bath for 10 minutes. The resulting mixture was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected, and the absorbance was measured at 268 nm, the previously identified maximum absorbance wavelength of the oil, using a UV spectrophotometer (Sotirova et al., 2025).

In vitro drug release was investigated by using a dialysis bag to investigate release kinetics. The *in vitro* release of *Hypericum perforatum* oil from the prepared HPMC-based vaginal gel was evaluated using a dialysis membrane (molecular weight limit 12,000–14,000 Da). Before use, the membrane was soaked in release medium overnight to ensure proper hydration and permeability. The receptor compartment was filled with phosphate-buffered saline (PBS, pH 4.5) containing 20% (v/v) ethanol, selected to enhance the solubility of the lipophilic oil while mimicking the vaginal pH environment. The temperature was maintained at 37 ± 0.5 °C using a water-jacketed system, and the receptor medium was continuously stirred at 100 rpm to ensure homogeneous mixing. Approximately 1g of the gel formulation was placed in the donor compartment directly on the membrane. At predetermined time intervals (2, 6, 10, 15, 20, and 24 h), 1-mL aliquots of receptor medium were withdrawn and replaced with an equal volume of fresh, prewarmed release medium to maintain sedimentation conditions. The amount of *Hypericum perforatum* oil released at each time point was determined by UV-visible spectrophotometry at 268 nm, based on a previously established standard calibration curve. Cumulative percent release was calculated relative to the amount of oil initially loaded into the gel formulation (Thapa et al., 2022).

The formulations were investigated further under accelerated conditions for the initial stability by investigating changes in appearance, pH, and viscosity with time (Ali et al., 2020a).

RESULTS

Results of the physicochemical and morphological characterization of niosomal formulations

The physicochemical characterization results of the niosomal formulations prepared with three different Span®60 and Tween®80 ratios (1:1, 1:2, and 2:1) are presented in Table 2.

Table 2. Physicochemical properties of niosomal formulations with varying Span®60:Tween®80 ratios (n=3)

Niosomal formulations	Particle size (nm) ± SD	Zeta potential (mV) ± SD	EE(%)±SD	PDI±SD	pH±SD
F1	302.9±0.1	-42.40±0.3	60.5±0.6	0.457±0.1	5.6±0.1
F2	190.8±0.1	-27.30±0.1	72.4±0.5	0.205±0.1	5.3±0.3
F3	327.1±0.3	-33.80±0.5	51.7±0.8	0.244±0.3	5.5±0.2

EE (%): percentage of encapsulation efficiency

PDI: Polydispersity index

SD: Standard deviation

In this study, niosomal formulations were developed using varying molar ratios of Span®60 and Tween®80 (1:1, 1:2, and 2:1) to evaluate the effect of surfactant composition on particle characteristics. Among the tested ratios, the 1:1 Span®60:Tween®80 formulation exhibited the smallest average particle size and the lowest polydispersity index (PDI), indicating a more uniform vesicle population. This result can be attributed to the optimal balance between the hydrophilic (Tween®80) and lipophilic (Span®60) surfactants, which likely promoted the formation of stable and homogeneous bilayer structures. In contrast, increasing the concentration of either surfactant alone disrupted this balance, leading to larger and more polydisperse vesicles. The 1:1 ratio may thus represent a critical point where the surfactant mixture provides the most favorable interfacial tension and membrane curvature for the self-assembly of stable, nano-sized niosomes.

The zeta potential values of the niosomal formulations varied according to the ratio of Span®60 to Tween®80, reflecting differences in surface charge distribution and colloidal stability. The formulation with a 1:1 Span®60:Tween®80 ratio exhibited a moderately negative zeta potential, indicative of a balanced bilayer organization that favors electrostatic repulsion between vesicles and supports dispersion stability. When the proportion of Tween®80 was increased (1:2 ratio), the zeta potential tended to decrease in magnitude,

possibly due to the enhanced hydrophilicity of the surface, leading to a reduction in surface charge density and, consequently, colloidal repulsion. Conversely, increasing the Span®60 content (2:1 ratio) resulted in a relatively more negative zeta potential, which may be attributed to the predominance of lipophilic moieties contributing to a denser bilayer structure and stronger surface charge. However, excessive Span®60 can also disrupt vesicle integrity and lead to aggregation. Therefore, the 1:1 ratio appeared to offer the most favorable surface charge profile for maintaining niosome stability.

The 1:1 Span®60:Tween®80 ratio provided the highest encapsulation efficiency among the tested formulations. This is likely due to the optimal amphiphilic balance, which facilitated the formation of well-organized bilayers capable of efficiently entrapping the lipophilic Hypericum oil. Formulations with higher Tween®80 content showed lower EE% values, presumably due to increased membrane fluidity and potential drug leakage. Conversely, while higher Span®60 ratios enhanced bilayer rigidity, excessive concentrations may have led to partial exclusion of the active compound, slightly reducing EE%.

The hydration medium consisted of 0.1 M sodium citrate buffer adjusted to pH 5.0 ± 0.3, ensuring both the physicochemical stability of the vesicles and compatibility with the intended vaginal application site.

Morphological results of niosome formulation

Based on the evaluation of the physicochemical properties of the niosome, including particle size, PDI, and zeta potential, formulation F2 was identified as the most optimal. The surface morphology of this formulation was further examined by SEM, and the resulting image is presented in Figure 1.

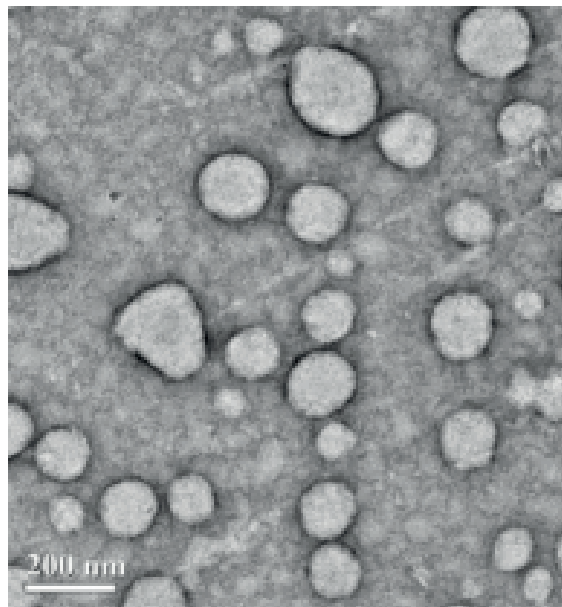


Figure 1. SEM image of the optimized niosomal formulation (F₂)

As shown in Figure 1, the particles exhibit a spherical shape and are uniformly distributed, indicating successful formation of stable vesicles with the desired niosomal structure. Although particles of varying sizes are present in the image, the majority appear to fall within the 100–200 nm range.

Results of physicochemical properties of niosome-loaded HPMC vaginal gel

The HPMC gel prepared with the optimized F₂ niosomal formulation was evaluated in terms of its visual appearance, viscosity, and pH. Additionally, the content of *Hypericum perforatum* oil and its release rate from the gel matrix were determined.

The prepared gel exhibited an opaque white color, consistent with the visual characteristics of the incorporated niosomal formulation. The formulation

displayed a homogeneous appearance and a viscosity of $17,000 \pm 52$ cP, as determined from three parallel measurements. The pH of the gel was measured at 5.5 ± 0.2 using a calibrated pH meter, aligning well with the physiological range for vaginal applications. The content of *Hypericum perforatum* oil, quantified via UV spectrophotometry, was found to be $71.6 \pm 0.4\%$, indicating efficient incorporation within the gel matrix. A UV spectrophotometric method developed for the quantitative analysis of *Hypericum perforatum* oil has been validated in accordance with the ICH Q2(R1) guidelines. The analysis was performed at a wavelength of 268 nm using methanol as the solvent, and the method was found to be linear in the concentration range of 0.5–10 $\mu\text{g/mL}$ ($R^2 = 0.9995$). Accuracy was evaluated at 80%, 100%, and 120% concentration levels, yielding recovery values between 99.1% and 101.3%. The method demonstrated reliable precision with intra-day and inter-day relative standard deviations (RSD) of 1.2% and 1.5%, respectively. The limit of detection (LOD) was calculated to be approximately 0.1 $\mu\text{g/mL}$, and the limit of quantification (LOQ) was 0.3 $\mu\text{g/mL}$. Specificity tests showed no interference from other excipients present in the formulation at the absorbance peak of the analyte. The method was found to be robust under small variations in wavelength (± 2 nm), solvent composition, and temperature. Stability testing showed that the sample solution remained stable for 24 hours, with absorbance changes not exceeding $\pm 2\%$. These findings confirm that the method is suitable for routine quantitative analysis.

In vitro release profile of *Hypericum perforatum* oil from the HPMC-based gel formulation

The release rate of *Hypericum perforatum* oil from the optimized niosomal gel formulation was compared to that of the non-niosomal HPMC gel containing only *Hypericum perforatum* oil, and the comparative release profile is presented in Figure 2.

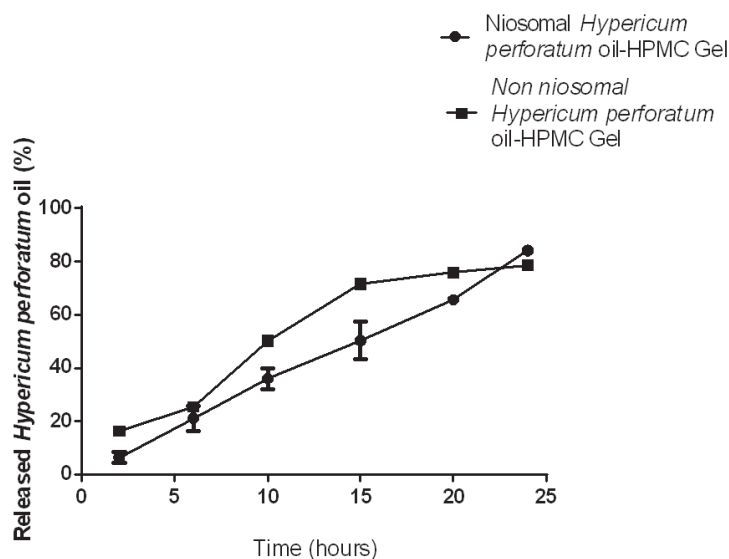


Figure 2. Comparative *in vitro* release profile of *Hypericum perforatum* oil from niosomal and non-niosomal HPMC gels

Stability of niosomal *Hypericum perforatum* HPMC gel

The stability conditions for the prepared formulation were selected according to ICH Q1A(R2) guidelines. This was done to evaluate the physical and chemical stability of the niosomal gel formulation during long-term and accelerated storage. To evaluate the physicochemical stability of the optimized niosomal HPMC gel formulation, a systematic stability study was conducted following ICH Q1A(R2) guidelines. The gel samples were stored under two different conditions: accelerated ($40 \pm 2^\circ\text{C}$ / $75 \pm 5\%$ RH) tem-

perature for three months. Analyses were performed at predetermined intervals: 0, 15, 30, 60, and 90 days.

At each time point, samples were evaluated for pH, viscosity, physical appearance, *Hypericum perforatum* oil content, and zeta potential value. All measurements were taken in triplicate, and the results were expressed as mean $\pm$ SD. Specifically, pH and viscosity remained within $\pm 5\%$ of initial values, the color and homogeneity of the gel were preserved, and the drug content and release behavior stayed within acceptable pharmaceutical limits. The results of stability tests are summarized in Table 3.

Table 3. Stability parameters of optimized niosomal HPMC gel

Duration (day)	Physical appearance	pH (mean $\pm$ SD)	Viscosity (mean $\pm$ SD (cP))	<i>Hypericum perforatum</i> oil content (mean $\pm$ SD) (%)	Particle size (mean $\pm$ SD) (nm)	PDI (mean $\pm$ SD)	Zeta potential (mean $\pm$ SD) (mV)
0	opaque white odorless	5.3 $\pm$ 0.3	17000 $\pm$ 52	71.6 $\pm$ 0.4	190.8 $\pm$ 0.1	0.205 $\pm$ 0.1	-27.30 $\pm$ 0.1
15	opaque white odorless	5.1 $\pm$ 0.3	16200 $\pm$ 47	70.4 $\pm$ 0.7	194.8 $\pm$ 0.3	0.222 $\pm$ 0.3	-20.40 $\pm$ 0.2
30	opaque white odorless	5.2 $\pm$ 0.1	16600 $\pm$ 22	70.1 $\pm$ 0.5	189.8 $\pm$ 0.5	0.192 $\pm$ 0.3	-22.40 $\pm$ 0.5
60	opaque white odorless	5.5 $\pm$ 0.2	17200 $\pm$ 41	68.7 $\pm$ 0.4	192.1 $\pm$ 0.2	0.208 $\pm$ 0.1	-26.10 $\pm$ 0.4
90	opaque white odorless	5.6 $\pm$ 0.1	16800 $\pm$ 32	69.7 $\pm$ 0.2	190.2 $\pm$ 0.3	0.231 $\pm$ 0.2	-24.50 $\pm$ 0.2

DISCUSSION

The current research introduces a novel niosomal vaginal gel containing *Hypericum perforatum* oil, encapsulated in an HPMC matrix, for controlled release and enhanced mucosal delivery. The results, including particle size, encapsulation efficiency (EE%), *in vitro* release, and stability over 90 days, verified the value of the niosomal system over regular gels.

The optimized formulation (F2; Span 60 : Tween 80 = 1:1) yielded vesicles with an average diameter of approximately 190 nm, low PDI (PDI < 0.3), and a moderately negative zeta potential (~ -27 mV), all collectively indicative of colloidal dispersion stability and little likelihood of aggregation. This finding agrees with recent literature reporting increased vesicle stability and homogeneity by way of equilibrated surfactant ratios (Yasamineh et al., 2022).

The EE% of the optimized preparation was 72%, reflecting good entrapment of the lipophilic *Hypericum* oil. High EE% values have also been reported in current niosomal gel systems with herbal extracts, which supports the viability of employing niosomal carriers for hydrophobic actives (Mahale et al., 2021). *In vitro* release profiles were remarkably different between the niosomal gel and the control non-niosomal HPMC gel: prolonged release up to 24 h with significantly less burst release was recorded for the niosomal preparation, whereas the plain gel released $\sim 90\%$ of the oil over the same period. The sustained release behavior seen for the niosomal gel is consistent with findings demonstrating that vesicular systems can be used as diffusion barriers and hence alter release kinetics and enhance retention (Ali et al., 2020b).

The HPMC gel demonstrated desirable physical properties: it had a viscosity of $\sim 17,000$ cP and pH 5.5, within the physiological range of the vagina, and is easy to apply, ensuring proper residence at the mucosal surface. Such rheological properties are related to some recent reports on the importance of viscosity for sustaining drug residence time and permitting controlled delivery (Gaynanova et al., 2025).

Three-month accelerated stability testing at accelerated (40 ± 2 °C / 75% RH) revealed very low pH variations (≤ 0.1 units), viscosity ($< 2\%$ change), and *Hypericum* oil content loss ($\leq 3\%$), with no measurable appearance change. These results indicate stable physicochemical integrity, which is an achievement that is generally lost with non-vesicular systems (Yasamineh et al., 2022).

Collectively, the results suggest that the addition of niosomal vesicles to an HPMC gel matrix enhances formulation stability, determines release behavior, and preserves physico-biological compatibility. The approach is an encouraging vehicle for vaginal delivery of lipophilic herbal actives and merits additional exploration in *in vivo* and clinical studies.

CONCLUSION

In this study, an innovative vaginal gel dosage form was developed through the encapsulation of *Hypericum perforatum* oil-loaded niosomes into an HPMC gel system. Among the three developed formulations of varying Span®60:Tween®80 ratios, the F2 formulation (1:1) displayed the most suitable physicochemical characteristics in the form of optimum particle size, narrow PDI, and stable zeta potential values. The niosomal formulation was optimized and entrapped in the gel matrix to produce a homogenous, stable formulation with acceptable viscosity and pH for vaginal use. The drug release pattern exhibited a sustained and controlled release profile in the niosome-loaded gel compared to the conventional formulation, suggesting that encapsulation effectively modulates the release kinetics of *Hypericum* oil. Furthermore, stability studies conducted over 90 days supported the stability of the formulation under 40 ± 2 °C / 75% RH condition. In summary, the findings highlight the promise of niosomal gels as appropriate vehicles for localized vaginal administration of lipophilic herbal medications like *Hypericum perforatum* oil. The approach could lead to enhanced bioavailability, diminished irritation, and prolonged therapeutic effect, an indication that such systems can be further developed in clinical and translational research in the future.

AUTHOR CONTRIBUTION STATEMENT

Hypothesis development, literature research, experimentation, data analysis, and preparation and correction of the manuscript were carried out by EDÖ.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Exploring the Antibacterial and Antioxidant Activities of *Ruta chalepensis* Before, During, and After Flowering

Mounir SELLOUM[°], Rachid BELHATTAB^{**}

Exploring the Antibacterial and Antioxidant Activities of *Ruta chalepensis* Before, During, and After Flowering

***Ruta chalepensis*'in Çiçeklenme Öncesi, Sırasında ve Sonrasında Antibakteriyel ve Antioksidan Aktivitelerinin Araştırılması**

SUMMARY

This study investigated the phenolic content, as well as the antibacterial and antioxidant activities of hydromethanolic extracts from *Ruta chalepensis* (R. chalepensis) collected at three different periods: before flowering (BF), during flowering (DF), and after flowering (AF). Three hydromethanolic extracts were prepared: the before flowering extract (BFE), the during flowering extract (DFE), and the after flowering extract (AFE). A thorough hydromethanolic extract analysis was conducted regarding their total phenolics and flavonoids contents. The AFE displayed the maximum polyphenol and flavonoid levels: $84.54 \pm 1.66 \mu\text{g GAE/mg extract}$ and $25.15 \pm 0.88 \mu\text{g QE/mg extract}$, respectively. Antibacterial activity was evaluated against seven bacterial strains using the disc diffusion (DD) method, and the minimal inhibitory concentration (MIC) was defined. The results of the studied plant showed an important antibacterial activity against both Gram-negative and Gram-positive bacteria. The bacterial strains showed sensitivity ranging from moderate to high. On the other hand, the antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and the total antioxidant capacity (TAC) assays. All extracts exhibited potent scavenging activity with IC₅₀ (Half maximal inhibitory concentration) values ranging between 14.18 ± 1.02 and $30.96 \pm 1.80 \mu\text{g/mL}$. The strongest antioxidant capacity was exerted by DFE ($160.59 \pm 5.78 \mu\text{g AAE/mg extract}$). In conclusion, the results revealed that *R. chalepensis* hydromethanolic extracts have strong antibacterial and antioxidant properties, highlighting their potential as natural sources for developing novel antibacterial and antioxidant agents.

Keywords: *Ruta chalepensis*, medicinal plants, phenolic compounds, antibacterial activity, antioxidant activity.

ÖZ

Bu çalışmada, *Ruta chalepensis* (R. chalepensis) bitkisinden çiçeklenme öncesi (BF), çiçeklenme dönemi (DF) ve çiçeklenme sonrası (AF) olmak üzere üç farklı büyüme döneminde toplanan hidrometanolik ekstraktların fenolik içeriği ile antibakteriyel ve antioksidan aktiviteleri araştırılmıştır. Üç hidrometanolik ekstre hazırlanmıştır: çiçeklenme öncesi ekstresi (BFE), çiçeklenme dönemi ekstresi (DFE) ve çiçeklenme sonrası ekstresi (AFE). Hidrometanolik ekstraktların toplam fenolik ve flavonoid içerikleri kapsamlı bir şekilde analiz edilmiştir. AFE özütü, maksimum polifenol ve flavonoid seviyelerini göstermiştir; sırasıyla $84,54 \pm 1,66 \mu\text{g GAE/mg özüt}$ ve $25,15 \pm 0,88 \mu\text{g QE/mg özüt}$. Antibakteriyel aktivite, yedi bakteri suşuna karşı disk difüzyon (DD) yöntemi kullanılarak değerlendirilmiş ve minimum inhibitör konsantrasyonu (MİK) tanımlanmıştır. İncelenen bitkinin sonuçları, hem Gram negatif hem de Gram pozitif bakterilere karşı önemli bir antibakteriyel aktivite göstermiştir. Bakteri suşları, orta ile yüksek arasında değişen bir hassasiyet sergilemiştir. Öte yandan, antioksidan aktivite DPPH (2,2-difenil-1-pikrilhidrazil) ve toplam antioksidan kapasite (TAC) analizleri kullanılarak değerlendirildi. Tüm Ekstreler, $14,18 \pm 1,02$ ile $30,96 \pm 1,80 \mu\text{g/mL}$ arasında değişen IC₅₀ (Maksimum inhibisyonun %50'sini sağlayan konsantrasyon) değerleriyle güçlü antioksidan aktivite gösterdi. En güçlü antioksidan kapasite DFE tarafından uygulandı ($160,59 \pm 5,78 \mu\text{g AAE/mg ekstre}$). Sonuç olarak, elde edilen veriler, *Ruta chalepensis* hidrometanolik ekstraktlarının güçlü antibakteriyel ve antioksidan özelliklere sahip olduğunu ortaya koymuştur. Bu bulgular, söz konusu ekstraktların yeni antibakteriyel ve antioksidan ajanların geliştirilmesinde doğal kaynak olarak kullanım potansiyelini vurgulamaktadır.

Anahtar Kelimeler: *Ruta chalepensis*, tıbbi bitkiler, fenolik bileşikler, antibakteriyel aktivite, antioksidan aktivite.

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[°] ORCID: 0000-0002-0521-6842, Department of Microbiology and Biochemistry, Faculty of Sciences, University of M'sila, University Pole, Road Bordj Bou Arreridj, M'sila 28000, Laboratory of Applied Microbiology, Faculty of Nature and Life Sciences, Ferhat Abbas Setif1 University, 19000, Setif, Algeria.

^{**} ORCID: 0000-0001-8154-156X, Department of Biochemistry, Faculty of Nature and Life Sciences, Ferhat Abbas Setif1 University, 19000, Setif, Algeria.

INTRODUCTION

Foodborne infections remain a significant global health concern with major impacts on public health systems, economic stability, and food safety. These illnesses primarily originate from two mechanisms: microbial contamination by pathogenic organisms capable of causing severe infections and intoxications despite the protective presence of the human microbiome (Severino et al., 2025), and oxidative spoilage leading to food deterioration (Sökmen et al., 2004). Such hazards affect all nations regardless of development status, with contaminated food consumption enabling pathogen proliferation that results in acute poisoning and significant health risks (Celiktaş et al., 2007). Compounding this challenge, the emergence of multidrug-resistant organisms – driven by antibiotic overuse in human medicine, agriculture, and livestock production – has created a parallel public health crisis that further complicates foodborne disease management (Elshobary et al., 2025; Marino et al., 2025). This situation has accelerated the search for new treatment approaches, particularly the investigation of safer bioactive substances, driven by increasing microbial virulence and the potential toxicity of synthetic antimicrobial drugs (Magri et al., 2023; Veiko et al., 2023).

Medicinal plants have been a basis of human and healthcare systems for a long time, with an important position in food, therapeutics, and hygiene in the earliest civilizations of Egypt, China, and Greece (Jindal & Seth, 2022). Recent epidemiological data collected by the World Health Organization (WHO) indicate that 80% of the global population continues to use plant-based medicines in their primary healthcare practices (WHO, 2019). Medicinal and edible plants represent a valuable source of bioactive compounds with multifunctional applications, serving as natural food additives, pharmaceutical agents, and industrial components. Their enduring pharmacological relevance is evidenced by continued incorporation in both traditional medicine systems and modern

therapeutic approaches, with phytochemical analyses validating the scientific basis for their historical use (Chihomvu et al., 2024; Pallarés et al., 2025).

Plant extracts are showing renewed promise as effective antimicrobials, especially against antibiotic-resistant pathogens (Manso et al., 2021). Their bioactive components include such as phenolics as flavonoids and phenolic acids that have therapeutic effects, including anticancer, antidiabetic, antioxidant, anti-inflammatory, and antimicrobial activities (Fraga et al., 2019; Gutiérrez-del-Río et al., 2018). These natural compounds are especially valuable as antioxidants, as they diminish free radical damage through mechanisms such as hydrogen donation, singlet oxygen quenching, metal chelation and direct radical scavenging (Kacem et al., 2015).

The *Ruta* genus, native to the Mediterranean region, encompasses around 40 species of fragrant shrubs. Among these are *Ruta graveolens*, *Ruta chalepensis*, and *Ruta montana* (Jianu et al., 2021). *Ruta chalepensis*, commonly known as fringed rue, is widely used in Mediterranean traditional medicine (Yesuf et al., 2023). It was known to treat a variety of ailments, including gastrointestinal disorders (Molares et al., 2023; Usman et al., 2022), migraine and tired eyes (Ahmed et al., 2023), neuralgia, rheumatism, and menstrual disorders (Martínez-Pérez et al., 2017). It has analgesic, antipyretic, anti-inflammatory, spasmolytic, and convulsion treatment properties (Althaher et al., 2024). Several extracts of *Ruta chalepensis* are remarkably rich in diverse bioactive compounds, predominantly phenolic compounds, flavonoids, alkaloids, and essential oils. Phytochemical analyses have confirmed the presence of key compounds like rutin, quercetin, and furanocoumarins such as xanthotoxin and bergapten (Bekkar et al., 2021). These compounds are well-regarded for their potent antioxidant and antimicrobial properties. While traditional applications of *R. chalepensis* are widespread, ongoing research continues to validate and explore its purported therapeutic benefits, including its insecticidal, antioxidant, and antimicrobial activities (Szewczyk et al., 2022; Yücel et al., 2023).

This study investigates the phenolic content, antibacterial activity, and antioxidant potential of *Ruta chalepensis* extracts. To achieve this, we analyzed extracts collected at three distinct phenological stages: before, during, and after flowering. Antibacterial activity was assessed using the disc diffusion method, and the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined. Antioxidant potential was evaluated using two established methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and total antioxidant capacity (TAC). Our findings aim to provide valuable insights into the potential of *R. chalepensis* as a natural food preservative.

MATERIALS AND METHODS

Plant material

Aerial parts of *R. chalepensis* were collected from Djebel Messaad, Algeria (Latitude 34° 59' 28" N and Longitude 4° 5' 33" East), during three periods: before flowering (BF), during flowering (DF), and after flowering (AF) in 2021. A voucher specimen (N°: RC-1726QS28DjM) was placed in the Department of Nature and Life Sciences' herbarium after the plant was recognized by botanist Dr. Sarri Djamel.

Preparation of *Ruta chalepensis* extracts

The aerial parts of three samples were air-dried at room temperature in dark conditions for one week, and then they were powdered using a blender apparatus. 50 g of each powdered sample was mixed with 500 mL of a methanol/water solution (3:1) and macerated for 24 hours at room temperature in the shade under continuous agitation. The extraction was performed three times. The resulting mixture was then filtered through Whatman No. 4 filter paper and concentrated using a rotary evaporator. The yield of the extraction was then measured, and the dried extracts were stored at 4°C until use (Nouasri et al., 2022).

Determination of the total phenolic content

For the purpose of determining the total phenolic content of each sample, the colorimetric method described by Li et al., (2007) using the Folin-Ciocalteu

reagent was adopted. 500 µL of 10% Folin-Ciocalteu reagent was combined with the samples (100 µL). 400 µL of a 7.5% sodium carbonate (Na_2CO_3) solution was added after 4 minutes. After that, the mixture was incubated for 2 hours at room temperature. A wavelength of 765 nm was used to measure the absorbance. Gallic acid (0-140 µg/mL) was used as a standard, and all tests were executed in triplicate. The amount of gallic acid equivalents in micrograms per milligram of extract (µg GAE/mg extract) was used to calculate the quantity of total phenolic compounds in the extracts.

Determination of the total flavonoid content

The total flavonoid content was determined using the method described by Bahorun et al., (1996). Briefly, 1 mL of 2% AlCl_3 reagent in methanol was added to an equal volume of extract or quercetin. Following that, the absorbance was measured at 430 nm using quercetin (0–20 µg/mL) as the reference. The extracts' flavonoid concentration was measured in micrograms of quercetin per milligram of extract.

Antibacterial activity

Strains

Bacterial strains subjected to the antibacterial activity of the plant extracts were four Gram-positive: *Staphylococcus aureus* (ATCC 65380), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis*, *Bacillus cereus*, and three Gram-negative: *Escherichia coli* (ATCC 7835), *Klebsiella pneumonia* (ATCC 13076), and *Proteus vulgaris*. These strains were generously supplied by the University Hospital Center (UHC) of Sétif, Algeria.

Disc diffusion method

The disc diffusion method was used to evaluate the antibacterial activity of *R. chalepensis* extracts. Bacterial dilution at 0.5 McFarland turbidity reference suspensions were inoculated into Müller-Hinton agar (MHA) plates. Afterwards, filter paper discs (6 mm) infused with 20 µL of each extract (200 mg/mL) in dimethyl sulfoxide (DMSO) were placed on the inoculated agar. DMSO-soaked discs served as neg-

active controls, and Ampicillin antibiotic discs (AMP) were used as positive controls. All plates were then incubated at 37°C for 24 h. The inhibition zone (IZ) diameters were measured to determine the antibacterial efficiency of the extracts. Each experiment was performed in triplicate (Szewczyk et al., 2022).

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC values are the minimum plant extract concentrations that are vital to totally stop the microorganism development. Those values were determined by the microdilution method using 96-well plates as described by Yèhouenou et al., (2010). Briefly, 100 µL of Müller-Hinton broth (MHB) medium with phenol red (0.2%) was added to each well of a 96-well plate, 100 µL of extract (500 mg/mL) was added to the first column wells, except the second row. Half-dilutions were successively applied to wells, up to the 12th well in each row, and 100 µL from the last well was discarded. The first well of the second row was filled with 100 µL of MHB that did not include phenol red. After that, successive dilutions of 2 wells per well were carried out up to the 12th well in each row and, the 100 µL of the last well was discarded. All wells were inoculated with 100 µL of bacterial suspension (10⁵ CFU/mL) except for the first row which was replaced by MHB without phenol red. Finally, the incubation was carried out at 37°C for 18 to 24h. Bacterial growth is recognized by a yellow color, while the persistence of the original red color indicates the absence of microbial growth. To determine the MBC, 0.1 mL from each well with no visible growth was sown onto MHA and incubated for 24 h at 37°C. The concentrations on plates that showed no signs of bacterial growth were determined to be the MBCs.

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) test

The antioxidant action of *R. chalepensis* extracts was determined regarding the hydrogen atom-donating ability of the plant or the plant radical-scaveng-

ing ability, using the stable radical DPPH (Que et al., 2006). Following the addition of 500 µL of the DPPH solution (0.1 mM) to 500 µL of extract solution or the standard butylated hydroxytoluene (BHT) at different concentrations (0-120 µg/mL), the mixture was allowed to stand at room temperature for 30 min in the dark. After that, the absorbance was measured at 517 nm. Based on the following formula, the DPPH scavenging activity was determined:

$$\text{Scavenging activity (\%)} = (A_0 - A_1) \times 100 / A_0$$

where A_0 and A_1 represent the absorbances of the control and the test /BHT, respectively.

Total antioxidant capacity (TAC)

The phosphomolybdenum technique was used to assess this test for *R. chalepensis* extracts (Ben Abbes et al., 2021). In Brief, 0.1 mL of each extract sample was added to 0.9 mL of reagent solution, which is composed of the following: 0.6M sulphuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate. The mixes were left at 95°C for 90 min, then allowed to cool to room temperature. Absorbance of the solution was determined at a wavelength of 695 nm against a blank. The amount of the standard ascorbic acid (AA) equivalent per milligram of extract (µg AAE/mg extract) was used to express the total antioxidant capability.

Statistical analysis

Results are mean $\pm$ SD. An ANOVA test and a Dunnett/Tukey test for multiple comparisons were used to assess the significance of the differences between tests/the control and the different tests at $p \leq 0.05$. Prism 9.00 computer software (GraphPad, Boston, USA) was used.

RESULTS AND DISCUSSION

Extraction yield and phenolic contents

Secondary metabolites are organic components found in any life form and are widely exploited in medicinal plants. They are not directly involved in the growth, reproduction, and development of the organism; however, they increase its survival and

fecundity. There are numerous advantages to using medicinal plants, including the ability to treat illnesses, to produce pharmaceutical medications, and to create cosmetics (Aboalhaija et al., 2022). Extraction of these compounds is necessary to evaluate their biological activities. The present study investigated three extracts from the *R. chalepensis* medicinal plant: before (BFE), during (DFE), and after (AFE) flowering. The yield of extraction varied between 16% and 28.97% depending on the collection period, with the following order: AFE > DFE > BFE. These results exhibited greater yields compared to those reported by Bekkar et al., (2021), with the *Ruta* plant grown in Mascara, western Algeria. They were also higher than those shown in similar studies in Tunisia by Khlifi et al., (2013) and Kacem et al., (2015). Moreover, they showed values slightly higher compared to those mentioned in a study conducted in Morocco (Neighboring country) on the same plant (Barbouchi et al., 2024). In a study by El Ouardi et al., (2025), the yield of the hydro-methanolic extract from Moroccan *Ruta montana* was 17.91%, which is comparable to the yields obtained in our DFE and AFE samples. As these results clearly indicate, the extraction yield

is subject to variation due to a complex interplay of factors. These include both the plant species itself and external factors, such as environmental conditions, climate, and the extraction solvent.

Table 1 lists the total amounts of flavonoids and polyphenolics in the *R. chalepensis* extracts under study. These contents depend on the collection period, with levels being high before flowering, decreasing during flowering, and rising again after flowering. The extracts contained polyphenols ranging from 70.36 to 84.54 µg GAE/mg extract. These are lower than those reported in the Tunisian *R. chalepensis* study by Kacem et al., (2015). Indeed, a strong polyphenolic content was mentioned in wild-grown *R. chalepensis* (168.91 mg caffeic acid E/g) cited by Ouerghemmi et al., (2017). The plant content of flavonoids was found to be between 17.15 and 27.15 µg QE/mg. The BFE of *R. chalepensis* showed the main amount, followed by AFE with a slightly lower amount, while DFE exhibited the lowest content. These results values are, in contrast to the polyphenols, higher than those mentioned by Kacem et al., (2015), and lower than those cited by Benchikh et al., (2019) and Althaher et al., (2024).

Table 1. Extraction yields, and polyphenolic and flavonoid contents of *R. chalepensis* extracts.

Extract	Yield (% w/w)	Polyphenols (µg GAE/mg)	Flavonoids (µg QE/mg)
BFE	28.97 ± 0.15	78.00 ± 0.12	27.15 ± 0.28
DFE	18.54 ± 0.21	70.36 ± 0.04	17.15 ± 0.34
AFE	16.00 ± 0.18	84.54 ± 1.66	25.15 ± 0.88

Values are mean ± SD (n = 3). Significant difference was found between values in each column according to Tukey's multiple comparisons test, p<0.05.

Antibacterial activity of *R. chalepensis* extracts

The pathogens' development of antibiotic resistance has prompted the scientific community to undertake significant research to identify alternative treatment approaches. The rich diversity of plant secondary metabolites has been acknowledged as a valuable resource in this context (Vaou et al., 2021). In this study, the antibacterial activity of the *R. chalepensis*

extracts was evaluated against Gram-positive and Gram-negative bacteria. The inhibition zone (IZ) diameters and the MIC values were determined by the disk diffusion and microdilution techniques, respectively. Based on the relevant results (Table 2), and the bacterial inhibition halo criteria mentioned by Hendel et al., (2024), generally, a bacterial strain is classified as sensitive when the inhibition zone (IZ) ranges

from 8 to 14 mm, at 14 to 20 mm, it is very sensitive, and when exceeding 20 mm, it is extremely sensitive. According to these classifications, all tested bacterial strains were sensitive to *R. chalepensis* extracts, except for *E. coli*, which was resistant to the BFE, and *P. vulgaris*, which was resistant to both BFE and AFE. The Gram-positive bacterial strains *E. faecalis* and *B. cereus* were the most sensitive, generally classified as very sensitive, except for *B. cereus* with the BFE, which exhibited a 10 mm inhibition zone. In terms of extract potency, the *R. chalepensis* DFE extract was found to be the most effective, exhibiting the strongest inhibitory action against all tested bacterial strains, and in particular versus gram-negative strains. This finding aligns with the data reported by Althaher et al., (2024); the ethanolic extract of *R. chalepensis* exhibited antibacterial effect against *B. cereus*, *S. aureus*, *E. coli*, and *P. penneri*. A study conducted by Mokhtar et al., (2022) reported antibacterial activity of a *Ruta*

graveolens extract against *Staphylococcus aureus*, with an inhibition zone of 14 mm, which is slightly higher than that observed in our results.

The antibacterial activity of the plant extracts is likely mediated by alkaloids, flavonoids, polysaccharides, and other phenolic substances (Álvarez-Ordóñez et al., 2013; Musyimi et al., 2007). One of the actions acts on the plasma membrane H⁺-ATPase (Trigui et al., 2013). Polyphenols exhibit antibacterial properties by targeting bacterial cell structures. They can trigger membrane condensation and morphological alterations, such as cell deformation and cell wall rupture. This disruption of the cell envelope leads to the release of cytoplasmic material and membrane debris. The fundamental process is the disruption of the bacterial cell wall and membrane, leading to increased permeability (Álvarez-Martínez et al., 2020; Wang et al., 2017).

Table 2. Antibacterial activity of *R. chalepensis* extracts and the ampicillin antibiotic (AMP), as shown with inhibition zone diameter (mm).

Bacterial strains	Inhibition zone diameter (mm)			
	BFE	DFE	AFE	AMP
<i>S. aureus</i>	11.67 ± 0.58 ^a	12.67 ± 1.08 ^a	10.00 ± 1.00 ^a	29.67 ± 1.53
<i>B. subtilis</i>	8.67 ± 0.58 ^b	8.67 ± 0.58 ^b	8.67 ± 0.58 ^a	19.67 ± 1.53 ^a
<i>E. faecalis</i>	15.33 ± 0.58 ^a	17.00 ± 1.00 ^c	14.67 ± 0.58 ^{a,b}	23.00 ± 1.00 ^b
<i>B. cereus</i>	10.33 ± 0.58 ^{a,b}	18.00 ± 1.00 ^c	17.67 ± 0.58 ^a	24.33 ± 1.53 ^b
<i>E. coli</i>	6 ± 0.47 ^c	12.67 ± 0.47 ^a	9.67 ± 0.47 ^a	17.67 ± 0.47 ^{a,c}
<i>K. pneumonia</i>	11.58 ± 0.47 ^a	10.00 ± 1.00 ^b	8.67 ± 0.58 ^a	17.33 ± 1.53 ^c
<i>P. vulgaris</i>	6 ± 0.47 ^c	12.67 ± 1.00 ^a	6 ± 0.47 ^a	18.67 ± 1.53 ^{a,c}

Values are mean ± SD (n = 3). No significant difference when the same superscript letter appears in the same column, at p<0.05 of Tukey’s multiple comparisons test.

Minimum inhibitory concentration (MIC)

Our investigation into the antibacterial activity of *Ruta chalepensis* extracts revealed MIC values ranging from 1.95 to 31.25 mg/mL. The DFE and AFE were particularly effective against *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus aureus* (Table 3). These findings align with previous research, such as Althaher et al., (2024), who reported an *R. chalepen-*

sis ethanolic extract with an *E. coli* MIC of 50 µg/mL, and Haddouchi et al., (2013), who observed MICs from 18-163 µg/mL for the essential oil against various bacteria. While MBC values were generally higher than MICs, indicating a bactericidal effect at higher concentrations, notably for *Enterococcus faecalis* and *Bacillus subtilis*, MICs and MBCs were equal, demonstrating a direct bactericidal action at growth-inhib-

itory concentrations. This aligns with Nazzaro et al., (2013), who noted stronger bactericidal effects of plant extracts against Gram-positive bacteria. The potent antibacterial activity against key pathogens, along with favorable MICs, highlights *R. chalepensis* as a promising natural antimicrobial agent, especially

relevant in the context of rising antibiotic resistance. The observed variations in activity across phenological stages (DFE and AFE being more efficacious) suggest that harvest timing significantly influences the extract's antimicrobial potential, likely due to differences in bioactive compound profiles.

Table 3. MIC and MBC values of *R. chalepensis* hydromethanolic extracts.

Bacterial strains	MIC (mg/mL)			MBC (mg/mL)		
	BFE	DFE	AFE	BFE	DFE	AFE
<i>S. aureus</i>	15.62	1.95	1.95	31.24	7.8	7.8
<i>B. subtilis</i>	1.95	7.81	15.62	3.9	15.62	15.62
<i>E. faecalis</i>	3.9	7.81	7.81	3.9	15.62	15.62
<i>B. cereus</i>	3.9	7.81	7.81	7.8	15.62	15.62
<i>E. coli</i>	31.25	1.95	1.95	62.5	7.8	15.62
<i>K. pneumonia</i>	7.81	1.95	1.95	15.62	3.9	7.8
<i>P. vulgaris</i>	–	3.9	–	–	7.8	–

– Not defined

Antioxidant activity

DPPH test

The present study investigated the free radical scavenging activity of *R. chalepensis* extracts using the DPPH assay, a widely recognized method for evaluating the ability of extracts to neutralize stable radicals through electron or hydrogen atom donation (Tepe et al., 2005). Our results demonstrate that all three *R. chalepensis* extracts-BFE, DFE, and AFE-exhibited concentration-dependent inhibition of the DPPH radical (Figure 1). This indicates a clear dose-response relationship, where higher concentrations of the extracts lead to increased scavenging activity.

A crucial finding of this investigation is the significant influence of harvesting time on the DPPH free radical scavenging power. The AFE showed the highest antioxidant activity with an IC₅₀ value of 14.18 ± 1.02 µg/mL, followed by BFE (IC₅₀ = 22.04 ± 1.16

µg/mL) and DFE (IC₅₀ = 30.96 ± 1.80 µg/mL). These values were statistically distinct, highlighting that the plant's antioxidant potential varies considerably throughout its phenological stages. Interestingly, the DFE's antioxidant activity (IC₅₀ = 30.96 ± 1.80 µg/mL) did not differ significantly (p < 0.05) from that of the synthetic antioxidant BHT (IC₅₀ = 12.89 ± 0.38 µg/mL). This suggests that the DFE possesses a comparable level of antioxidant efficacy to a well-established synthetic standard.

When compared to other studies on *R. chalepensis*, our observed DPPH scavenging activities appear to be more potent. For instance, an ethanolic extract from Jordanian *R. chalepensis* reported an IC₅₀ of 41.2 ± 0.1 µg/mL (Althaher et al., 2024), while a Tunisian *R. chalepensis* extract showed an IC₅₀ of 83.42 ± 3.71 µg/mL (Kacem et al., 2015). The significantly lower IC₅₀ values obtained in our study, particularly for AFE,

indicate a superior free radical scavenging capacity compared to those previously reported extracts. This enhanced activity could be attributed to variations in geographical origin, environmental factors, extraction methods, or indeed, the specific phenological stage at which the plant material was harvested, as our results strongly suggest. Furthermore, the outcomes reported by Ouerghemmi et al., (2017) for *R. chalepensis* extracts were similar to ours, reinforcing the notion that *R. chalepensis* is a rich source of natural antioxidants.

The robust radical scavenging activity demonstrated by *R. chalepensis* extracts is primarily attributed to their rich content of phenolic compounds. Phenolic compounds, including flavonoids, coumarins, and phenolic acids, are well-known for their antioxidant properties due to their ability to donate electrons or hydrogen atoms to neutralize free radicals (Mokhtar

et al., 2022; Nouasri et al., 2022). These compounds possess hydroxyl groups that enable them to scavenge reactive oxygen species (ROS), chelate metal ions, and inhibit lipid peroxidation, thereby protecting cellular components from oxidative damage (Althaher et al., 2023; Barbouchi et al., 2024). The high levels of total phenolic and flavonoid content previously identified in *Ruta* species (Althaher et al., 2024; Barbouchi et al., 2024) directly correlate with their observed antioxidant capacities. The variations in antioxidant activity across different harvesting times (BFE, DFE, AFE) further support the idea that the biosynthesis and accumulation of these phenolic compounds are dynamic processes influenced by the plant's developmental stage. This suggests that optimizing harvest timing could be a critical factor in maximizing the antioxidant potential of *R. chalepensis* for various applications, including nutraceuticals and pharmaceuticals.

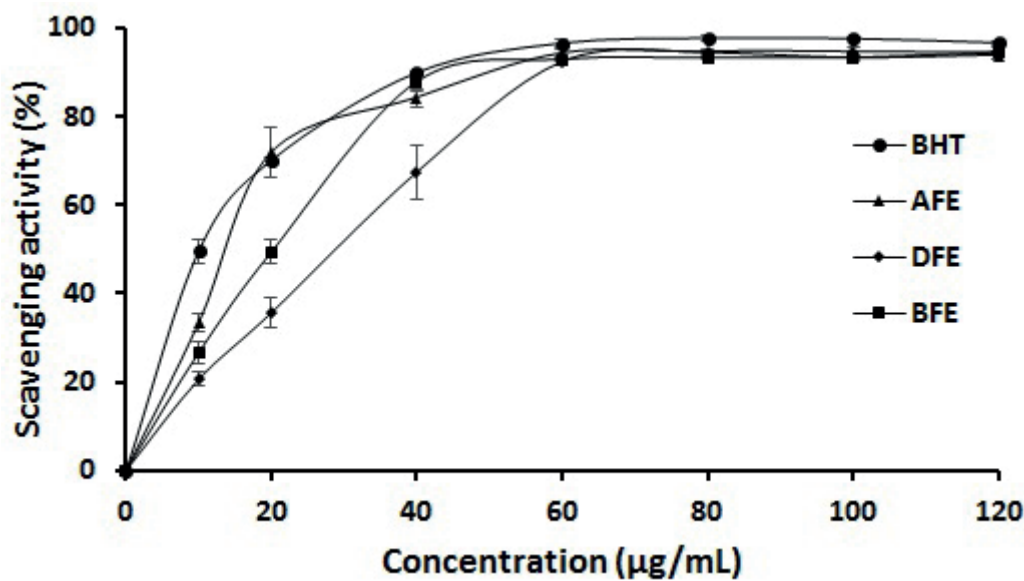


Figure 1. Free radical scavenging activity of BFE, DFE and AFE of *R. chalapensis*, and BHT. Values are mean $\pm$ SD (n = 3).

Total antioxidant capacity

The total antioxidant capacity (TAC) of *Ruta chalepensis* extracts varied significantly depending on the phenological stage of plant collection, as presented

in Table 4. The highest TAC was observed in the DFE with a value of 160.59 ± 5.78 μ g AAE/mg extract. This was followed by the AFE at 136.03 ± 1.46 μ g AAE/mg extract, and the BFE exhibited the lowest capacity at 125.12 ± 2.47 μ g AAE/mg extract.

Table 4: Total antioxidant capacity of *R. chalepensis* extracts.

Extract	TAC (µg AAE/mg extract)
BFE	125.12 ± 2.47
DFE	160.59 ± 5.78
AFE	136.03 ± 1.46

Values are mean ± SD (n = 3).

The observed variation in TAC across different phenological stages highlights the dynamic nature of bioactive compound accumulation in *Ruta chalepensis*. The maximal antioxidant capacity recorded during the flowering stage suggests that this period is optimal for the synthesis and accumulation of compounds responsible for antioxidant activity in the plant. This aligns with the general understanding that plants, as a rich source of secondary metabolites such as polyphenols, flavonoids, and tannins, produce these compounds as part of their defense mechanisms or physiological processes, which can be influenced by developmental stages (Barbouchi et al., 2024).

Previous studies on *Ruta* species and other medicinal plants have similarly demonstrated that antioxidant capacities can fluctuate based on plant parts, environmental conditions, and growth stages. For instance, Barbouchi et al., (2024) reported a broad range of TAC values for *Ruta* crude extracts (37.67 to 396.80 mg AAE/g CE), indicating the variability in antioxidant potential within the genus and across different preparation methods. Our results, falling within this broad spectrum, reinforce the notion of *R. chalepensis* as a significant botanical source of antioxidants. The enhanced antioxidant activity during flowering might be attributed to an increased synthesis of specific phenolic compounds and flavonoids, which are well-known for their radical scavenging, metal chelating, reducing, and hydrogen-donating properties (Kacem et al., 2015; Ouerghemmi et al., 2017). For example, studies on *R. chalepensis* have confirmed the presence of various polyphenols, with total phenolic content varying significantly between extracts (Ouerghemmi et al., 2017). The correlation between high polyphenol levels and strong antioxidant activity has been previ-

ously established (Gali and Bedjou, 2019). Ouerghemmi et al., (2017) also found that *R. chalepensis* leaves and flowers exhibited high total polyphenol content and strong total antioxidant activity, which supports our findings of higher activity during or after the flowering period (DFE and AFE compared to BFE). The pronounced antioxidant potential of *R. chalepensis*, particularly during its flowering stage, reinforces its traditional medicinal uses and suggests its promising application in the food and pharmaceutical industries as a natural preservative and health-promoting agent.

CONCLUSION

This study thoroughly investigated the phenolic content, antibacterial activity, and antioxidant properties of *Ruta chalepensis* extracts across three growth stages (pre-flowering, flowering, and post-flowering). It confirmed that *Ruta chalepensis* is a rich source of polyphenols (70.36 to 84.54 µg GAE/mg extract), with concentrations varying significantly depending on the growth stage. The extracts showed significant antibacterial and antioxidant properties. They exhibited potent broad-spectrum antibacterial activity, with MIC values from 1.95 to 31.25 µg/mL, though *E. coli* showed resistance to the BFE, while *P. vulgaris* was resistant to both BFE and AFE. Antioxidant activity was growth-stage-dependent, with AFE showing the highest DPPH scavenging capacity (IC₅₀ of 14.18 ± 1.02 µg/mL) and DFE exhibiting the highest total antioxidant capacity. These findings firmly establish *R. chalepensis* as a promising natural source of bioactive agents for pharmaceutical, nutraceutical, or food preservation applications. Future research should focus on isolating and characterizing the specific bioactive compounds responsible for these beneficial effects to optimize their use in health and food quality formulations.

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AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis (MS, RB), experimenting and statistics (MS), study text, literature research, interpretation of the data, and reviewing (MS, RB).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Hydrocortisone-Loaded Polyvinyl Alcohol-Based Films for Buccal Delivery in the Treatment of Oral Aphthous Ulcers

Beyza AKYÜZ^{*/**}, Sinem SAAR^{***}, Fatma Nur TUĞCU DEMİRÖZ^{****}

Hydrocortisone-Loaded Polyvinyl Alcohol-Based Films for Buccal Delivery in the Treatment of Oral Aphthous Ulcers

Oral Aftöz Ülserlerinin Tedavisinde Bukkal Uygulama İçin Hidrokortizon Yüklü Polivinil Alkol Bazlı Filmler

SUMMARY

Oral aphthous ulcers are recurrent, painful lesions of the oral mucosa that adversely affect patients' quality of life. Buccal films are crucial to enhance drug retention, achieve sustained therapeutic effect, and ultimately improve clinical outcomes in patients suffering from oral aphthous ulcers. The aim of this study was to develop and evaluate polyvinyl alcohol (PVA)-based buccal film formulations loaded with hydrocortisone (HC) for the local treatment of oral aphthous ulcers. Buccal films were prepared using the solvent casting method. The film formulations were characterized in terms of film thickness, moisture content, and contact angle. Mechanical properties were evaluated by determining tensile strength and elongation at break using a texture analyzer. Thermal behavior was assessed via differential scanning calorimetry (DSC), while chemical interactions were examined using Fourier-transform infrared spectroscopy (FTIR). Mucoadhesive performance was tested ex vivo using cow buccal mucosa, and in vitro drug release studies were conducted using Franz diffusion cells with phosphate buffer (pH 6.8) as the receptor medium. The PVA-based formulation exhibited suitable mechanical strength and high elongation at break. Ex vivo mucoadhesion studies showed that the films demonstrated adequate mucoadhesive properties in cow buccal tissue. In vitro release studies demonstrated a sustained release profile of HC over 8 hours. The findings indicate that PVA-based buccal films are promising candidates for effective localized therapy of oral aphthous ulcers and enhanced patient compliance.

Keywords: Buccal film, Hydrocortisone, Oral aphthous ulcers, Solvent casting.

ÖZ

Oral aftöz ülserler, ağız mukozasında tekrarlayan ve ağrılı lezyonlar olup hastaların yaşam kalitesini olumsuz etkiler. Bukkal filmler, ilacın bölgede kalış süresini artırmak, sürekli bir terapötik etki sağlamak ve nihayetinde oral aftöz ülser hastalarında klinik sonuçları iyileştirmek açısından önemlidir. Bu çalışmanın amacı, oral aftöz ülserlerin lokal tedavisi için hidrokortizon (HC) yüklü polivinil alkol (PVA) temelli bukkal film formülasyonlarının geliştirilmesi ve değerlendirilmesidir. Bukkal filmler, çözücü döküm yöntemi kullanılarak hazırlanmıştır. Film formülasyonları; film kalınlığı, nem içeriği ve temas açısı açısından karakterize edilmiştir. Mekanik özellikler, bir doku analiz cihazı kullanılarak çekme dayanımı ve kopma uzaması belirlenerek değerlendirilmiştir. Termal özellikleri diferansiyel taramalı kalorimetri (DSC) ile, kimyasal etkileşimler ise Fourier dönüşümlü kızılötesi spektroskopisi (FTIR) ile incelenmiştir. Mukoadezif performans, inek bukkal mukozası kullanılarak ex vivo olarak test edilmiş; in vitro ilaç salım çalışmaları ise fosfat tamponu (pH 6,8) içeren Franz difüzyon hücrelerinde yürütülmüştür. PVA temelli formülasyon, uygun mekanik dayanım ve yüksek kopma uzaması göstermiştir. Ex vivo mukoadesyon çalışmaları, filmlerin inek bukkal dokusunda yeterli mukoadesif özellik sergilediğini ortaya koymuştur. In vitro salım çalışmaları, HC'nin 8 saat boyunca sürdürülebilir salım profilini göstermiştir. Elde edilen bulgular, PVA temelli bukkal filmlerin oral aftöz ülserlerin etkili lokal tedavisi ve hasta uyuncunun artırılması için umut verici adaylar olduğunu göstermektedir.

Anahtar Kelimeler: Bukkal film, Hidrokortizon, Oral aftöz ülser, Çözücü döküm metot.

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* ORCID: 0000-0001-9677-6740, Gazi University, Department of Pharmaceutical Technology, Ankara, Türkiye

** ORCID: 0000-0001-9677-6740, Kırıkkale University Faculty of Medicine Hospital, Department of Pharmacy, Kırıkkale, Türkiye

*** ORCID: 0000-0001-6892-5497, Gazi University, Department of Pharmaceutical Technology, Ankara, Türkiye

**** ORCID: 0000-0002-9468-3329, Gazi University, Department of Pharmaceutical Technology, Ankara, Türkiye

INTRODUCTION

Buccal drug delivery refers to the administration of medication to the buccal mucosa on the inner lining of the cheek (Shipp, Liu, Kerai-Varsani, & Okwuosa, 2022). The oral cavity is a preferred site for drug delivery because of its simplicity, safety, high patient compliance, improved bioavailability, and avoidance of first-pass metabolism (Dalei & Das, 2022). Oral aphthous ulcers are painful lesions associated with numerous disorders that develop in the oral cavity and are known to cause severe pain and difficulty in chewing and speaking, which has a significant impact on the quality of life of patients (Noor, Menzel, & Gasmi, 2021).

The primary goals in the treatment of oral aphthous ulcers are to alleviate symptoms, minimize the number and size of ulcers, and prolong the intervals between recurrences (Tarakji, Gazal, Al-Maweri, Azzeghaiby, & Alaizari, 2015). Topical treatment is the first-line approach and includes agents with antiseptic, anti-inflammatory, antibiotic, and steroid properties. Systemic treatment is initiated when these measures are insufficient due to the severity of the lesions or unidentified underlying causes (Belenguier-Guallar, Jiménez-Soriano, & Claramunt-Lozano, 2014). The use of topical corticosteroids in the treatment of oral aphthous aims to limit the inflammatory process. Corticosteroids may act on T lymphocytes or alter the effector cell response to the causes of immunopathogenesis (Barrons, 2001). Hydrocortisone is used to treat aphthous ulcers and to reduce the inflammatory cycle associated with the development of aphthae (Sanjana, Ahmed, & Bh, 2021).

In buccal drug delivery, mucoadhesive tablets, films/patches, gels/lozenges, and nano/micro carrier systems (such as liposomes, solid lipid nanoparticles, and niosomes) are utilized to enhance drug retention on the mucosal surface, enable controlled release, and improve bioavailability (Shirvan, Bashari, & Hemmatinejad, 2019). Buccal films have the

advantages of being flexible, easily transportable, having a rapid onset of action, not requiring chewing and swallowing, bypassing first-pass metabolism, and providing protection against degradation by the drug, enzymes in the gastrointestinal tract, and acidic environment. They are also suitable for pediatric and geriatric patients, as well as for individuals with intellectual disabilities, physical impairments, or difficulty swallowing (Haneef, Sultana & Shahidulla, 2024). The film casting method is one of the most widely used methods for buccal film production, with the advantages of being easy to prepare, inexpensive, and easily adopted on a laboratory scale (Hanif, Zaman, & Chaurasiya, 2015). Polyvinyl alcohol (PVA) is a water-soluble, biocompatible, and biodegradable polymer with high film formation and plasticity (Aygün, 2025; Saar & Demiröz, 2023). Polyvinylpyrrolidone (PVP) is a water-soluble and biodegradable polymer. This study aims to develop hydrocortisone (HC)-loaded film formulations for the local treatment of oral ulcers. These films were designed for buccal application and characterized to ensure enhanced drug retention, controlled release, and improved patient compliance through the use of PVA and PVP polymers with different plasticizers.

MATERIALS AND METHODS

Materials

Hydrocortisone was kindly gifted by Gen İlaç, Türkiye. Polyviol 26/140 (80,000 Da, Wacker Chemie), PVP (M.W. 40,000, BASF, Germany), glycerine, Polyethylene glycol (PEG), and propylene glycol (PG) were used for the preparation of different formulations. NaCl, KH_2PO_4 , NaHPO_4 , HCl, distilled water and ethanol were used for simulated saliva fluid (pH 6.8). All chemicals were of analytical grade.

Preparation of buccal films

The buccal films were prepared using the solvent casting method. PVA and PVP were employed as film-forming polymers, while glycerine, PEG, and PG were used as plasticizers. PVA was dissolved in hot water at 90 °C under stirring at 500 rpm. Once the PVA was

completely dissolved, ethanol and PVP were added, and the mixture was stirred until a homogeneous solution was obtained. Subsequently, the plasticizer was incorporated into the solution and stirred to ensure uniform distribution. Entrapped air bubbles

were removed by sonication. The formulations were then poured into Petri dishes and dried in an oven at 40 °C. The compositions and codes of the formulations are presented in Table 1.

Table 1. Content and codes of buccal film formulations

Formulation Code	HC (%w/v)	PVA (%w/v)	PVP (%w/v)	PEG (%w/v)	Glycerine (%w/v)	PG (%w/v)
F1	-	10	-	3		
F2	-	10	-		3	
F3	-	10	-			3
F4	-	7.5	2.5			3
F5	-	5	5			3
F6	0.2	10	-			3

Characterization studies of buccal films

Thermal analysis of buccal films

The thermal properties of HC, PVA, PVP, and the film formulations were evaluated using differential scanning calorimetry (DSC) (Shimadzu, DSC-60, Japan). Approximately 2 mg of each sample was weighed and pressed into aluminum pans. DSC analyses were conducted with heating rate of 10 °C/min under a nitrogen atmosphere up to 300 °C.

Fourier transform infrared (FT-IR) spectroscopy studies

The chemical interactions among HC, PVA, PVP, and plasticizers, as well as potential structural changes were investigated using Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer, Spectrum 400, USA). Spectra were recorded over the range of 600 - 4400 cm⁻¹ at room temperature with an ATR probe.

Weight and thickness of buccal films

The thickness of the films was measured at different points using a digital micrometer (Mitutoyo Digital Micrometer, Japan), and the mean values were calculated. For weight determination, film specimens of 1 × 1 cm² were cut and weighed on a digital scale. The average values were reported. All experiments were performed in triplicate on films, and results are

reported as mean ± standard deviation (SD).

Contact angle measurements

The wettability of the films was evaluated by measuring the contact angle with distilled water. An optical tensiometer (Attension, Theta Lite, Finland) equipped with a convex sample holder was used for the measurements. The shape of the droplet and the contact angle were determined using the Young–Laplace method via the instrument software (Gajewski, 2017). The experiment was conducted in three replicates (n = 3).

Mechanical properties of buccal films

The mechanical characterization of the buccal films was determined using a texture analyzer (TA, XTPlus Texture Analyzer, Stable Micro Systems, UK). Tensile strength and elongation at break were measured with a tensile grip apparatus. Film samples (3 × 1 cm) were mounted between the miniature tensile grips, and stress–strain curves were recorded. Rectangular strips were used for tensile testing to match the apparatus. Each experiment was performed in triplicate for all film formulations.

Ex vivo mucoadhesion studies of buccal films

The mucoadhesive properties of the buccal films were evaluated using cow buccal mucosa and TA-

XT Plus Texture Analyzer (Stable Micro Systems, UK). The films were attached to the upper probe with double-sided adhesive tape, while the buccal mucosa was fixed on the lower platform. The Study was carried out at a speed of 1 mm/s, 60 seconds contact time of the probe, and probe force of 0.2 N (Tuğcu-Demiröz, Acartürk, & Erdoğan, 2013; Tuğcu-Demiröz, Acartürk, & Özkul, 2015). The work of mucoadhesion (W , mJ/cm²) was calculated from the area under the force–distance curve (AUC) generated during the detachment of the mucoadhesion test. In this curve, the x-axis represents the probe displacement (distance, mm) and the y-axis represents the detachment force (mN). The AUC corresponds to the total energy required to separate the film from the mucosal surface. The work of mucoadhesion values were calculated with the formula ($\text{mJ/cm}^2 = \text{AUC} / (\pi r^2)$) (Cevher, Sensoy, Taha, & Araman, 2008). *Ex vivo* mucoadhesion studies were conducted in triplicate ($n = 3$), and the results are expressed as mean $\pm$ SD.

***In vitro* drug release of buccal films**

In vitro release of hydrocortisone (HC) from the buccal films was evaluated using Franz diffusion cells equipped with a dialysis membrane (12 kDa, Sigma®, USA). The films were placed in the donor compartment, while the receptor phase was filled with pH 6.8 phosphate buffer. Samples were taken from the receptor phase at certain time intervals (0.25, 0.5, 1, 1.5, 2, 3, and 4 h) and an equal volume of fresh buffer was immediately added to maintain sink conditions. Ultraviolet (UV) spectrometer (Cary 60 UVvis, Agilent Technologies, US) was used to determine the amount of HC at 248 nm. The analytical method was validated according to ICH guidelines in terms of linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). Release studies were conducted on three films ($n = 3$).

Statistical analysis

GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Comparisons across ≥ 3 formulations, one-

way ANOVA with Tukey's post hoc test were used when parametric assumptions were met. Two-group comparisons employed Student's t-test or Mann–Whitney U where appropriate. The significance threshold was $\alpha = 0.05$.

RESULTS AND DISCUSSION

Thermal properties of buccal film formulation

Thermal analysis provides valuable information about the physical state of the drug within the formulations, polymer compatibility, and potential interactions in the film matrix. The DSC thermograms of HC, the polymers, and the film formulations are presented in Figure 1. The DSC analysis revealed an endothermic peak at 227.2 °C corresponding to the melting point of HC. Ahmed and Adinarayana reported a sharp endothermic peak for pure HC at 220.26 °C, which is consistent with our findings (Ahmed & Adinarayana, 2019). Compared with pure hydrocortisone, the melting point in the film formulation thermograms appeared to be reduced, and the presence of a broader endothermic peak indicated the amorphous nature of hydrocortisone. The DSC curve of PVA showed a peak around 195 °C, corresponding to its melting point. Literature values report a melting peak of approximately 202.7 °C for pure PVA; thus, the observed melting peak at 195 °C in this study is slightly lower but still consistent with reported literature values. Propylene glycol exhibited thermal events at lower temperatures due to its low molecular weight, hygroscopic nature, and partial volatility under heating. In the thermograms of the F3 and F6 films, the sharp melting endotherm of hydrocortisone was either shifted or significantly reduced in intensity. The absence of a distinct HC peak in F6 also supports the formation of an amorphous state. No thermal degradation or chemical incompatibility observed in the film formulation. These findings demonstrate that the selected polymer–plasticizer combination successfully incorporated hydrocortisone in a stable, amorphous form, which is favorable for uniform drug release from the buccal film.

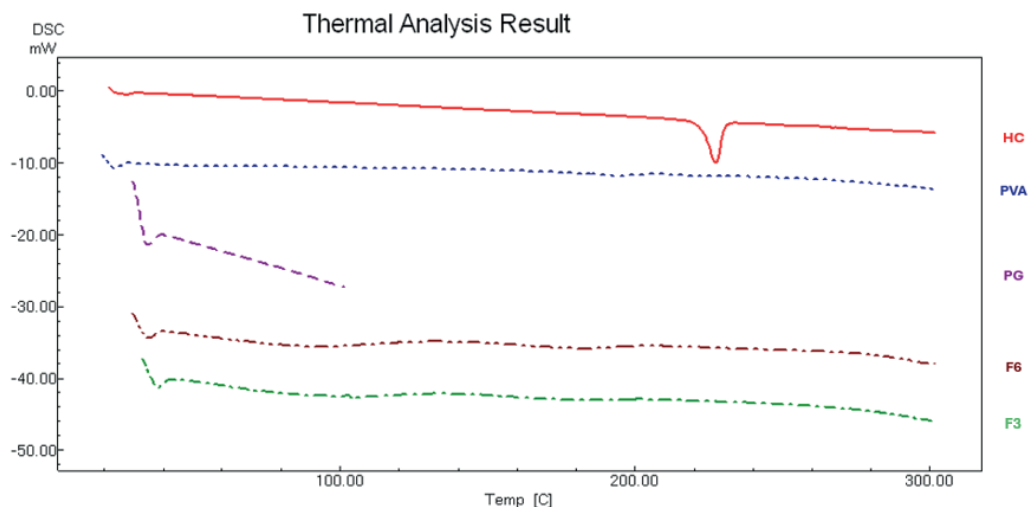


Figure 1. DSC thermograms of HC, PVA, PG, F3, and F6 formulation.

FTIR analyses

The FTIR spectrum of hydrocortisone exhibited characteristic absorptions at approximately 3410 cm^{-1} (O–H stretching), 2936 cm^{-1} (aliphatic C–H stretching), 1706 cm^{-1} (C=O carbonyl stretching), $1630\text{--}1641\text{ cm}^{-1}$ (C=C conjugated bond stretching), 1453 cm^{-1} ($-\text{CH}_2$ bending), and 1046 cm^{-1} (C–O stretching), which are consistent with previously reported values in the literature (Ahmed & Adinarayana, 2019; Altamimi et al., 2019). Characteristic peaks of PVA, F1 and F6 formulations were identified in the FT-IR spectra at approximately 3316 cm^{-1} (O–H stretching due to intra- and intermolecular hydrogen bonding), 2901 cm^{-1} (asymmetric CH_2 stretching), $1432\text{--}1420\text{ cm}^{-1}$ (CH_2 symmetric bending), and 1377 cm^{-1} (CH–OH bending vibration)

which are consistent with the literature (Reguieg, Ricci, Bouyacoub, Belbachir & Bertoldo, 2020; Saar & Demiröz, 2023). These results confirm that the film fabrication process did not alter the chemical integrity of PVA. The FTIR spectrum of PG showed a broad O–H stretching band around 3315 cm^{-1} , C–H stretching bands at 2970 cm^{-1} , and a C–O stretching peak at 1076 cm^{-1} (Ledniowska et al., 2022). The FTIR analyses confirmed the chemical compatibility and integrity of all components within the buccal film formulations (Figure 2). The characteristic peaks of hydrocortisone, PVA, and propylene glycol were clearly identified without the appearance of new peaks or significant peak shifts, indicating the absence of chemical interactions or degradation during the solvent casting process.

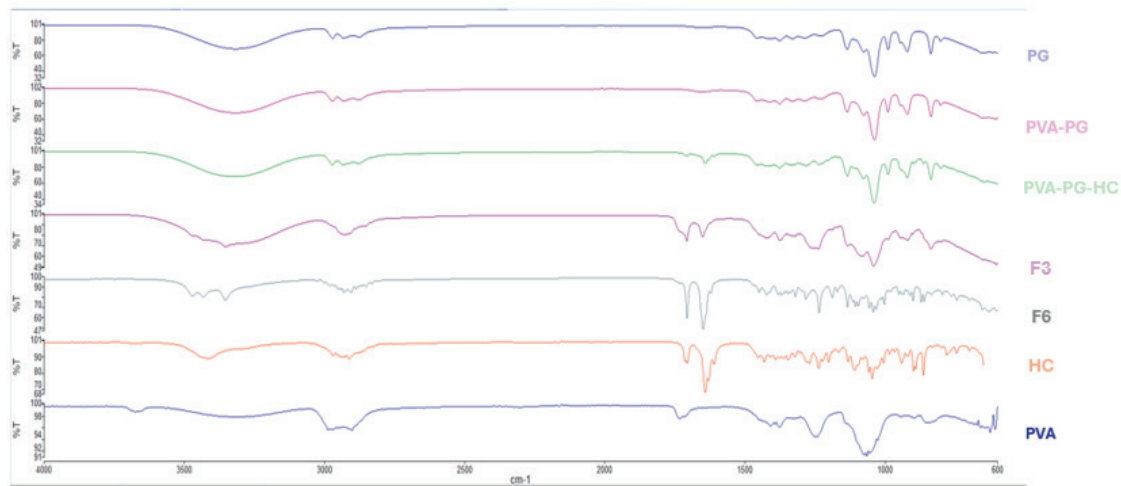


Figure 2. FTIR spectrum of HC, PVA, PG, PVA: PG, HC: PVA: PG, F3, and F6 formulation.

Weight and thickness of buccal film formulation

The uniformity in weight and thickness across different film samples indicates homogeneity of the production process, which is essential for dose accuracy and consistent drug release in buccal film formulations. In terms of weight, the lowest value was recorded as F2, and the highest as F6. In terms of thickness, the thinnest film was determined as F5, and the thickest as F6. As seen in Table 2, although there are certain differences between the weight and thickness values of all formulations, the low standard deviations indicate that the production process is homogeneous

and repeatable. The low variability within each formulation batch confirms the homogeneity of the solvent casting method, supporting its suitability for producing reproducible buccal film dosage forms. The F6 film contains both the hydrocortisone and propylene glycol, which together increase total solids and solution viscosity relative to placebo films. PG is less volatile and more hygroscopic than PEG, and also reduces drying shrinkage. These factors plausibly lead to the slightly higher weight-per-area and thickness recorded for F6.

Table 2. Weight variation and thickness measurements of buccal film formulations (mean $\pm$ SD, n=3).

Formulation Code	Variation in mass (g)	Thickness (μm)
F1	0.0422 ± 0.0070	424.9640 ± 4.0825
F2	0.0322 ± 0.0013	282.3333 ± 5.4365
F3	0.0508 ± 0.0023	433.6667 ± 6.3421
F4	0.0466 ± 0.0031	376.3333 ± 2.8674
F5	0.0500 ± 0.0033	244.6667 ± 2.4944
F6	0.0626 ± 0.0052	463.3333 ± 5.3125

Mechanical properties of buccal film formulation

Buccal films should have sufficient tensile strength and high elongation at break to ensure mechanical stability and ease of application (Preis, Knop, &

Breitkreutz, 2014). The tensile strength and elongation at break values are given in Figure 3. Both the type and concentration of the polymer affect these mechanical properties. Among all the formulations, F4 showed the highest tensile strength and elongation at break. These results suggest that the film can be applied

without damaging the mucosa during administration and are likely to support good patient compliance. The choice of plasticizer also affected the mechanical properties of the formulations. The F2 formulation, which uses glycerin as a plasticizer, showed the highest tensile strength. Statistical analysis revealed that the tensile strength of F2 was significantly higher than all other formulations ($p < 0.05$). Glycerin, when combined with PVA, provides both mechanical strength and flexibility. By contrast, the use of PEG reduced the elongation at break, while PG-containing formulations exhibited higher tensile strength and elongation compared with PEG-based films. The use of PEG in film formulations reduced elongation at break value. Formulations with PG showed higher tensile strength and elongation at break compared to

PEG and PG-containing formulations. Mechanical characterization shows that all film formulations have sufficient tensile strength and flexibility for buccal application. Mechanical properties are crucial for ease of application, patient comfort, and retention. Preis and colleagues reported elongation values ranging from 4-26% for buccal film samples they prepared and 1-6% for commercially available products (Preis et al., 2014). The mechanical parameters of films are crucial factors not only in production or development but also in proper patient use. Buccal films require sufficient mechanical integrity to prevent breakage during application while maintaining flexibility to accommodate the dynamic movements of the oral cavity.

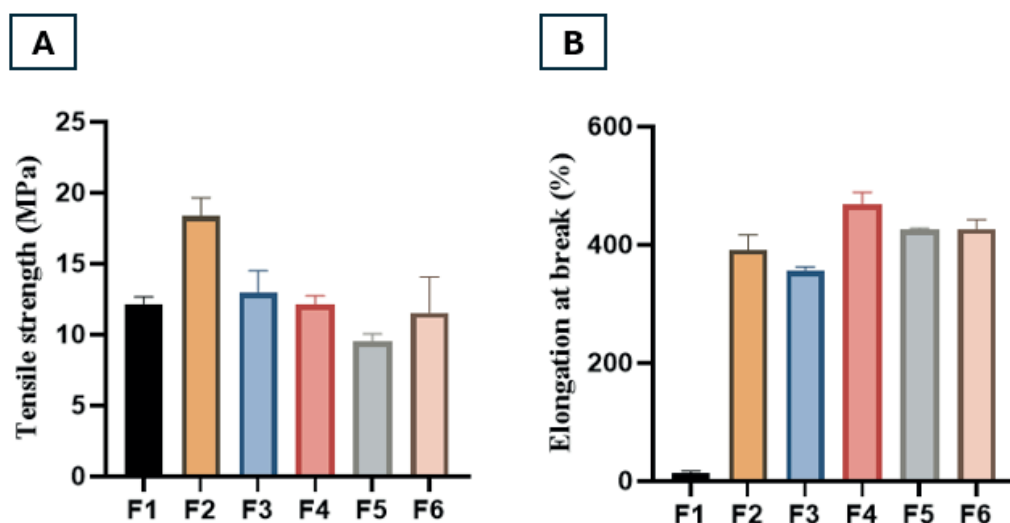


Figure 3. Mechanical properties of buccal film formulations: (a) tensile strength (MPa), and (b) elongation at break (%), measured using a texture analyzer with tensile grip setup ($n = 3$, mean $\pm$ SD).

Contact angle measurements of films

As shown in Figure 4, all film formulations exhibited contact angle values below 90° , indicating their hydrophilic character. Contact angle values were consistent with the properties of PVA-based materials, which are characterized by strong water affinity due to the abundance of hydroxyl groups in the polymer

structure. This parameter plays a crucial role in enhancing the wettability and potential mucoadhesive performance of the films upon buccal application. Contact angle results align with literature reports on PVA-based films: for instance, Wu et al. (2020) found that pure PVA films have been shown to exhibit contact angles of approximately $45.5^\circ \pm 1.1^\circ$ (Wu et

al., 2020). Similarly, in our study, contact angle values ranged from 55° to 67°, demonstrating hydrophilic characteristics and supporting the suitability of the films for buccal delivery. Statistical analysis revealed no significant differences among the contact angle values of the different formulations ($p > 0.05$).

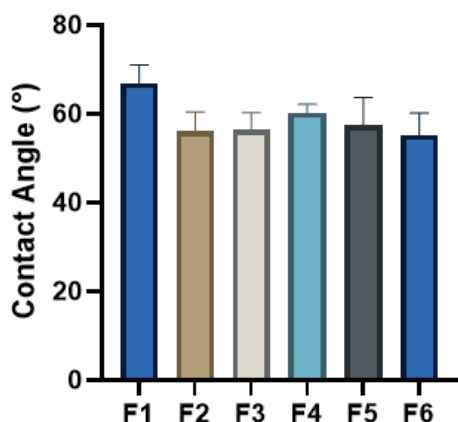


Figure 4. Contact angle values of buccal film formulations measured using distilled water ($n = 3$, mean $\pm$ SD)

Ex vivo mucoadhesion studies of buccal film formulation

Mucoadhesive polymer systems that exhibit similar structures and functional groupings to the mucus layer can result in a greater degree of diffusibility of the polymer across the mucosal surface (Andrews, Laverty, & Jones, 2009). Plasticizers can alter the flexibility, tensile strength, and adhesion properties of film formulations (Saringat, Alfadol, & Khan, 2005). The presence of plasticizers may improve chain mobility and promote interpenetration with mucosal surfaces, but excessive plasticizers can also reduce cohesive strength and lead to lower mucoadhesion. In formulations containing PG, mucoadhesion was acceptable but slightly lower compared to those containing glycerin or PEG (Figure 5). Glycerin is known to increase hydrophilicity and plasticity, but it can cause excessive swelling of the hygroscopic structure, potentially compromising its structural integrity. PEG contributes to flexibility

but can reduce adhesive interactions within the film matrix and potentially limit mucosal retention when used at high concentrations. Shojaei and Li examined the effects of hydration on mucoadhesion observed in PEG copolymers and reported that films with higher hydration had lower mucoadhesive strengths. This is because the mucoadhesive properties of the polymer film are reduced due to excessive elongation of the mucoadhesive bonds at higher hydration (Shojaei & Li, 1997). F5 exhibited the highest mucoadhesion performance, demonstrating the importance of optimal polymer-plasticizer ratios. F4 exhibited the lowest mucoadhesion performance despite its high mechanical properties. Although all formulations exhibited mucoadhesive properties, statistical analysis showed a significant difference between F4 and F5 ($p < 0.05$), suggesting that the polymer-plasticizer combination in F5 enhanced mucoadhesion. All film formulations exhibited adequate mucoadhesive strength for buccal application. These findings demonstrate the potential of PVA-based films to achieve effective and prolonged residence time in the buccal mucosa, which is essential for local drug delivery in oral ulcer treatment.

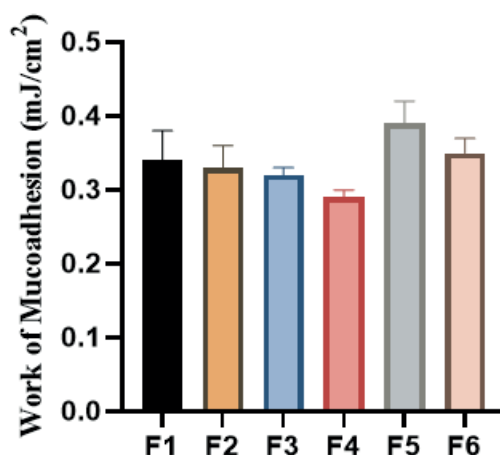


Figure 5. Ex vivo mucoadhesion performance of buccal film formulations evaluated on cow buccal mucosa using a texture analyzer ($n = 3$, mean $\pm$ SD).

***In vitro* drug release of buccal film formulation**

The *in vitro* release profile of the film formulations showed a controlled release without a burst effect within the first few hours. Among the prepared formulations, only F6 contained hydrocortisone (0.2% w/v) and therefore was selected for *in vitro* drug release studies (Figure 6). The other formulations (F1–F5) were primarily used to optimize polymer–plasticizer ratios, mechanical properties, and mucoadhesion performance before drug loading. Based on these evaluations, F6 exhibited the most favorable combination of mechanical strength, flexibility, and mucoadhesive performance, making it the optimal candidate for drug loading and subsequent release studies. The F6 formulation released HC at a rate of $93.2 \pm 3.1\%$ over 8 hours. This suggests that the film matrix has a high drug retention capacity and a structure that supports controlled

release. This controlled release was achieved by a combination of factors such as the PVA polymer and the type of plasticizer. HC release from PVA films occurs via a diffusion-based mechanism controlled by polymer swelling and dissolution. The 8-hour release duration suggests that once-daily dosing can achieve therapeutic efficacy and increase patient compliance. The release profile is suitable for maintaining local anti-inflammatory effects by providing sufficient residence time in the buccal mucosal area. In a study by Khan et al. (2024) the development of diclofenac sodium mucoadhesive films achieved a cumulative release of $80 \pm 5\%$ over 8 hours, meeting the therapeutic criteria for anti-inflammatory efficacy (Khan et al., 2024). Extended release up to 8 hours supports reduced dosing frequency, thereby enhancing patient compliance.

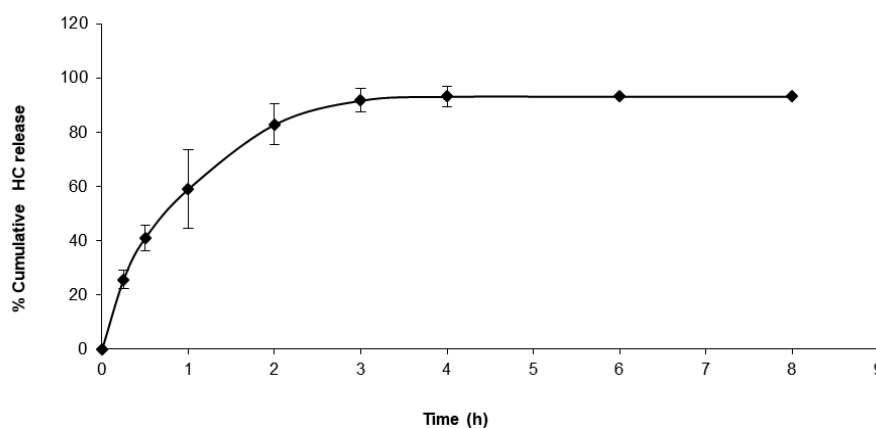


Figure 6. *In vitro* release profile of HC from F6 buccal film formulation using Franz diffusion cells over 8 hours (n=3, Mean±SD)

CONCLUSION

The films were successfully fabricated via the solvent casting method. In conclusion, PVA-based hydrocortisone-loaded buccal films exhibited promising physicochemical and mechanical properties suitable for buccal application. Blank films were characterized against mechanical integrity, surface wettability suitable for buccal use, and mucoadhesive performance. The formulation containing PVA and propylene gly-

col exhibited optimal tensile strength and high elongation at break for buccal application. The PG-plasticized blank formulation (F3) provided the best overall balance for robust processing and buccal retention and was therefore selected as the lead placebo. F6 was then generated by incorporating hydrocortisone into the F3 matrix, and *in vitro* release studies were conducted. The films demonstrated adequate mucoadhesion and controlled drug release, both of which

are critical for effective local treatment of oral ulcers. These findings suggest that film formulations could contribute to improved therapeutic outcomes in the management of oral aphthous lesions. Future studies should focus on *in vivo* performance, stability profiling, and patient-centric evaluations to further advance clinical applicability.

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AUTHOR CONTRIBUTION STATEMENT

BA: Concept, Design, Data Collection or Processing, Analysis or Interpretation, Literature Search, Writing. SS: Design, Data Collection or Processing, Analysis or Interpretation, Literature Search, Writing. FNT: Concept, Design, Data Collection or Processing, Literature Search, Writing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Comparative Antioxidant Activities and Total Phenolic Content of *Hypericum perforatum*, *H. scabrum*, and *H. heterophyllum* from Different Regions of Türkiye

Osman Tuncay AGAR^{**}, Bekir TOSUN^{**}, Tunahan DEMIRCI^{***}

Comparative Antioxidant Activities and Total Phenolic Content of *Hypericum perforatum*, *H. scabrum*, and *H. heterophyllum* from Different Regions of Türkiye

SUMMARY

The genus *Hypericum* is a rich source of bioactive compounds, particularly phenolics and flavonoids, which play a key role in antioxidant activity. While *H. perforatum* (St. John's Wort) has been extensively investigated, comparative evaluations of Turkish representatives of the genus, including the widespread *H. scabrum* and the endemic *H. heterophyllum*, remain limited. This study compared the antioxidant capacities and total phenolic content (TPC) of these three species collected from different regions of Türkiye. Aerial parts were extracted with 70% ethanol using ultrasonic-assisted extraction, and the TPC was determined by the Folin–Ciocalteu method. Antioxidant activities were assessed by DPPH and ABTS radical scavenging assays, with Trolox as the reference standard. *H. perforatum* exhibited the highest TPC (201.4 ± 2.8 mg GAE/g extract) and antioxidant activity (IC_{50} : 9.82 ± 0.76 µg/mL for DPPH; 7.01 ± 0.98 µg/mL for ABTS), followed by *H. heterophyllum* (141.9 ± 1.9 mg GAE/g; 17.16 ± 0.67 and 10.10 ± 1.15 µg/mL). *H. scabrum* showed the lowest TPC (129.1 ± 2.1 mg GAE/g) and weakest activity (24.17 ± 0.65 and 9.21 ± 1.22 µg/mL). Pearson's correlation analysis revealed strong negative associations between TPC and IC_{50} values ($r = -0.94$ for DPPH; $r = -0.90$ for ABTS), confirming phenolics as the main contributors to radical scavenging potential. These findings highlight clear interspecific differences in antioxidant capacity within *Hypericum*, reaffirming the pharmacological value of *H. perforatum* and pointing to the underexplored potential of *H. heterophyllum*.

Keywords: *Hypericum perforatum*, *Hypericum scabrum*, *Hypericum heterophyllum*, total phenolic content, DPPH, ABTS.

Türkiye'nin Farklı Bölgelerinden Toplanan *Hypericum perforatum*, *H. scabrum* ve *H. heterophyllum* Türlerinin Karşılaştırmalı Antioksidan Aktiviteleri ve Toplam Fenolik İçeriği

ÖZ

Hypericum cinsi, özellikle fenolikler ve flavonoidler açısından zengin biyoaktif bileşikler içermekte olup, bu bileşikler antioksidan aktivitede önemli rol oynamaktadır. *H. perforatum* (Sarı Kantaron) kapsamlı şekilde araştırılmış olsa da, cinsin Türkiye'deki temsilcileri arasında yaygın olarak bulunan *H. scabrum* ve endemik *H. heterophyllum* üzerine yapılan karşılaştırmalı çalışmalar sınırlıdır. Bu çalışmada Türkiye'nin farklı bölgelerinden toplanan bu üç türün toplam fenolik içerikleri (TFİ) ve antioksidan aktiviteleri karşılaştırılmıştır. Bitkilerin toprak üstü kısımları %70 etanol ile ultrasonik destekli ekstraksiyon yöntemiyle ekstrakte edilmiş, TFİ Folin–Ciocalteu yöntemiyle belirlenmiştir. Antioksidan aktiviteler DPPH ve ABTS radikal süpürme testleri ile değerlendirilmiş, Trolox referans standart olarak kullanılmıştır. *H. perforatum* en yüksek TFİ'ye ($201,4 \pm 2,8$ mg GAE/g ekstre) ve antioksidan aktiviteye (IC_{50} : DPPH için $9,82 \pm 0,76$ µg/mL; ABTS için $7,01 \pm 0,98$ µg/mL) sahip tür olmuştur. Bunu *H. heterophyllum* ($141,9 \pm 1,9$ mg GAE/g; $17,16 \pm 0,67$ ve $10,10 \pm 1,15$ µg/mL) izlemiş, *H. scabrum* ise en düşük TFİ'ye ($129,1 \pm 2,1$ mg GAE/g) ve en zayıf aktiviteye ($24,17 \pm 0,65$ ve $9,21 \pm 1,22$ µg/mL) sahip olmuştur. Pearson korelasyon analizi, TFİ ile IC_{50} değerleri arasında güçlü negatif ilişkiler olduğunu göstermiştir (DPPH için $r = -0,94$; ABTS için $r = -0,90$). Bu sonuçlar, fenolik bileşiklerin antioksidan potansiyelin başlıca belirleyicileri olduğunu doğrulamaktadır. Bulgular, *Hypericum* türleri arasında belirgin antioksidan kapasite farklılıklarını ortaya koymakta, *H. perforatum*'un farmakolojik önemini yeniden vurgularken *H. heterophyllum*'un da keşfedilmemiş potansiyeline işaret etmektedir.

Anahtar Kelimeler: *Hypericum perforatum*, *Hypericum scabrum*, *Hypericum heterophyllum*, toplam fenolik içerik, DPPH, ABTS.

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* ORCID: 0000-0002-1676-2443, Department of Pharmacognosy, Faculty of Pharmacy, Suleyman Demirel University, Isparta, 32260, Türkiye.

** ORCID: 0000-0002-2470-3865, Department of Plant and Animal Production, Burdur Vocational School of Food, Agriculture and Livestock, Burdur Mehmet Akif Ersoy University, Burdur, Türkiye.

*** ORCID: 0000-0001-8516-911X, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Suleyman Demirel University, Isparta, 32260, Türkiye.

INTRODUCTION

The genus *Hypericum* (family Hypericaceae) comprises more than 500 species distributed worldwide, many of which have long-standing use in traditional medicine for their antidepressant, anti-inflammatory, antimicrobial, and wound-healing properties (El-Chaghaby et al., 2024; Özkan & Mat, 2013). Türkiye represents one of the main centers of *Hypericum* diversity. Floristic surveys report between 98 and 106 taxa distributed across 19–20 sections, with approximately 45–49 species being endemic to the country (Ersoy, Eroglu Ozkan, Boga, Yilmaz, & Mat, 2019; Özkan & Mat, 2013; Uskutoglu & Coşge Şenkal, 2021). This high diversity and endemism highlight the importance of Türkiye as a reservoir of *Hypericum* species and justify comparative phytochemical studies. Among these, *Hypericum perforatum* L. (St. John's Wort) is the most extensively investigated, with standardized extracts rich in phenolics, flavonoids, and phloroglucinols such as hyperforin, which are considered key contributors to its pharmacological effects, including antioxidant activity (Orcic, Mimica-Dukic, Franciskovic, Petrovic, & Jovin, 2011). In particular, the abundance of phenolic compounds in *Hypericum* is regarded as a critical determinant of bioactivity, and total phenolic content (TPC) has been widely employed as a reliable indicator of antioxidant potential in plant extracts.

Antioxidant activity in *Hypericum* species is closely linked to their secondary metabolites, particularly phenolic acids and flavonoids, which exert radical-scavenging and metal-chelating properties (Öztürk, Tunçel, & Potoğlu-Erkara, 2009). Numerous studies have shown that *H. perforatum* extracts display potent antioxidant capacity across multiple assay systems, often correlating strongly with high total phenolic content (Seyrekoglu, Temiz, Eser, & Yildirim, 2022). Other taxa distributed in Türkiye, such as *H. scabrum* and *H. heterophyllum*, have also been reported to contain diverse phenolic compounds with promising biological activity. *H. scabrum* is known to possess notable levels of phenolics and flavonoids associated with antioxidant and cytotoxic effects (Jiang

et al., 2015), while *H. heterophyllum*, a species endemic to Türkiye, has demonstrated significant radical scavenging potential supported by its phenolic composition (Hazman et al., 2022; Yaman, Erenler, Atalar, Adem, & Çalışkan, 2024). These observations highlight the central role of phenolic richness, measurable through TPC assays, in determining the antioxidant potential of *Hypericum* extracts.

Although these species have each been studied individually, direct comparative evaluations across multiple *Hypericum* taxa remain limited. Most prior work has emphasized either single-species assessments or broad surveys without detailed cross-species comparisons (Özdemir, Uzun, Gül, Gül, & Çon, 2020). Furthermore, while TPC has frequently been used to estimate antioxidant potential in plants, systematic comparisons of TPC values across *Hypericum* species remain relatively rare — among the few are studies of nine *Hypericum* taxa in Greece comparing TPC/total flavonoid content (TFC) and antioxidant assays (Kakouri et al., 2023), and phenolic profiling among seven species showing consistent interspecific differences in Europe (Zdunic, Godjevac, Savikin, & Petrovic, 2017). Understanding interspecific differences is therefore crucial, as antioxidant activity may vary not only with environmental influences but also with intrinsic phytochemical diversity and total phenolic content among taxa.

In this context, the present study aimed to compare the antioxidant activities and TPC of three *Hypericum* species distributed in Türkiye—*H. perforatum*, *H. scabrum*, and *H. heterophyllum*. These taxa were evaluated using two widely applied radical scavenging assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), alongside TPC determination to better explain the observed bioactivities. By situating these findings within the broader literature on *Hypericum* phytochemistry, this study seeks to clarify interspecific variation in antioxidant capacity, establish correlations with phenolic content, and highlight the pharmacological relevance of lesser-studied species alongside the well-known *H. perforatum*.

MATERIAL and METHOD

Plant material

Aerial parts of three *Hypericum* species were collected from different regions of Türkiye during their flowering period. The species were identified by Dr. Osman Tuncay Agar, and voucher specimens were

deposited in the Herbarium of the Department of Pharmaceutical Botany (HUEF), Hacettepe University, Ankara, Türkiye. Collection details are summarized in Table 1, and voucher specimens are preserved at HUEF for future reference. These authenticated materials provided the basis for subsequent TPC and antioxidant evaluations.

Table 1. Collection details of the *Hypericum* species investigated in this study

Species	Herbarium No.	Locality & Province	Coordinates	Altitude (m)	Habitat
HH	HUEF 14013	Ankara, Kızılcahamam District, between Gebeler and Berçinçatak Villages (5 km past Gebeler Village)	40°39'39" N, 32°30'58" E	1288	Rocky slopes, montane vegetation
HS	HUEF 14038	Sivas, Yenicebucuk, 2 km before Bengiser Plateau, mountain road	36°23'45" N, 36°02'10" E	1750	High mountain steppe
HP	HUEF 14035	Hatay, Samandağ, Çevlik, Musa Mountain, Kapısıyü Village	36°8'2.28" N, 35°57'6.59" E	376	Mediterranean scrub

HP: *H. perforatum*; HS: *H. scabrum*; HH: *H. heterophyllum*

Extraction

Dried aerial parts (5 g) of each *Hypericum* species were extracted with 40 mL of 70% (v/v) aqueous ethanol. This solvent system was selected for its proven efficacy in solubilizing both polar and moderately non-polar phytochemicals, particularly phenolic acids and flavonoids. Extraction was performed in an ultrasonic water bath, a method known to enhance cell wall disruption and improve the recovery of bioactive constituents, including phenolics, in *Hypericum* species (Gao, Hu, Shen, Zheng, & Liang, 2023; Tahmasebi-Boldaji, Hatamipour, Khanahmadi, Sadeh, & Najafipour, 2019).

Each sample was processed in triplicate to ensure reproducibility, and the combined extracts were concentrated under reduced pressure at 40 °C using a rotary evaporator to remove the ethanol fraction. The residues were re-dissolved in distilled water, frozen, and subsequently lyophilized to obtain dry powdered extracts suitable for both antioxidant and phenolic content analyses. The extraction yields, expressed as % (w/w) of dry extract relative to initial plant material, were 18.4% for *H. perforatum*, 15.7% for *H. heterophyllum*, and 12.9% for *H. scabrum*, with *H. perfora-*

tum giving the highest yield. Comparable ethanol–ultrasonic–lyophilization protocols, with minor modifications in solvent ratios and extraction times, have been successfully applied in studies of *Hypericum* taxa (Charalambous et al., 2025; Kakouri et al., 2023).

Determination of total phenolic content

TPC was quantified using the Folin–Ciocalteu colorimetric method, with minor modifications from Lucas, Dalla, Boeira, Verruck, and Rosa (2022). Briefly, 0.5 mL of each 70% aqueous ethanol extract was mixed with 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent and allowed to react for 5 min. Subsequently, 2.0 mL of 7.5% (w/v) sodium carbonate solution was added. The mixtures were vortexed and incubated in the dark at room temperature for 30 min.

Absorbance was measured at 765 nm using a UV–Vis spectrophotometer. A gallic acid calibration curve was employed for quantification, and results were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g extract). All measurements were carried out in triplicate and reported as mean ± standard deviation (SD).

Although Folin–Ciocalteu is a non-specific reagent that may react with other reducing substances,

it remains one of the most widely accepted methods for estimating total phenolic levels in plant extracts. In this study, TPC determination was additionally performed to assess its relationship with antioxidant activity measured by DPPH and ABTS assays.

DPPH radical scavenging assay

The antioxidant capacity of the extracts was determined using the DPPH radical scavenging method, with minor modifications from previously described protocols for *Hypericum* species (Orcic et al., 2011; Öztürk et al., 2009). Briefly, 40 µL of each extract solution was added to 260 µL of freshly prepared DPPH methanolic solution in a 96-well microplate. The mixtures were incubated at 25 °C in the dark for 30 min, after which absorbance was measured at 517 nm using a microplate reader.

Radical scavenging activity was calculated as the percentage inhibition of DPPH relative to a blank control. IC₅₀ values (µg/mL) were determined from concentration–response curves by nonlinear regression. Trolox was used as a standard antioxidant for calibration. All experiments were performed in triplicate, and results were expressed as mean ± SD.

The DPPH assay was selected due to its reproducibility and widespread application in evaluating lipophilic antioxidant activity in plant extracts. In this study, DPPH results were also compared with TPC values to assess potential correlations between phenolic levels and radical scavenging capacity.

ABTS radical cation scavenging assay

The antioxidant activity of the extracts was further assessed using the ABTS radical cation decolorization assay, with minor modifications from established protocols (Kakouri et al., 2023; Öztürk et al., 2009). Briefly, 10 µL of each extract solution was added to 290 µL of ABTS radical solution in a 96-well microplate. The mixtures were incubated at 25 °C in the dark for 6 min, after which absorbance was recorded at 734 nm using a microplate spectrophotometer.

Radical scavenging activity was expressed as percentage inhibition relative to a blank control. IC₅₀ val-

ues (µg/mL) were calculated from concentration–response curves using Trolox as a standard antioxidant. All measurements were carried out in triplicate and reported as mean ± SD.

The ABTS assay was included because of its broader solubility range, allowing evaluation of both hydrophilic and lipophilic antioxidant compounds. Together with the DPPH method, it provided complementary insights into radical scavenging potential. In addition, ABTS IC₅₀ values were compared with TPC to explore correlations between phenolic content and antioxidant activity.

Statistical analysis

For both DPPH and ABTS assays, Trolox was used as a reference antioxidant to generate calibration curves, and results were expressed as half-maximal inhibitory concentration (IC₅₀) values in µg/mL. IC₅₀ values were obtained from concentration–response curves using a four-parameter logistic nonlinear regression model. All experiments were performed in triplicate, and data were reported as mean ± SD.

Interspecific differences in antioxidant activity were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. A significance level of $\alpha = 0.05$ was applied in all cases.

To explore the relationship between phenolic content and radical scavenging capacity, Pearson's correlation coefficients (*r*) were calculated between TPC values and IC₅₀ values obtained from both DPPH and ABTS assays. Statistical analyses were performed using software packages suitable for nonlinear regression, correlation analysis, and post-hoc testing.

RESULTS and DISCUSSION

Total phenolic content

TPC of 70% aqueous ethanol extracts from *Hypericum perforatum*, *H. scabrum*, and *H. heterophyllum* was determined using the Folin–Ciocalteu method (Table 2). Among the three taxa, *H. perforatum* exhibited the highest TPC (201.4 ± 2.8 mg GAE/g extract), followed by *H. heterophyllum* (141.9 ± 1.9 mg GAE/g),

while *H. scabrum* showed the lowest content (129.1 ± 2.1 mg GAE/g). Statistical analysis (one-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$) confirmed that all interspecific differences were significant.

These findings are consistent with previous reports, which likewise identified *H. perforatum* as phenolic-rich (Chimshirova, Karsheva, Diankov, & Hinkov, 2019) and *H. scabrum* as comparatively poorer in polyphenols (Kızıl, Kızıl, Yavuz, Emen, & Haki-moğlu, 2008). *H. heterophyllum* showed intermediate

levels, similar to values reported by Yaman (2020). Such variation reflects both species-specific biosyn-thetic capacity and ecological influences on second-ary metabolism.

Importantly, the observed ranking (*H. perforatum* > *H. heterophyllum* > *H. scabrum*) parallels trends later confirmed in antioxidant assays, supporting TPC as a reliable marker of radical scavenging potential in *Hypericum* extracts (Öztürk et al., 2009; Zdunic et al., 2017).

Table 2. Total phenolic content (TPC) of *Hypericum* species.

Species	TPC (mg GAE/g extract)
<i>H. perforatum</i>	201.4 ± 2.8^a
<i>H. heterophyllum</i>	141.9 ± 1.9^b
<i>H. scabrum</i>	129.1 ± 2.1^c

Values are expressed as mean $\pm$ SD ($n \geq 3$). Different superscript letters (a–c) indicate statistically significant differences between species according to Tukey's post-hoc test ($p < 0.05$).

Antioxidant activity

The antioxidant activities of *H. perforatum* (HP), *H. scabrum* (HS), and *H. heterophyllum* (HH) were evaluated using DPPH and ABTS radical scavenging assays, with IC_{50} values summarized in Table 3 and Figure 1. Trolox was used as the standard reference.

In the DPPH assay, HP exhibited the strongest activity (9.82 ± 0.76 μ g/mL), followed by HH (17.16 ± 0.67 μ g/mL), while HS was the least active ($24.17 \pm$

0.65 μ g/mL). Trolox, as expected, displayed the highest potency (3.72 ± 0.43 μ g/mL).

In the ABTS assay, a similar trend was observed, though the interspecific differences were narrower: HP (7.01 ± 0.98 μ g/mL) remained the most active, while HS (9.21 ± 1.22 μ g/mL) and HH (10.10 ± 1.15 μ g/mL) did not differ significantly from each other. Overall, IC_{50} values were consistently lower in ABTS compared to DPPH, reflecting higher assay sensitivity.

Table 3. IC_{50} values (μ g/mL, mean $\pm$ SD, $n \geq 3$) of *Hypericum* species and Trolox in DPPH and ABTS assays

Assay	HP	HS	HH	Trolox
DPPH	9.82 ± 0.76^a	24.17 ± 0.65^c	17.16 ± 0.67^b	3.72 ± 0.43^d
ABTS	7.01 ± 0.98^a	9.21 ± 1.22^b	10.10 ± 1.15^b	2.25 ± 0.66^c

HP: *H. perforatum*; HS: *H. scabrum*; HH: *H. heterophyllum*. Different superscript letters (a–d) indicate statistically significant differences between species according to Tukey's post-hoc test ($p < 0.05$).

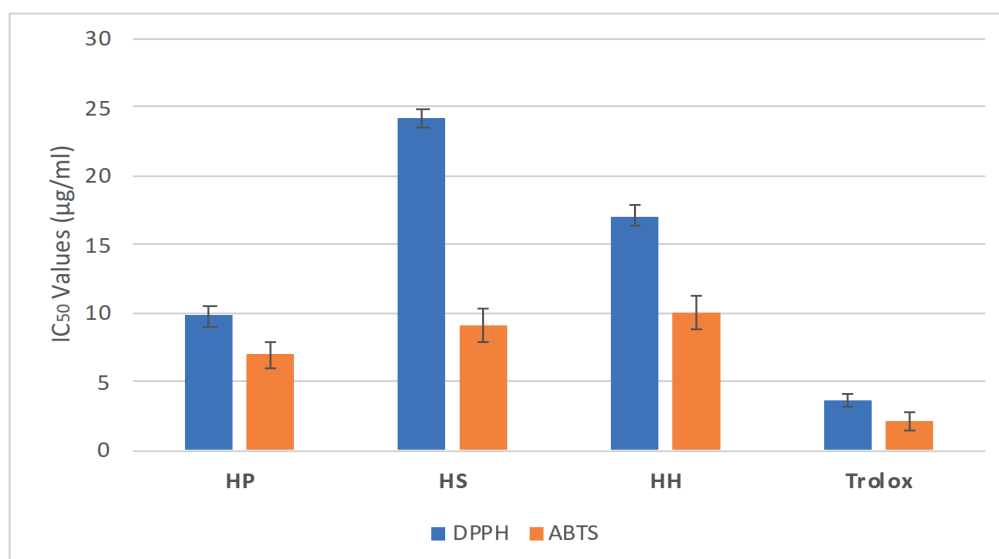


Figure 1. IC₅₀ values (µg/mL) of *Hypericum perforatum* (HP), *H. scabrum* (HS), *H. heterophyllum* (HH), and Trolox determined by DPPH and ABTS assays. Data are expressed as mean ± SD (n ≥ 3).

The observed ranking of antioxidant potency (HP > HH > HS) corresponds well with the known phytochemical richness of these taxa. *H. perforatum* is widely recognized as a phenolic- and flavonoid-rich species, containing chlorogenic acid, caffeic acid, hyperoside, rutin, and quercetin derivatives, all of which are strongly associated with radical scavenging capacity (Orcic et al., 2011; Öztürk et al., 2009). Its dominance in antioxidant assays has also been demonstrated in other comparative studies of Turkish *Hypericum*, where *H. perforatum* consistently outperformed co-occurring taxa (Seyrekoglu et al., 2022; Zdunic et al., 2017).

By contrast, *H. scabrum* is generally reported to contain lower concentrations of total phenolics and flavonoids, consistent with its weaker activity observed here. Previous phytochemical analyses identified modest levels of hyperoside and quercetin glycosides in *H. scabrum* (Jiang et al., 2015), and studies of other Turkish species show similar lower-level phenolic profiles (Cirak et al., 2017; Özdemir et al., 2020). Although *H. scabrum* has demonstrated other pharmacological properties, such as cytotoxic and antimicrobial activities (Stojanovic, Dordevic, &

Smelcerovic, 2013), its antioxidant potential appears comparatively limited.

H. heterophyllum occupied an intermediate position, displaying higher activity than HS but lower than HP. Recent work on *H. heterophyllum* flowers has shown significant TPC and TFC, with strong antioxidant activity in DPPH, ABTS, superoxide, and hydroxyl radical assays (Erenler, Yaman, Demirtas, & Hakki Alma, 2023; Eruygur et al., 2024). This species' phytochemical profile, including the presence of chlorogenic acid and quercetin derivatives (Hazman et al., 2022; Yaman et al., 2024), likely underlies its moderate antioxidant profile. This balanced composition suggests that *H. heterophyllum* may represent a promising but underexplored source of natural antioxidants.

A clear methodological distinction was also evident between the two radical scavenging assays. IC₅₀ values were consistently lower in the ABTS assay than in the DPPH assay, reflecting greater sensitivity of ABTS under the tested conditions. This is consistent with the physicochemical properties of the radicals employed. DPPH is a stable nitrogen-centered radical soluble primarily in organic solvents, and thus more suitable for assessing lipophilic antioxidants (Orcic

et al., 2011; Öztürk et al., 2009). By contrast, ABTS can be dissolved in both aqueous and organic media, allowing it to interact with a broader spectrum of hydrophilic as well as lipophilic compounds (Zheleva-Dimitrova, Nedialkov, & Kitanov, 2010).

Similar patterns have been reported in other studies of *Hypericum*. In Eruygur et al. (2024), extracts of *H. heterophyllum* showed significantly lower (i.e., more potent) IC_{50} values in ABTS compared to DPPH, alongside high phenolic/flavonoid contents. In a more recent study of *H. scabrum* leaves and flowers, multiple assay methods, including DPPH and ABTS, similarly suggested that ABTS may pick up polar antioxidants more efficiently (Muhammad, 2019). Studies on *H. empetrifolium* and *H. lydiu*m also reveal assay-dependent variations, with ABTS often giving more favorable (lower) values than DPPH (Sut et al., 2025).

Taken together, these observations emphasize the importance of employing multiple radical scavenging assays when evaluating antioxidant potential. While both DPPH and ABTS confirmed the same activity ranking (HP > HH > HS), their complementary sensitivities provided a more complete view of the antioxidant spectrum in *Hypericum* extracts (Zdunic et al., 2017).

Correlation between TPC and antioxidant activity

A strong inverse relationship was observed between TPC and antioxidant capacity, as measured by IC_{50} values (Figure 2). Pearson's correlation coefficients were $r = -0.94$ for TPC versus DPPH IC_{50} and $r = -0.90$ for TPC versus ABTS IC_{50} , both indicating strong negative associations. These findings demon-

strate that higher phenolic concentrations were consistently associated with stronger radical-scavenging activity across the tested *Hypericum* species.

Comparable results have been obtained in several recent studies. For example, *Hypericum perforatum* under chitosan treatment showed increases in both TPC and DPPH radical scavenging activity in mild water stress conditions (Amooaghaie & Rajaie, 2025). Błńska-Sikora, Zielińska, Dobros, Paradowska, and Michalak (2025) compared different commercial *H. perforatum* extracts and found a strong correlation between TPC/TFC and antioxidant assays like DPPH and FRAP. In *H. spectabile* optimized extracts, Gürgeç, Sevindik, Krupodorova, Uysal, and Unal (2024) reported that phenolic compound abundance (including caffeic acid and quercetin derivatives) was mirrored by high antioxidant activity. Studies in other *Hypericum* taxa, such as *H. cordifolium* and *H. repens*, similarly underscore the relationship between phenolic/flavonoid levels and radical scavenging ability (Sapkota, Maharjan, Tiwari, & Rajbhandari, 2024).

This supports the role of phenolic compounds as key chemotaxonomic and pharmacological markers within the genus. Notably, the slightly stronger correlation with the DPPH assay in our data suggests that phenolics in these taxa may include both hydrophilic and moderately lipophilic molecules, which contribute differentially depending on radical type and assay environment.

Collectively, these results highlight TPC not only as a reliable predictor of antioxidant potential but also as a valuable screening parameter for guiding further phytochemical and pharmacological studies of *Hypericum* species.

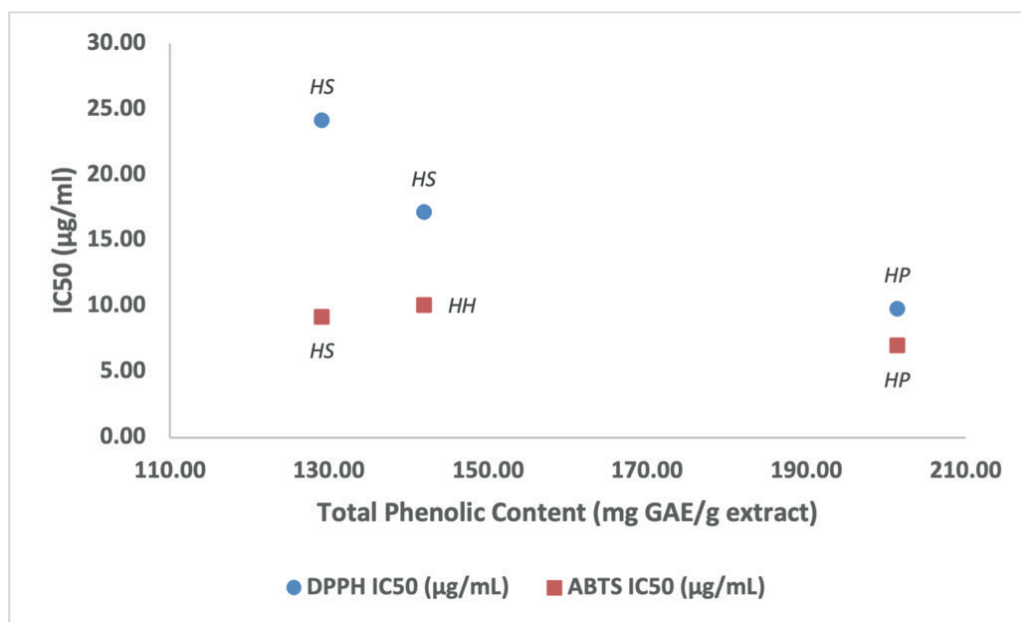


Figure 2. Correlation between total phenolic content (TPC, mg GAE/g extract) and antioxidant activity (IC₅₀ values for DPPH and ABTS assays) in *H. perforatum* (HP), *H. scabrum* (HS), and *H. heterophyllum* (HH). Pearson's correlation coefficients were $r = -0.94$ (DPPH) and $r = -0.90$ (ABTS), both indicating strong negative associations. Data represent mean $\pm$ SD ($n \geq 3$).

Limitations and future perspectives

While this study provides valuable comparative insights into the antioxidant potential of *H. perforatum*, *H. scabrum*, and *H. heterophyllum*, several limitations should be acknowledged. First, plant material was collected from single sites for each taxon. This constitutes a major limitation, as secondary metabolite accumulation in *Hypericum* species is strongly influenced by environmental and genetic factors such as altitude, soil structure, and climatic conditions. Consequently, the present findings should be interpreted with caution, as they may not fully represent the phytochemical and antioxidant profiles of these species across their broader geographical distribution. Previous studies have demonstrated substantial intraspecific variation in *Hypericum*, with phenolic content and antioxidant activity differing among populations depending on altitude, soil type, and climatic factors (Cirak et al., 2017; Özdemir et al., 2020). Similarly, Şanlı et al. (2015) reported marked location-dependent differences in hypericin content and quality

traits of Turkish *Hypericum*, further emphasizing the need for broader sampling. Broader multi-site and multi-seasonal sampling would therefore enhance the robustness of future comparative analyses.

Second, antioxidant capacity was assessed using only two radical scavenging assays (DPPH and ABTS). Although complementary, these methods capture only part of the antioxidant spectrum. Additional electron-transfer and hydrogen-donating assays, such as FRAP, CUPRAC, and ORAC, would provide a more comprehensive picture of redox-related activity (Zdunic et al., 2017).

Third, this work focused on bioactivity assays with only TPC analysis. Although TPC is a valuable screening parameter, it does not identify which specific metabolites are primarily responsible for the observed antioxidant activity. The absence of TFC measurements and compound-level profiling (e.g., HPLC-DAD or LC-MS/MS quantification of hyperoside, rutin, chlorogenic acid, quercetin derivatives) is a significant limitation. Therefore, while our results

provide useful comparative insights, they should be interpreted cautiously. Future studies should integrate TFC assays and chromatographic analyses to establish stronger correlations between specific metabolites and radical scavenging activity.

Future research should therefore integrate expanded chemical profiling, broader assay coverage, and multi-site ecological sampling to better capture the complexity of antioxidant diversity in *Hypericum*. Such integrative approaches will support the rational development of these species as pharmacologically relevant sources of natural antioxidants.

In summary, this study provides new comparative insights into interspecific differences in phenolic content and radical scavenging capacity among *H. perforatum*, *H. scabrum*, and the endemic *H. heterophyllum*. By including this underexplored taxon, our findings extend the phytochemical and pharmacological knowledge of Turkish *Hypericum* and strengthen the chemotaxonomic understanding of the genus.

CONCLUSION

This study comparatively evaluated the antioxidant activities of *Hypericum perforatum*, *H. scabrum*, and *H. heterophyllum* collected from different regions of Türkiye. Both DPPH and ABTS radical scavenging assays demonstrated clear interspecific differences, with *H. perforatum* exhibiting the strongest activity, *H. heterophyllum* showing intermediate activity, and *H. scabrum* being the least active. These results were consistent with TPC, which correlated strongly and negatively with IC₅₀ values ($r = -0.94$ for DPPH; $r = -0.90$ for ABTS), confirming phenolics as key contributors to the observed antioxidant potential.

While the study was limited by single-site sampling and the use of only two radical scavenging assays, the findings provide a valuable foundation for future investigations. Expanding chemical profiling to include flavonoid quantification and metabolite fingerprinting, alongside additional antioxidant assays, will be essential for clarifying structure–activity relationships and ecological influences within the genus.

In summary, this study contributes novel comparative data by linking phenolic content with antioxidant activity across three Turkish *Hypericum* taxa. The results reaffirm the pharmacological relevance of *H. perforatum* while also highlighting the underexplored potential of the endemic *H. heterophyllum* as a promising source of natural antioxidants.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization: O.T.A.; Investigation: O.T.A., B.T., T.D.; Methodology: O.T.A., B.T., T.D.; Validation: O.T.A.; Formal analysis: O.T.A., B.T.; Data curation: O.T.A., T.D.; Visualization: O.T.A.; Writing – original draft: O.T.A.; Writing – review & editing: O.T.A., B.T., T.D.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Liposomes and Chitosan-Coated Liposomes (Chitosomes) for the Delivery of Letrozole: A Comparative Study

Tahir Emre YALCIN^{**}, Nihal Tugce OZAKSUN^{**}, Cigdem YUCEL^{***},
Tuba INCECAYIR^{****}

Liposomes and Chitosan-Coated Liposomes (Chitosomes) for the Delivery of Letrozole: A Comparative Study

Letrozol İletimi için Lipozom ve Kitosan Kaplı Lipozomlar (Kitozomlar): Karşılaştırmalı Çalışma

SUMMARY

Letrozole (LTZ) is a first-line aromatase inhibitor for hormone-sensitive breast cancer in postmenopausal women, but its poor aqueous solubility and limited tissue selectivity reduce therapeutic efficacy. This study aimed to develop liposome and chitosan-coated liposome (chitosome) formulations as lipid-chitosan-based nanocarriers to enhance LTZ delivery, enable sustained release, and minimize side effects. LTZ-loaded liposomes were prepared by the thin-film hydration method, and the optimal formulation was selected for chitosan coating. The impact of chitosan on physicochemical properties was assessed through particle size, zeta potential, encapsulation efficiency, and stability studies. In vitro drug release was evaluated via dialysis at 37°C, and cytotoxicity was tested on MCF-7 breast cancer cells. Optimized liposomes and chitosomes exhibited mean particle sizes of 112 nm and 155 nm, respectively, with high encapsulation efficiency (>90%). Chitosan coating led to a slight reduction in the net negative zeta potential, decreasing it from -48.9 mV to -40.0 mV. Both formulations demonstrated sustained LTZ release and maintained their particle size stability after one month of storage at 2–8°C ($p>0.05$). Cytotoxicity assays indicated that both formulations exhibited similar effects to free LTZ on MCF-7 cells. In conclusion, LTZ-loaded liposome and chitosome formulations were successfully developed, offering a favorable particle size, high encapsulation efficiency, and sustained drug release. Chitosan coating modified surface properties and influenced formulation stability. These findings suggest that lipid-chitosan nanocarriers are promising platforms for LTZ delivery. Further in vivo studies are warranted to confirm their potential in breast cancer therapy.

Keywords: Letrozole, liposome, chitosome, MCF-7, cytotoxicity.

ÖZ

Letrozol (LTZ), postmenopozal kadınlarda hormon duyarlı meme kanserinin tedavisinde birinci basamak aromataz inhibitörüdür; ancak düşük suda çözünürlüğü ve sınırlı doku seçiciliği terapötik etkinliğini azaltmaktadır. Bu çalışmada, LTZ iletimini artırmak, kontrollü salım sağlamak ve yan etkileri en aza indirmek amacıyla lipid-kitosan bazlı nanotaşıyıcılar olan lipozom ve kitosan kaplı lipozom (kitazom) formülasyonlarının geliştirilmesi hedeflenmiştir. LTZ yüklü lipozomlar ince film hidrasyon yöntemi ile hazırlanmış ve en uygun formülasyon kitosan ile kaplanmıştır. Kitosan kaplamanın fizikokimyasal özelliklere etkisi; partikül boyutu, zeta potansiyeli, enkapsülasyon etkinliği ve stabilite çalışmaları ile değerlendirilmiştir. İn vitro ilaç salımı, 37°C'de diyaliz yöntemi ile; sitotoksikite ise MCF-7 meme kanseri hücrelerinde test edilmiştir. Optimum lipozom ve kitazom formülasyonlarının ortalama partikül boyutları sırasıyla 112 nm ve 155 nm olup, her iki formülasyon da yüksek enkapsülasyon etkinliği göstermiştir (>90%). Kitosan kaplaması, net negatif zeta potansiyelinde hafif bir azalmaya neden olmuş ve bu değer -48,9 mV'den -40,0 mV'ye düşmüştür. Her iki formülasyon da sürekli LTZ salımı sergilemiştir ve 2–8°C'de bir ay süreyle depolama sonrasında parçacık boyutunda anlamlı bir değişim göstermemiştir ($p>0.05$). Sitotoksikite testleri, her iki formülasyonun da serbest LTZ ile benzer etki gösterdiğini ortaya koymuştur. Sonuç olarak, LTZ yüklü lipozom ve kitozom formülasyonları başarıyla geliştirilmiş olup; bu formülasyonlar uygun bir parçacık boyutu, yüksek enkapsülasyon verimi ve sürekli ilaç salımı sunmuştur. Kitosan kaplama, yüzey özelliklerini değiştirmiş ve formülasyon stabilitesini etkilemiştir. Bulgular, lipid-kitosan bazlı nanotaşıyıcıların LTZ taşınmasında umut verici olduğunu ve potansiyellerinin doğrulanması için ileri in vivo çalışmalar gerektiğini göstermektedir.

Anahtar Kelimeler: Letrozol, lipozom, kitozom, MCF-7, sitotoksikite.

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^{*} ORCID: 0000-0001-5834-2335, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Türkiye

^{**} ORCID: 0000-0001-6276-9227, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Türkiye

^{***} ORCID: 0000-0002-0622-5150, Department of Pharmaceutical Technology, Faculty of Pharmacy, Erciyes University, 38039 Kayseri, Türkiye

^{****} ORCID: 0000-0002-7106-7929, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Türkiye

INTRODUCTION

Breast cancer is the most common type of cancer among women worldwide and accounts for the majority of cancer-related deaths in this population (Maajani et al., 2019). Estrogens have been shown to play a critical role in the development and progression of breast cancer (Samavat & Kurzer, 2015). Consequently, reducing circulating estrogen levels is a well-established therapeutic strategy aimed at limiting the growth of estrogen-dependent tumors (Simon et al., 2002). Several pharmacologically active compounds are used for this purpose, among which letrozole (LTZ) is a widely utilized agent.

LTZ is a third-generation non-steroidal aromatase inhibitor and has been approved for both primary and secondary treatment of breast cancer (Fenton et al., 2004). Clinical studies have demonstrated that LTZ significantly reduces circulating estrogen levels (Bhatnagar, 2007). However, LTZ is associated with a range of adverse effects, including hot flashes, headaches, dizziness, agitation, anxiety, insomnia, dry skin, and hypertension (Iqbal et al., 2019). Its limited therapeutic efficacy is primarily attributed to its excessive partitioning into red blood cells and insufficient accumulation within tumor tissues following intravenous administration (Ijaz et al., 2024). Moreover, LTZ's clinical utility is further constrained by dose-dependent toxicities, including neurotoxicity. The systemic delivery of LTZ and other chemotherapeutic agents often results in off-target effects, as these drugs impact both malignant and healthy cells. Considering these limitations, there is a clear need for an alternative drug delivery strategy that enhances antitumor efficacy while minimizing toxicity to normal tissues. One promising approach to overcome these challenges involves the use of nanocarriers for the targeted delivery of chemotherapeutic agents.

Various strategies have been developed to reduce the side effects of LTZ and improve its targeted de-

livery to tumor tissues. One such approach involves encapsulating LTZ within liposomes. Liposomes are spherical vesicles composed of a phospholipid bilayer surrounding an aqueous core. They exhibit several advantageous properties, such as small particle size (PS), high encapsulation efficiency (EE%) of hydrophobic drugs, and biocompatibility with human cells (Liu et al., 2016; Maja et al., 2020). Despite these favorable characteristics, liposomes are associated with certain stability issues (Sogut et al., 2021). To address this limitation, surface modification with different polymers has been explored to improve their physicochemical stability.

Chitosan is a natural aminopolysaccharide derived from the deacetylation of chitin (Motiei et al., 2017). Owing to its positive charge and bioadhesive properties, chitosan readily interacts with negatively charged drug delivery systems (Yuan et al., 2006). Chitosomes, a hybrid system formed by coating liposome surfaces with chitosan, can enhance the physicochemical stability of liposomes and promote improved drug retention and tumor-targeting efficiency (Esposto et al., 2021; Zhou et al., 2018).

In this study, LTZ-loaded liposomes were prepared using soy phosphatidylcholine (SPC) in combination with two different excipients: cholesterol (CHOL) and cholesteryl hemisuccinate (CHEMS). Based on PS and zeta potential (ZP) measurements, the most suitable liposome formulation was selected and subsequently coated with chitosan. The EE%, *in vitro* release profiles, and physicochemical stability of the prepared formulations were systematically evaluated. The cytotoxicity of the optimized liposome and chitosome formulations was further evaluated in MCF-7 cells via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In this context, LTZ-loaded chitosome formulations were developed and characterized for the first time, contributing novel findings to the literature.

MATERIAL AND METHODS

Materials

LTZ, the active pharmaceutical ingredient, was kindly provided by Deva Holding (Istanbul, Türkiye). CHOL, CHEMS, low molecular weight (MW) chitosan, and dialysis tubing cellulose membranes were purchased from Sigma-Aldrich (St. Louis, MO, USA). L- α -soy phosphatidylcholine (95%) (SPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Polysorbate 80 and sucrose were procured from Merck (Darmstadt, Germany). All other chemicals were of analytical grade, and all solvents were of high-performance liquid chromatography (HPLC) grade.

The MCF-7 human breast cancer cell line (ATCC CCL-222) was obtained from the American Type Culture Collection (Manassas, VA, USA) for cytotoxicity studies. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Grand Island, NY, USA).

Preparation of liposomes

LTZ-loaded liposomes were prepared using the thin-film hydration method. Briefly, LTZ (10 mg), SPC, and either CHOL or CHEMS were dissolved in a chloroform: methanol mixture (2:1, v/v) (Vu et al., 2020). The organic solvents were subsequently

removed under reduced pressure at 60°C using a rotary evaporator (Rotavapor® R-3, Büchi, Switzerland), and a thin lipid film was formed. This film was hydrated with phosphate buffer solution (PBS, pH 7.4) and subjected to probe sonication (Sonics VCX 130 FSJ, Newtown, CT, USA) for 15 minutes with a 2-second /3-second on/off pulse cycle to reduce PS. The resulting liposomal dispersions were then extruded through polycarbonate membranes with a pore size of 200 nm using an extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA) to obtain uniform vesicle sizes.

Preparation of chitosomes

Different concentrations of low molecular weight chitosan were dissolved in a 1% v/v aqueous solution of glacial acetic acid. The liposome suspension was then added dropwise into an equal volume of the chitosan solution under continuous magnetic stirring at 500 rpm for 1 hour (Lee et al., 2019). Subsequently, both liposomes and chitosomes were transferred into dialysis bags (molecular weight cut-off: 14,000 Da) to remove unencapsulated LTZ prior to lyophilization. The dialysis bags were immersed in 400 mL of PBS (pH 7.4) and stirred at 100 rpm for 2 hours at room temperature. Following dialysis, the formulations were collected from the bags and lyophilized. The compositions of all formulations are detailed in Table 1.

Table 1. Composition of various liposome and chitosome formulations.

Formulation code	SPC (mg)	CHOL (mg)	CHEMS (mg)	Chitosan concentration (%)
LP1	140	60	-	-
LP2	140	-	60	-
CCL1	140	-	60	0.01
CCL2	140	-	60	0.025
CCL3	140	-	60	0.05

*SPC: Soy phosphatidylcholine, CHOL: Cholesterol, CHEMS: Cholesteryl hemisuccinate

Lyophilization of formulations

All formulations (liposomes and chitosomes) containing sucrose as a cryoprotectant were transferred into glass vials and frozen at –80°C. The frozen samples were then lyophilized at –55°C for 40 hours using a freeze dryer (Christ Alpha 1-2 LD plus, Osterode am Harz, Germany) (Yalcin et al., 2021). The resulting

lyophilized powders were stored at 2-8°C for further analysis.

Characterization of formulations

PS, polydispersity index (PDI), and ZP measurements

The lyophilized formulations were dispersed in deionized water, and the PS, PDI, and ZP of both li-

posomes and chitosomes were measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) (Yalcin et al., 2022; Yalcin et al., 2025) ($n = 3$).

Determination of EE%

The EE% of both liposomes and chitosomes was determined using an indirect method. Briefly, the formulations were placed in dialysis bags to separate the unencapsulated LTZ and stirred magnetically for 1 hour. Following dialysis, 1 mL of the external medium was collected and analyzed by HPLC ($n = 3$). The EE% was calculated using the following equation:

$$EE\% = \frac{\text{total added drug} - \text{free drug}}{\text{total added drug}} \times 100 \quad (1)$$

HPLC assay for quantification

The amount of LTZ in the samples was quantified using HPLC (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a reverse-phase C18 column (250 mm $\times$ 4.6 mm, 5 μ m; XSelect, Waters, Ireland). The ultraviolet (UV) detection and column temperature were 242 nm, and 30°C, respectively. The mobile phase consisted of acetonitrile and water (65:35, v/v) under isocratic conditions, with a flow rate of 1 mL/minute (Hooshyar et al., 2021). The injection volume was 20 μ L. The retention time was approximately 11 minutes. The standard deviation (SD) of the y-intercepts (σ) and the slopes (S) of the calibration curves were obtained from three independent replicate experiments. These values were then used to calculate the limits of detection (LOD) and quantification (LOQ) using the formulas: $LOD = 3.3 (\sigma/S)$ and $LOQ = 10 (\sigma/S)$, respectively. The LOD and LOQ were 0.035 and 0.106 μ g mL⁻¹, respectively.

In vitro drug release studies

Drug release from the formulations was evaluated by measuring the drug concentration in the release medium using the HPLC method. For this purpose, a specified amount of lyophilized LTZ-loaded formulations, adjusted based on the EE% to contain 0.5 mg of LTZ, was dispersed in PBS containing 2% Polysorbate 80 and transferred into dialysis bags (cellulose

membranes, MW cut-off: 14,000 Da). Polysorbate 80 was included to maintain sink conditions during the release process (Kaplan et al., 2022), as LTZ exhibits poor water solubility in aqueous media (Mohammadi et al., 2022). The dialysis bags were then immersed in 200 mL of release medium composed of PBS (pH 7.4) with 2% Polysorbate 80, maintained at $37 \pm 0.5^\circ\text{C}$. At predetermined time points (0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hours), 1 mL aliquots were withdrawn and immediately replaced with an equal volume of fresh medium. All experiments were performed in triplicate.

Differential scanning calorimetry (DSC)

Approximately 2 mg of each sample was accurately weighed and placed into standard aluminum pans, which were then sealed. Differential Scanning Calorimetry (DSC) measurements were performed using a DSC-60 instrument (Shimadzu, Japan) under a continuous nitrogen purge at a flow rate of 20 mL/min. Samples were heated from 20°C to 250°C at a controlled rate of 10°C/min. An empty aluminum pan was used as the reference to ensure baseline correction and accurate thermal signal detection. The analysis was conducted to investigate possible interactions between the active pharmaceutical ingredient and excipients, as well as to evaluate their thermal behavior in physical mixtures.

Transmission electron microscopy (TEM)

Liposome and chitosome morphologies were examined using transmission electron microscopy (TEM). For sample preparation, a drop of each sample was placed onto a carbon-coated copper grid and negatively stained with 1% (w/v) phosphotungstic acid. The grids were air-dried and kept at room temperature until analysis. TEM observations were performed under low vacuum using a FEI Tecnai G2 Spirit BioTwin CTEM microscope (Hillsboro, OR, USA) at 120 kV.

Stability of liposome and chitosome formulations under different temperature conditions

The stability of the formulations (liposomes and chitosomes) was assessed by monitoring PS, PDI, and ZP. The stability tests for both liposome and chitosome formulations were performed on samples in dis-

persion form (non-lyophilized). Samples were stored at 2-8°C and 25°C for one month prior to analysis. Before measurements, the samples were diluted with deionized water (n = 3).

***In vitro* cytotoxicity studies**

The estrogen receptor (ER)-positive MCF-7 (ATCC CCL-222, Human Breast Cancer Cell Series) cell line model, which is often used to demonstrate the estrogen dependence of breast cancer development (Xu et al., 2017), was cultured in DMEM supplemented with 1% penicillin, streptomycin mix solution (Gibco-Invitrogen, Grand Island, NY, USA), and 10% FBS.

Cell viability was assessed with MTT assay. MCF-7 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and allowed to attach overnight. Control groups containing only culture medium without substance were determined. Then, the solutions of the test substances in DMEM (100 μ L) were added to the wells, and after 24 hours of incubation, MTT solution (13 μ L/well) in fresh DMEM (100 μ L/well) (Mitropoulou et al., 2003) was added to all wells whose contents were emptied and kept for 4 hours. Then, 100 μ L of DMSO was added to the wells to solubilize formazan crystals. The resulting purple color was determined spectrophotometrically at 570 nm. The mean absorbance of the control wells was considered as corresponding to 100% cell viability. Different absorbance values were obtained for different test substances, and viability percentages were calculated

(Yalcin et al., 2022).

Statistical analysis

Results were expressed as mean $\pm$ SD for all parameters. A paired t-test was used to test the statistical significance when comparing data between two groups, and more than two groups were compared using one-way analysis of variance (ANOVA) with post hoc Tukey's test. A difference was considered statistically significant at $p < 0.05$. For this purpose, GraphPad Prism 9.3.0 (GraphPad Software, Inc.) was used (Yalcin et al., 2025).

RESULTS AND DISCUSSION

PS, PDI, and ZP measurements

The mean PS of the LTZ-loaded liposomes were found to be 164 nm for the CHOL-containing formulation (LP1) and 112 nm for the CHEMS-containing formulation (LP2). The ZP values were -14.9 mV and -48.9 mV for LP1 and LP2, respectively (Table 2). Chitosan-coated liposomes (chitosomes) prepared using LP2 formulation, which had a smaller PS and higher surface charge, exhibited increased PS values, indicating successful coating. The mean particle sizes of the chitosomes were 155 nm (CCL1, 0.01% chitosan), 198 nm (CCL2, 0.025% chitosan), and 1088 nm (CCL3, 0.05% chitosan). Chitosomes exhibited less negative ZP values ranging from -40.0 mV to -27.5 mV, confirming surface modification. All formulations, except CCL3, exhibited low PDI values (< 0.5), indicating monodisperse particle distribution.

Table 2. Characteristic properties of various liposome and chitosome formulations (mean $\pm$ SD, n=3).

Formulation code	PS (nm)	PDI	ZP	EE%
LP1	164 $\pm$ 10	0.283 $\pm$ 0.028	-14.9 $\pm$ 2.1	95.6 $\pm$ 0.1
LP2	112 $\pm$ 3	0.286 $\pm$ 0.010	-48.9 $\pm$ 1.4	95.1 $\pm$ 0.2
CCL1	155 $\pm$ 19	0.302 $\pm$ 0.056	-40.0 $\pm$ 2.3	93.7 $\pm$ 0.5
CCL2	198 $\pm$ 12	0.245 $\pm$ 0.007	-27.5 $\pm$ 1.7	93.8 $\pm$ 0.7
CCL3	1088 $\pm$ 323	0.876 $\pm$ 0.175	-29.3 $\pm$ 2.0	92.7 $\pm$ 1.5

*LP1 contains 140 mg SPC and 60 mg CHOL. LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1, CCL2, and CCL3 are liposomal formulations based on LP2, coated with 0.01%, 0.025%, and 0.05% chitosan solutions, respectively. SPC: Soy phosphatidylcholine, CHOL: Cholesterol, CHEMS: Cholesteryl hemisuccinate. For PS and PDI value, CCL3 was significantly different from LP1, LP2, CCL1, and CCL2 ($p < 0.05$), while no significant differences were observed among the other formulations. For ZP, CCL2 and CCL3 did not differ significantly from each other ($p > 0.05$), while all other pairwise comparisons showed significant differences. For EE%, significant differences were observed between LP1 and CCL3; and between LP2 and CCL3 ($p < 0.05$), while no significant differences were found among the other formu-

lations ($p>0.05$).

The smaller PS observed in the CHEMS-containing formulation (LP2) compared to LP1 may be attributed to the increased net negative surface charge provided by the carboxylic acid group of CHEMS, enhancing repulsion between particles and thus preventing aggregation. These findings align with previous reports (Kulig et al., 2015). The chitosan coating process was performed on LP2 due to its favorable physicochemical properties (smaller size and higher ZP). The subsequent increase in PS after chitosan coating further supports the formation of a polymeric layer on the liposome surface. As the chitosan concentration increased, the PS also increased significantly—particularly in CCL3—suggesting that chitosan self-assembled into thicker or possibly aggregated structures (Park et al., 2014). The observed decrease in the magnitude of negative ZP upon chitosan coating—from -48.9 mV (LP2) to values between -40.0 mV and -27.5 mV—supports the interaction between the positively charged chitosan molecules and the negatively charged liposomal surface (Table 2). While CCL1 and CCL2 remained within monodisperse limits ($PDI<0.5$), CCL3 exhibited a markedly higher PDI value, likely due to aggregation induced by the excess polymer concentration. This finding suggests that high chitosan concentrations may compromise colloidal stability by promoting interparticle bridging or aggregation (Soo et al., 2016).

Determination of EE%

Drug EE% in liposomal formulations is influenced by several physicochemical factors, including liposome size, surface charge, bilayer rigidity, aqueous core volume, and the preparation method (Hooshyar et al., 2021). In this study, the EE% of LTZ in uncoated liposomes was found to be high and not significantly affected by the presence of either CHOL (LP1) or CHEMS (LP2). The average EE% ranged between 92.7% and 95.6% for all tested formulations (Table 2). However, a slight decrease in EE% was observed as the chitosan concentration increased during the coating process. Notably, a statistically significant

reduction in EE% was recorded for CCL3 compared to LP2 ($p<0.05$), while the EE% of CCL1 and CCL2 remained comparable to the uncoated liposome formulation (LP2).

The consistently high EE% (92.7–95.6%) across all liposomal and chitosomal formulations indicates the favorable incorporation of LTZ within the lipid bilayer. This is likely due to the lipophilic nature of LTZ and the optimized lipid composition, which promotes drug retention. The lack of significant difference in EE% between LP1 and LP2 suggests that the inclusion of CHOL or CHEMS did not disrupt bilayer packing to an extent that would affect drug loading. This finding aligns with previous studies indicating that CHOL and CHEMS, although they influence membrane rigidity and surface charge, do not necessarily affect the EE% of hydrophobic drugs (Haeri et al., 2017). The slight reduction in EE% with increasing chitosan concentration may be attributed to partial drug leakage during the coating step. This phenomenon is likely due to the mechanical stress introduced by stirring and the potential disruption of the lipid bilayer interface during the formation of the chitosan shell (Trapani et al., 2018). The statistically significant drop in EE% in the CCL3 formulation (0.05% chitosan) supports this explanation, and aligns with earlier research suggesting that excessive polymer concentration can destabilize vesicle structure or displace encapsulated drug molecules (Haeri et al., 2017).

In vitro drug release studies

The *in vitro* release profile of LTZ from conventional liposomes (LP1 and LP2) and chitosomes (CCL1 and CCL2) was evaluated over 24 hours. Due to its significantly large PS, the CCL3 formulation was excluded from the release study. As shown in Figure 1, CCL1 and CCL2 exhibited a markedly lower cumulative release than the uncoated liposomes at all measured time points. At 6 hours, cumulative LTZ release from LP1 and LP2 was 70.2% and 58.7%, respectively. In contrast, CCL1 and CCL2 showed release rates of 46.9% and 38.2%, respectively. The overall maximum

release observed for CCL1 and CCL2 was 53.5% and 46.6%, respectively. Additionally, all formulations displayed a biphasic release pattern characterized by

an initial burst phase followed by a sustained release phase. Within the first 2 hours, LTZ release was 40.2% for LP2, 35.7% for CCL1, and 23.6% for CCL2.

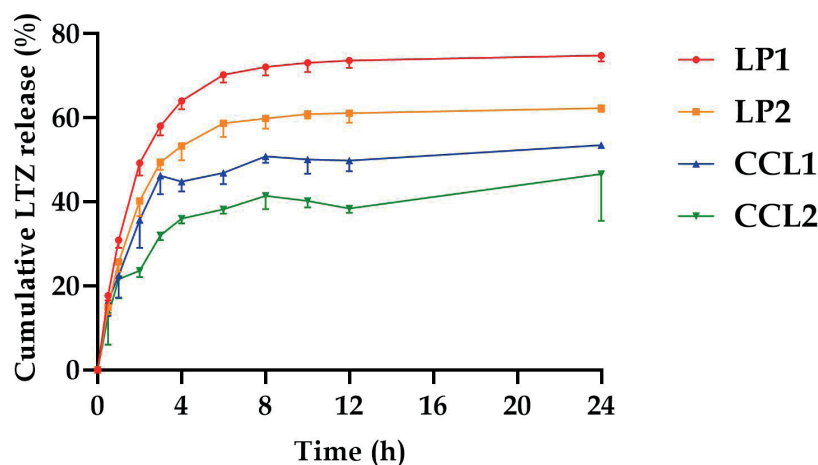


Figure 1. *In vitro* drug release profiles of LTZ-loaded liposomes and chitosomes (mean $\pm$ SD, $n=3$).

*LP1 contains 140 mg SPC and 60 mg CHOL, while LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 and CCL2 are LP2-based liposomes coated with 0.01% and 0.025% chitosan solution, respectively.

The higher release rates observed in uncoated liposomes (LP1 and LP2) suggest a more rapid diffusion of LTZ, potentially due to the absence of a polymeric barrier. The difference between LP1 and LP2 may stem from the influence of CHEMS on bilayer rigidity. As previously reported (Xu et al., 2017), CHEMS enhances membrane stability more effectively than CHOL, potentially limiting drug diffusion and resulting in slower release from LP2. Similar trends were reported by Ding et al (Ding et al., 2005). The reduced release rates from chitosomes, especially CCL2, suggest that higher chitosan concentrations result in greater restriction of drug diffusion. The polymeric chitosan coating likely decreases bilayer fluidity and acts as an additional diffusional barrier (Alshamsan et al., 2019). This effect is more pronounced at higher chitosan concentrations, as demonstrated by the lower release rate of CCL2 compared to CCL1. The initial burst release observed in all formulations may be attributed to the release of LTZ adsorbed on or near the surface of the liposomes and chitosomes (El-Hammadi et al., 2017). Following the burst phase, the sustained release is likely governed by a combination of drug diffusion through the lipid bilayer and the gradual degradation or swelling of the chitosan

coating (Hasan et al., 2019). These findings support the hypothesis that chitosan coating modulates drug release from liposomal carriers, enabling controlled delivery profiles, which depend on polymer concentration.

Differential scanning calorimetry (DSC)

The DSC thermogram of pure LTZ exhibited a sharp endothermic peak at 187.14°C, while sucrose and CHEMS showed endothermic peaks at 190.94°C and 187.11°C, respectively (Figure 2A and B). These thermal analysis results were found to be consistent with previous studies (Hegde et al., 2023; Zhang et al., 2020; Verma et al., 2020). In the physical mixtures, all combinations except the mixture with CHEMS showed shifts of less than 5°C, indicating minimal interactions between LTZ and the excipients (Yalcin et al., 2025). In the LTZ–CHEMS mixture, however, the endothermic peaks of both LTZ and CHEMS shifted by more than 5°C, which was attributed to the solubilization of LTZ into CHEMS or a solid-state interaction induced by the input of energy and dilution during heating, although the interaction was not complete. Similar findings have been reported in the study by Zhang et al (Zhang et al., 2020).

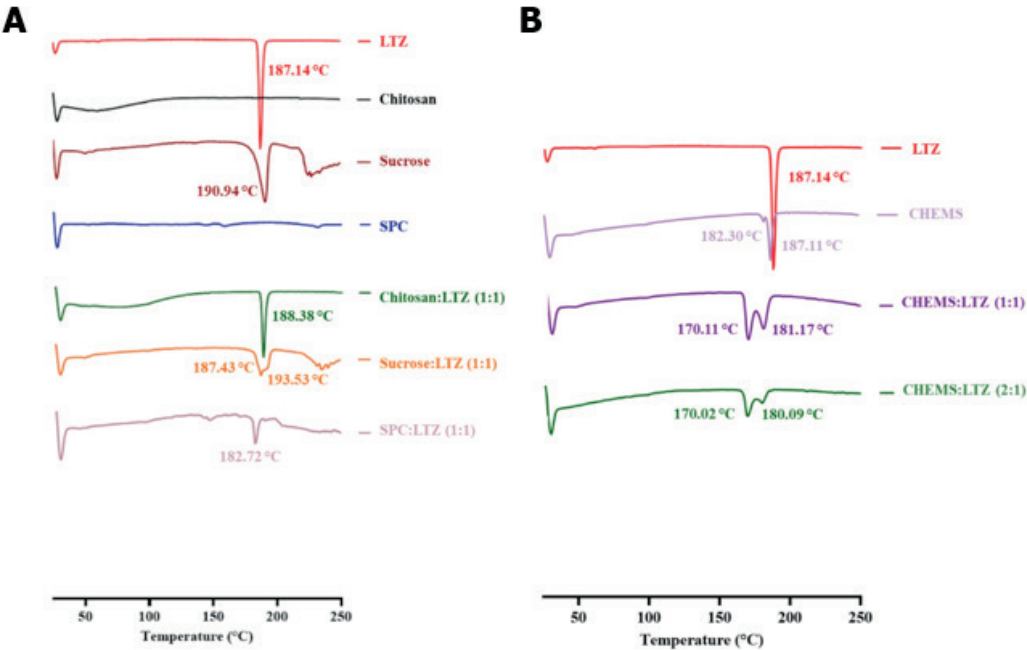


Figure 2. DSC thermograms of (A) LTZ, chitosan, sucrose, SPC, and their physical mixtures with LTZ; (B) LTZ, CHEMS, and their physical mixtures.

Transmission electron microscopy (TEM)

TEM images (Figure 3) revealed that both the liposome (LP2) and chitosome (CCL1) formulations exhibited predominantly spherical morphology. The vesicles appeared well-dispersed with smooth surfaces, indicating uniform particle formation. No sig-

nificant aggregation or deformation was observed, suggesting that the preparation methods successfully produced stable and structurally intact vesicles. These morphological characteristics align with the expected behavior of lipid- and chitosan-based vesicular systems, confirming their suitability for potential drug delivery applications.

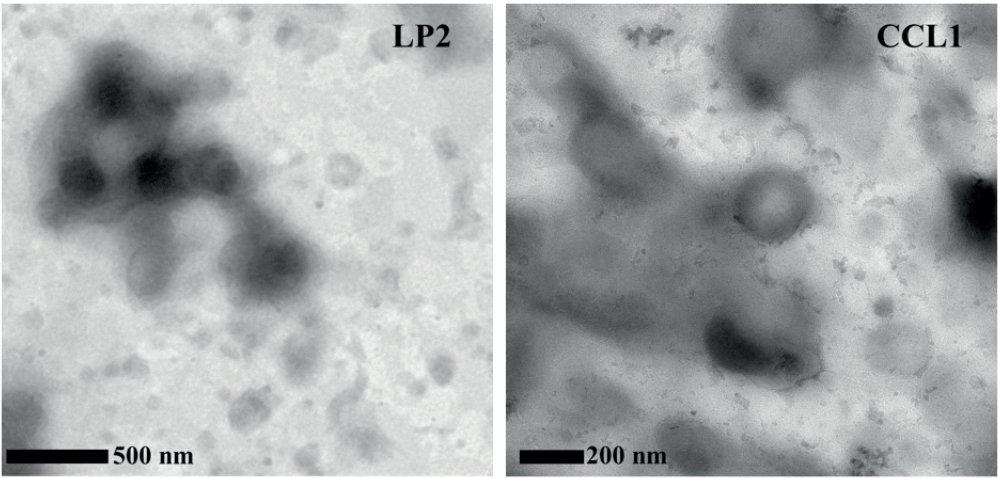


Figure 3. TEM images of liposome (LP2) and chitosome (CCL1) formulations,

Stability of liposome and chitosome formulations under different temperature conditions

The stability profiles of liposome (LP2) and chitosome (CCL1) formulations stored at 2-8°C and 25°C for one month are summarized in Table 3. The LP2 formulation showed no statistically significant changes in mean PS at either storage condition compared to its initial measurements ($p > 0.05$). The changes in ZP

and PDI were also minimal, indicating good physical stability over time. In contrast, the CCL1 formulation exhibited a noticeable increase in PS after storage, particularly at 25°C. The observed increase was accompanied by a corresponding decrease in the net negative ZP value. Notably, PS analysis could not be performed for CCL1 stored at 25°C due to severe aggregation and poor PS distribution.

Table 3. Characteristic properties of LP2 and CCL1 formulations after a month of storage at 2-8°C and 25°C (mean $\pm$ SD, $n=3$).

Formulation code	Time and storage conditions	PS (nm)	PDI	ZP
LP2	Initial	112 $\pm$ 3	0.286 $\pm$ 0.010	-48.9 $\pm$ 1.4
	1 month (2-8°C)	112 $\pm$ 3	0.244 $\pm$ 0.027	-55.1 $\pm$ 1.4
	1 month (25°C)	117 $\pm$ 13	0.285 $\pm$ 0.054	-48.6 $\pm$ 3.2
CCL1	Initial	155 $\pm$ 19	0.302 $\pm$ 0.056	-40.0 $\pm$ 2.3
	1 month (2-8°C)	206 $\pm$ 39	0.322 $\pm$ 0.060	-32.1 $\pm$ 0.8
	1 month (25°C)	Remarkable aggregation observed		

*LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 is an LP2-based liposome coated with 0.01% chitosan solution. SPC: Soy phosphatidylcholine, CHEMS: Cholesteryl hemisuccinate

The stability results indicate that the uncoated liposome formulation (LP2) retained its physicochemical integrity over the storage period, with minimal variations in PS, ZP, and PDI. These findings suggest that the incorporation of CHEMS contributes to the structural stability of the liposomal bilayer, even at elevated temperatures. In contrast, the chitosome formulation (CCL1) demonstrated instability during storage, especially at 25°C. The observed increase in PS and decrease in ZP may indicate weakened electrostatic repulsion between particles, leading to aggregation. The inability to measure PS for CCL1 after storage at 25°C further supports this conclusion. Similar observations were reported by Ezzat et al. (Ezzat et al., 2019), who noted that elevated temperatures could compromise the integrity of chitosan-coated vesicles. The high purity of SPC used in this formulation may have contributed to weak chitosan adhesion on the vesicle surface

(Guo et al., 2003), thus reducing the stability of the chitosan coating. As temperature increases, this fragile interaction may further deteriorate, promoting the formation of large aggregates.

In vitro cytotoxicity studies

The cytotoxic potential of LTZ and its nanoformulations was evaluated using the MTT assay on MCF-7 breast cancer cells. The IC_{50} value of free LTZ was determined to be 61.3 nM. Drug-free liposome and chitosome formulations showed cell viability rates above 90% after 24 hours of treatment, indicating minimal cytotoxicity in the absence of the active drug. LTZ-loaded LP2 and CCL1 formulations, when administered at an LTZ concentration of 61.3 nM, reduced cell viability to 48.9% and 48.5%, respectively, indicating a relatively higher cytotoxic effect compared to the free LTZ solution (Figure 4).

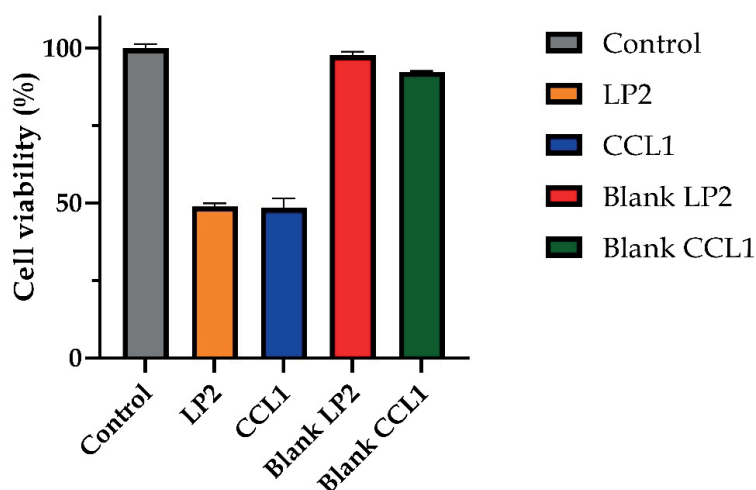


Figure 4. The percent cell viability of the MCF-7 cells after 24 h of incubation (mean $\pm$ SD, n=6).

*LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 is a liposomal formulation based on LP2, coated with 0.01% chitosan solution.

The IC_{50} value observed for LTZ (61.3 nM) was consistent with previous studies, confirming the reproducibility and reliability of the assay (Kijima et al., 2005). The high cell viability observed with drug-free formulations aligns with literature findings that demonstrate the biocompatibility of liposomal and chitosomal carriers (Patel et al., 2018). The slightly enhanced cytotoxicity observed in LTZ-loaded liposome (LP2) and chitosome (CCL1) formulations suggests improved intracellular delivery of LTZ. This may result from endocytosis-mediated uptake, a well-documented advantage of nanoparticulate drug delivery systems (Hassanzadeganroudsari et al., 2020; Kadari et al., 2017). Encapsulation within nanocarriers can also protect the drug from degradation and facilitate its accumulation in tumor cells, thus amplifying its therapeutic effect.

CONCLUSION

In this study, LTZ-loaded liposomal (LP2) and chitosomal (CCL1) formulations were successfully developed, demonstrating high EE% (>90%) and suitable particle sizes (<200 nm) suitable for passive targeting in cancer therapy. The chitosan coating process significantly altered key physicochemical characteristics of the formulations, including PS, ZP, and

drug release profiles. *In vitro* stability assessments revealed that the chitosome formulation (CCL1) was more sensitive to temperature variations than the uncoated liposome (LP2). The observed instability may be attributed to the use of highly pure phospholipids, potentially resulting in weaker interactions with the chitosan layer. To improve thermal stability, future studies may consider using phospholipid grades with lower purity or incorporating stabilizing agents during formulation development. Cytotoxicity studies performed on MCF-7 breast cancer cells demonstrated that LTZ-loaded liposomal and chitosomal formulations exhibited comparable IC_{50} values to the free LTZ solution. However, both LP2 and CCL1 induced a relatively higher cytotoxic effect, suggesting enhanced intracellular delivery of LTZ via nanocarrier-mediated uptake. Overall, these findings support the potential of LTZ-loaded liposome and chitosome systems as effective nanocarriers for the delivery of anticancer drugs. Further, *in vivo* studies are warranted to evaluate the pharmacokinetic profile, therapeutic efficacy, and safety of these formulations in animal models.

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AUTHOR CONTRIBUTION STATEMENT

Concept, design, supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (TEY), data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (NTO), Supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (CY), Concept, design, supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (TI).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Eudragit S100-based Floating Microballoons of Antitubercular Drug: Design, Preparation, and *In Vitro* Characterization

Gyati Shilakari ASTHANA^{*}, Krishna T.K.T.S^{**}, Abhay ASTHANA^{***},
Sri Rupa Bhoomika SATTI^{****}, Sameeksha POLDASARI^{*****}

Eudragit S100-based Floating Microballoons of Antitubercular Drug: Design, Preparation, and In Vitro Characterization

Eudragit S100-bazlı Antitüberküloz İlaç İçeren Yüzen Mikroküreler: Tasarım, Hazırlık ve İn Vitro Karakterizasyon

SUMMARY

The present study aimed to develop and characterise floating microspheres of Eudragit S100 containing rifampicin (RFN) to enhance bioavailability, control drug release, and achieve site-specific delivery. Rifampicin is an antitubercular drug commonly used in the treatment of tuberculosis. Floating microspheres were prepared using the solvent evaporation method with varying drug-to-polymer ratios (1:1–1:5), stirring speeds (300, 400, and 500 rpm), and stirring times (1, 1.5, and 2 hours). The developed formulations were evaluated for particle size, floating lag time, percentage buoyancy, surface morphology (via scanning electron microscopy), swelling index, drug content, entrapment efficiency, and *in vitro* drug release. The microspheres were spherical with smooth surfaces, and all formulations were found to float for up to 24 hours. Particle size and percentage buoyancy increased with rising polymer concentration. *In vitro* studies revealed that the drug was released from the floating microspheres over 24 hours and followed the Higuchi kinetic model. Among all formulations, FES4 (1:4 drug-to-Eudragit S100 ratio) exhibited the lowest floating lag time and the highest percentage buoyancy. Therefore, FES4 was selected as the optimised formulation based on its floating behaviour and other *in vitro* parameters. Further, antimicrobial activity studies demonstrated a clear zone of inhibition as compared to the control. The FES4 formulation remained stable under storage conditions. Overall, the optimised formulation (FES4) displayed excellent floating properties and antimicrobial activity, indicating the potential of rifampicin-loaded microspheres as an effective system for tuberculosis treatment.

Keywords: Floating microspheres, eudragit S 100, rifampicin, *in vitro* characterization, anti-microbial activity.

ÖZ

Bu çalışmanın amacı, biyoyararlanımı artırmak, kontrollü ilaç salımını sağlamak ve hedefe yönelik ilaç iletimini gerçekleştirmek amacıyla rifampisin (RFN) içeren Eudragit S100'den yapılmış yüzen mikroküreler geliştirmek ve karakterize etmektir. Rifampisin, tüberküloz tedavisinde yaygın olarak kullanılan bir antitüberküloz ilaçtır. Yüzen mikroküreler, çözücü buharlaştırma yöntemi kullanılarak farklı ilaç-polimer oranlarında (1:1–1:5), karıştırma hızlarında (300, 400 ve 500 rpm) ve karıştırma sürelerinde (1, 1,5 ve 2 saat) hazırlanmıştır. Geliştirilen formülasyonlar; parçacık boyutu, yüzme gecikme süresi, yüzdürme yüzdesi, yüzey morfolojisi (taramalı elektron mikroskopu ile), şişme indeksi, ilaç içeriği, tutma verimi ve *in vitro* ilaç salımı açısından değerlendirilmiştir. Mikrokürelerin küresel ve düzgün yüzeyli olduğu gözlenmiştir ve tüm formülasyonlar 24 saat boyunca yüzebilir durumda kalmıştır. Polimer konsantrasyonunun artmasıyla birlikte parçacık boyutu ve yüzdürme yüzdesi de artmıştır. *In vitro* çalışmalar, ilacın yüzen mikrokürelerden 24 saat boyunca salındığını ve Higuchi kinetik modeline uyduğunu göstermiştir. Tüm formülasyonlar arasında, FES4 (1:4 ilaç-Eudragit S100 oranı) en düşük yüzme gecikme süresini ve en yüksek yüzdürme yüzdesini göstermiştir. Bu nedenle, FES4 formülasyonu, yüzdürme davranışı ve diğer *in vitro* parametreler temelinde optimize edilmiş formülasyon olarak seçilmiştir. Ayrıca, antimikrobiyal aktivite çalışmaları, kontrol grubuna kıyasla belirgin bir inhibisyon zonu göstermiştir. FES4 formülasyonu depolama koşullarında stabil kalmıştır. Genel olarak, optimize edilmiş FES4 formülasyonu mükemmel yüzdürme özellikleri ve antimikrobiyal aktivite sergilemiş olup, rifampisin yüklü mikrokürelerin tüberküloz tedavisinde etkili bir sistem olma potansiyelini göstermektedir.

Anahtar Kelimeler: Yüzen mikroküreler, eudragit s100, rifampisin, *in vitro* karakterizasyon, anti-mikrobiyal aktivite.

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^{*} ORCID: 0000-0002-2895-2332, Department of Pharmaceutics, Gokaraju Rangaraju College of Pharmacy, affiliated to Osmania University, Hyderabad, India.

^{**} ORCID: 0009-0003-9231-3118, Department of Pharmaceutics, Gokaraju Rangaraju College of Pharmacy, affiliated to Osmania University, Hyderabad, India.

^{***} ORCID: 0000-0001-7103-488X, Sreeven Pharma, Bidar, Karnataka.

^{****} ORCID: 0009-0007-2667-7633, Department of Pharmaceutics, Gokaraju Rangaraju College of Pharmacy, affiliated to Osmania University, Hyderabad, India.

^{*****} ORCID: 0009-0008-6597-9113, Department of Pharmaceutics, Gokaraju Rangaraju College of Pharmacy, affiliated to Osmania University, Hyderabad, India.

[°] Corresponding Author; Dr. Gyati Shilakari Asthana, Professor
E-mail: gyatimmu@gmail.com

INTRODUCTION

Oral administration has garnered the greatest attention because it is the most convenient method of drug delivery and offers significant flexibility in dosage form design. However, limited bioavailability and short gastric retention time remain major drawbacks of conventional drug delivery systems. The gastrointestinal tract (GIT) still poses several challenges with standard drug delivery methods, such as insufficient drug release, reduced dose efficacy, and the need for frequent dosing. The inability of conventional systems to retain drugs in the stomach has led to the development of gastro-retentive drug delivery systems (GRDDS). These systems improve drug absorption and prolong gastric retention time. Gastric retention can be achieved through various approaches, including raft-forming systems, high-density systems, magnetic systems, super-porous hydrogels, low-density systems, expandable systems, and mucoadhesive or bio-adhesive systems (Mandal et al., 2016).

It is anticipated that floating drug delivery systems will remain buoyant upon reaching the stomach, thereby increasing the bioavailability of the drug. Several types of buoyant formulations exist, including hollow microspheres, granules, tablets, capsules, pills, laminated films, and powders. Among these, floating microspheres are being extensively studied due to their ability to deliver drugs directly to the stomach. They allow the continuous release of the drug while remaining afloat on the gastric contents for extended periods. Since these systems do not adversely affect the gastrointestinal tract (GIT) motility, they have been the focus of considerable research (Pawar et al., 2011).

Rifampicin (RFN) is a first-line antitubercular drug used to treat tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). It has a short half-life of 1.5 to 4.5 hours and belongs to BCS Class II. RFN exhibits gastric permeability, and site-specific sustained drug delivery in the stomach may be beneficial for enhancing its bioavailability. Because RFN is less soluble at higher pH values (pH 7.4; 1 in 100 of

phosphate buffer at 37°C) and more soluble at lower pH values (pH 1.5; 1 in 5 of 0.1 M HCl at 37°C), it is a suitable candidate for gastro-retentive drug delivery system (GRDDS) formulation. The development of a gastro-retentive controlled-release (CR) formulation of RFN is required to improve its biopharmaceutical properties, owing to its high solubility in acidic pH and optimal absorption window in the stomach. Eudragit S100, a methacrylic acid and methyl methacrylate anionic copolymer, is widely used as a sustained-release polymer (Patra et al., 2017). This polymer is soluble in intestinal fluid at pH values greater than 7 and has a low density (0.83–0.85 g/cm³). These properties make it suitable for use in sustained-release formulations, ensuring that the drug remains intact until it reaches a specific pH environment within the GIT. When the formulation reaches the duodenum or stomach, the drug is released and absorbed from the polymer matrix at these sites.

Floating microspheres containing Eudragit S100 have been reported for a wide range of drugs, such as itopride hydrochloride (Bansal et al., 2016), curcumin (Kumar et al., 2016), acyclovir (Junyaprasert & Pornsuwannapha, 2008), repaglinide (Sivaprasad et al., 2021), metformin hydrochloride (Verma et al., 2020), amoxicillin and rabeprazole sodium (Choudhary et al., 2016), and famotidine (Gupta et al., 2014). RFN has also been formulated using various technologies, including microspheres (Bhadouriya et al., 2012), microparticles (Goyal et al., 2011), biodegradable microcapsules (Maurya et al., 2012), solid lipid nanoparticles (Sarfaraz et al., 2010), and niosomes (Singh et al., 2013). Although several studies have explored rifampicin-loaded floating microspheres using polymers such as ethyl cellulose, hydroxypropyl methylcellulose (HPMC), and Eudragit RLPO (Bhise et al., 2010; Goyal et al., 2011; Pingale & Amrutkar, 2021), the use of Eudragit S100 to enhance gastric retention remains largely under-investigated (Kallepu et al., 2016; Pallam & Maruvajala, 2024; Raveendra et al., 2012). Traditionally, Eudragit S100 has been employed for colon-targeted drug delivery due to its pH-dependent

solubility—being soluble only at pH values above 7—making its application in gastric-floating systems both unconventional and novel.

In the present study, Eudragit S100 was innovatively employed to develop buoyant microspheres with prolonged gastric retention. This strategy was designed to optimize drug release at the primary absorption sites—the stomach and upper small intestine—thereby enhancing the bioavailability of rifampicin, a drug characterized by limited solubility and a short half-life.

Unlike previous formulations—such as those by Patel et al. (2006), which incorporated ethyl cellulose and HPMC, or Pingale & Amrutkar (2021), who utilized Eudragit RL/RS for rifampicin microspheres—our approach uniquely capitalizes on the gas-trapping capacity and low density of Eudragit S100. Despite its pH-dependent solubility, Eudragit S100 exhibits a remarkable ability to entrap air during the solvent evaporation process, enabling the microspheres to remain buoyant under gastric conditions.

This formulation strategy not only extends gastric residence time but also provides a controlled and sustained drug release profile, which is particularly advantageous for rifampicin due to its narrow absorption window and degradation in acidic media.

To the best of our knowledge, Eudragit S100 has not been previously explored in the development of floating microspheres for rifampicin delivery, underscoring the novelty and potential impact of this research in the field of gastro-retentive drug delivery systems.

The present study focused on the design and formulation of Eudragit S100-based floating microspheres for the delivery of RFN. The developed mi-

cro-spheres were characterized using various *in vitro* parameters, including particle size, floating lag time, total buoyancy time, scanning electron microscopy (SEM), *in vitro* drug release, and antimicrobial activity.

MATERIALS AND METHODS

Materials

Rifampicin was purchased from Yarrow Chemicals, Mumbai. Eudragit polymer was obtained as a gift sample from Evonik, Mumbai. All other chemicals, like ethanol, isopropanol, dichloromethane, and polyvinyl alcohol, were obtained from SD Fine Chemicals, Hyderabad.

Methods

Preparation of Rifampicin Floating Microspheres

Eudragit microspheres were prepared using various formulation variables, such as the drug-to-polymer ratio (1:1 to 1:5), and process variables, including stirring speed (300, 400, and 500 rpm) and stirring time (1, 1.5, and 2 hours), as shown in Table 1. The microspheres were fabricated by the solvent evaporation method, as reported earlier, with slight modifications. RFN (200 mg) and the required quantity of polymers, as mentioned in Table 1, were dissolved in a solvent mixture of dichloromethane (DCM), ethanol, and isopropanol in a 5:8:2 ratio. This organic phase was poured dropwise over 3–4 minutes into 1000 mL of an aqueous phase containing 0.6% polyvinyl alcohol (PVA) and stirred at room temperature using a mechanical stirrer for 1 hour at speeds of 300, 400, or 500 rpm. The resulting microspheres were then filtered and dried (Pallam & Maruvajala, 2024; Phutane et al., 2010).

Table 1. Formulation composition of floating microspheres using Eudragit S 100

Formulation codes	Drug (mg)	Polymer (mg)	Stirring speed (RPM)	Stirring time (Hrs)
Effect of polymer concentration				
FES1	200	200	300	1
FES2	200	400	300	1
FES3	200	600	300	1
FES4	200	800	300	1
FES5	200	1000	300	1
Effect of stirring speed				
FES6	200	800	400	1
FES7	200	800	500	1
Effect of stirring time				
FES8	200	800	300	1.5
FES9	200	800	300	2

Characterization of rifampicin floating microspheres

Fourier transformed infrared spectroscopy (FT-IR) analysis

An FTIR spectrophotometer was used to record the FTIR spectra of the drug and drug-polymer mixture using a KBr disc over the range of 4000–400 cm⁻¹.

Shape of floating microspheres

The shape and surface morphology of the floating microspheres were examined by spreading the samples on a clean glass slide and observing them under an optical microscope.

Production yield of microspheres

The total weight of the dried microspheres was measured, and the production yield was determined (Khan et al., 2020) by comparing the weight of the dried microspheres from each formulation with the initial dry weight of the drug and polymer combined, as calculated using the following equation:

$$\text{Yield}(\%) = \frac{\text{Weight of micro spheres}}{\text{Weight of drug + Polymer use in formulation}} \times 100$$

Particle size

Microspheres were carefully examined using a calibrated optical microscope to estimate their particle size. The diameters of a minimum of 100 microspheres from each batch were measured, and the results were recorded.

Drug content

To determine the drug content of the floating microspheres, 50 mg of a precisely weighed formulation was dissolved in 10 mL of ethanol and stirred with a magnetic stirrer to break down the polymer. The drug was then extracted and filtered. The concentration of the drug in the ethanol phase was measured using UV spectrophotometry at 475 nm (Nila et al., 2014).

$$\text{Drug content}(\%) = \frac{\text{Calculated amount of drug}}{\text{Total weight of floating microspheres}} \times 100$$

Entrapment efficiency

Entrapment efficiency was assessed using a previously reported method with slight modifications (Bakshi, 2016). Briefly, microspheres containing 10 mg of RFN were ground using a glass mortar and pestle, and the resulting powder was suspended in 10 mL of methanol. After an appropriate dilution, the drug content was analyzed at 475 nm using a UV spectrophotometer.

$$\text{Entrapment efficiency}(\%) = \frac{\text{Calculated drug concentration}}{\text{Theoretical Drug Concentration}} \times 100$$

In vitro buoyancy studies

A 50 ml beaker was filled with 50 mg of the microspheres. Then, 20 mL of 0.1N HCl containing 0.02% Tween 20 was added, and the mixture was stirred using a magnetic stirrer in a water bath maintained at 37 ± 0.5 °C. The time taken for the microspheres to float to the surface, referred to as the floating lag

time, was recorded. After 24 hours, the floating microspheres were collected.

$$\text{Buoyancy}(\%) = \frac{\text{Weight of Floating Microspheres}}{\text{Initial weight of hollow microspheres}} \times 100$$

Swelling index

With minor modifications, the swelling index was determined using the methodology described in the literature (Mukund et al., 2012). Precisely weighed microspheres (100 mg) were allowed to swell in 0.1N HCl for 24 hours. After excess surface-attached liquid was gently blotted away, the swollen microspheres were weighed using a microbalance. The swelling index was calculated using the following formula:

$$\text{Swelling index}(\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Final weight}} \times 100$$

Statistical Analysis

All experiments were performed in triplicate ($n = 3$), and the results are presented as mean $\pm$ standard deviation (SD). Statistical comparisons between different formulations (e.g., entrapment efficiency, particle size, buoyancy, drug release) were conducted using one-way analysis of variance (ANOVA). A p -value of less than 0.05 ($p < 0.05$) was considered statistically significant. All data were analyzed using GraphPad Prism 10. (GraphPad Software, Version 10.4.1).

Scanning electron microscopy

Scanning electron microscopy (S-3700N) was used to examine the surface characteristics of the microspheres. The dried microspheres were mounted on brass stubs using double-sided adhesive tape and then coated with gold. The samples were scanned at a voltage of 10 kV, with a magnification range of 100–2000X.

Differential scanning calorimetry (DSC)

DSC (Perkin-Elmer DSC 7, USA) was used to assess the thermal characteristics of the microspheres. Approximately 3–5 mg of each sample was placed in an aluminium pan and heated at a rate of 10 °C per minute up to 300 °C in a nitrogen atmosphere with a flow rate of 20 mL/min. Thermograms of the microspheres were recorded over the temperature range of 50–300 °C.

In vitro drug release studies

The drug release of RFN microspheres was evaluated using a USP Type I (basket) apparatus, following the method described by Devi et al. (2016). The apparatus was filled with 900 mL of simulated gastric fluid (SGF, pH 1.2) and maintained at 37 ± 0.5 °C with a rotation speed of 100 ± 2 rpm. Microspheres containing 50 mg of RFN were accurately weighed and placed in the basket. At predetermined intervals, 5 mL samples were withdrawn and replaced with an equal volume of fresh dissolution medium to maintain sink conditions. SGF (pH 1.2) was used as a blank, and the drug concentration in the samples was measured spectrophotometrically at 475 nm (Jain et al., 2015).

Release kinetic studies

To understand the kinetics of drug release, various release kinetic models were applied to analyze the drug release data. The best-fit model was selected by comparing the R^2 values obtained from the different kinetic models.

Antimicrobial activity/Antitubercular activity

Staphylococcus aureus (*S. aureus*) was used as a model strain due to its well-characterized nature and ease of handling under standard biosafety conditions. Testing against *M. tuberculosis* requires BSL-3 containment, which was not available during this study.

The antibacterial activity of RIF-loaded microspheres was evaluated against *S. aureus*. Bacterial spores were initially added to 5 mL of Mueller-Hinton broth and incubated for 12 hours. Subsequently, 0.1 mL of the culture was transferred into another 5 mL of Mueller-Hinton broth to achieve a final concentration of approximately 3×10^8 colony-forming units (cfu)/mL.

Meanwhile, molten Mueller-Hinton agar was poured into sterile Petri dishes and allowed to solidify. Sterile filter discs with a diameter of 1 cm were immersed in PBS containing the pure drug, while PBS containing RIF released from the RIF-loaded microspheres was placed at the centre of the plates. All samples were incubated at 37 °C for 18 hours, and the

results were compared with RIF-free microspheres. The diameter of the zone of inhibition was measured. All antibacterial activity experiments were performed in triplicate (Jain et al., 2008).

Stability study

Stability testing of the formulation was conducted under different storage conditions: $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ /60% RH $\pm$ 5% RH and refrigerated conditions ($2-8^{\circ}\text{C}$) for a period of three months, following ICH guidelines. The formulation was stored in aluminium foil throughout the study. At predetermined intervals, the samples were withdrawn and evaluated for physical appearance, buoyancy, and drug content. Drug content and buoyancy were measured on days 30, 60, and 90.

RESULTS AND DISCUSSIONS

Drug compatibility studies

By UV spectrophotometry

Drug–excipient compatibility was evaluated using UV spectrophotometry by scanning solutions of the drug, the excipient, and a physical mixture of the drug and excipient separately, over the wavelength range of 400–800 nm. The spectra were shown in Figure 1. The results reveal that the UV absorption maxima of the physical mixture overlapped with those of the drug, indicating no interaction between the drug and the polymer. Thus, the drug was found to be compatible with the excipient.

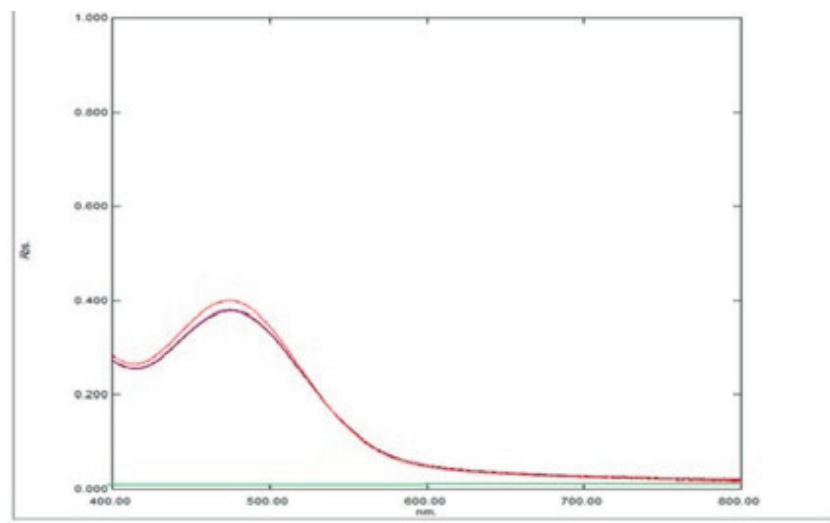


Figure 1. Compatibility study of Rifampicin and Eudragit S100 by using the UV spectrophotometric method

By fourier-transformed infrared spectroscopy (FT-IR)

The drug was identified using FTIR, and the spectra are shown in Figure 2 (A–D). The characteristic peaks for all functional groups of RFN—C=O acetyl stretching, CH_3O symmetric stretching, $-\text{CH}_3$ stretching, and $-\text{C}=\text{N}$ asymmetric bending—were observed at 1734.05, 2828.73, 2934.04, and 1654.03 cm^{-1} , respectively. Peaks corresponding to the functional groups C=O (1749 cm^{-1}) and OH (1248 cm^{-1})

were observed in the FTIR spectrum of Eudragit S100 (Figure 2B).

All the functional group peaks of the drug appeared in the FTIR spectra of the physical mixture of drug and polymer, indicating that there was no interaction between the drug and the polymer (Figure 2C and 2D). Similar findings of drug–polymer compatibility via FTIR have been reported for rifampicin microspheres (Goyal et al., 2011) and for other drugs in Eudragit-based systems (Verma et al., 2020).

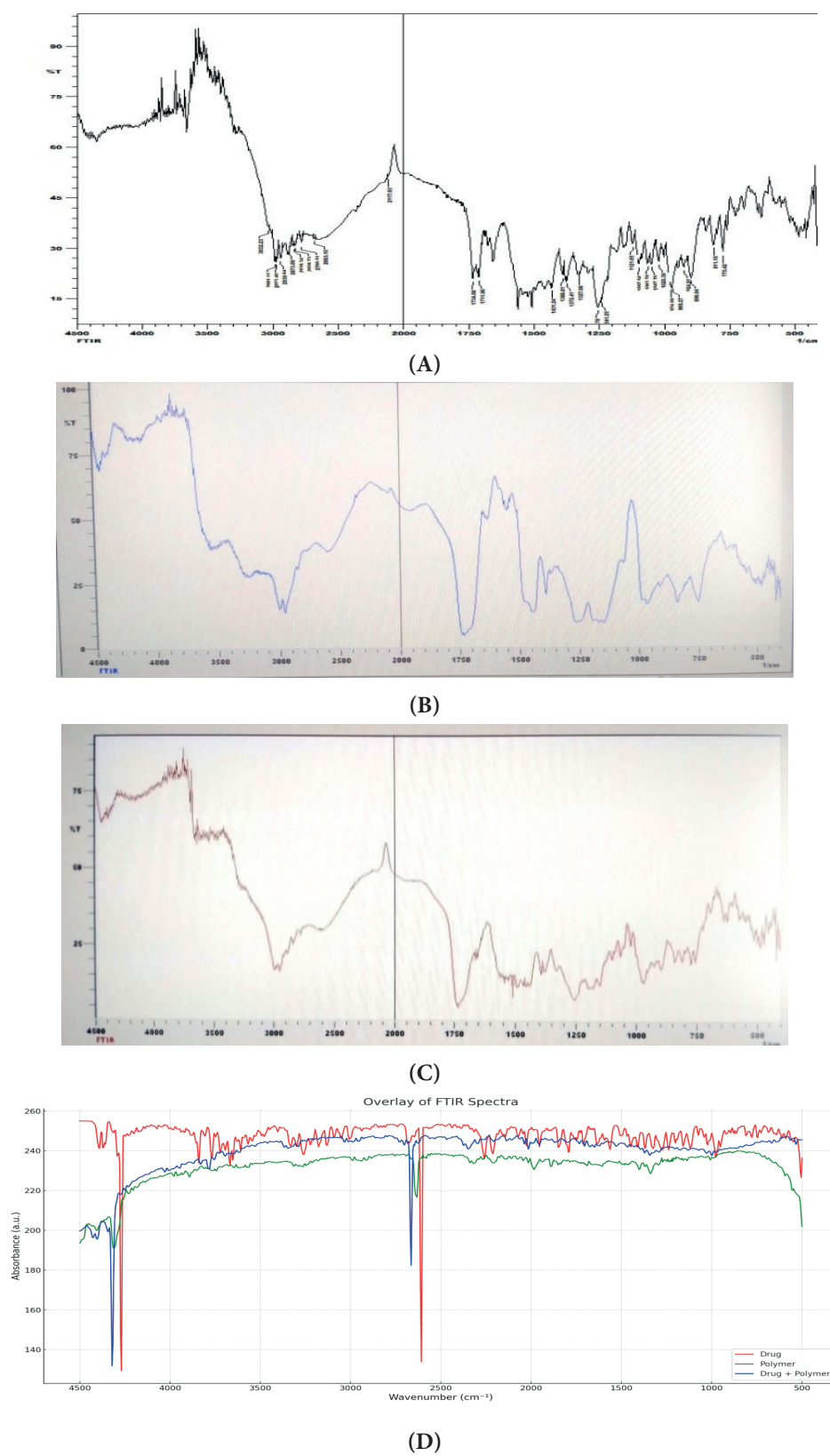
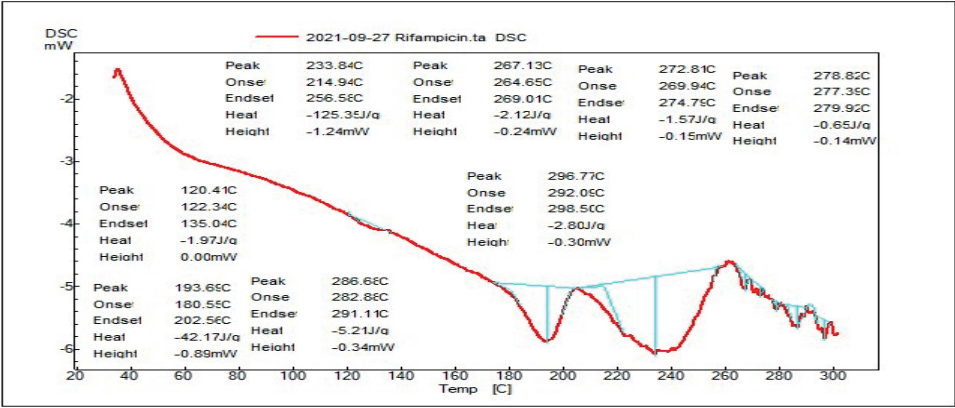


Figure 2. FTIR spectrum of (A) RFN, (B) Eudragit S100, (C) Physical mixture of Drug and Eudragit S100, (D) Overlay FTIR spectra of RFN, Eudragit S100, and Physical mixture of Drug and Eudragit S100

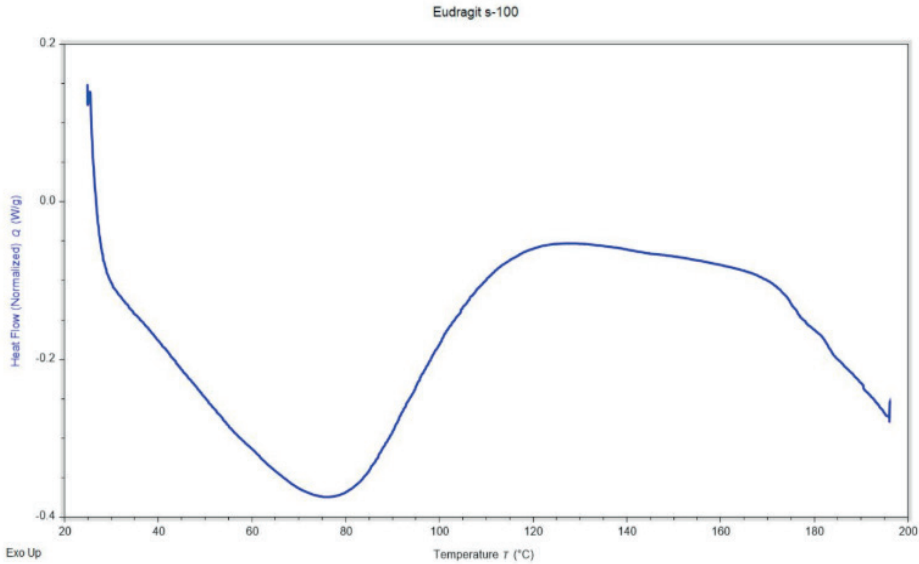
By differential scanning colourimetry (DSC)

RFN can exist in two polymorphic forms: form I and form II, consistent with previously reported transitions (Patel et al., 2006). Form I of RFN is less chemically stable at higher temperatures and decomposes before melting at 258 °C. The DSC thermogram of pure RFN displayed an endothermic peak at 193.69 °C, corresponding to the melting of form II, followed by an exothermic peak at 205.62 °C, indicating the crystallization of form I. An endothermic peak identified the decomposition of RFN at 233.84 °C (Figure 3A).

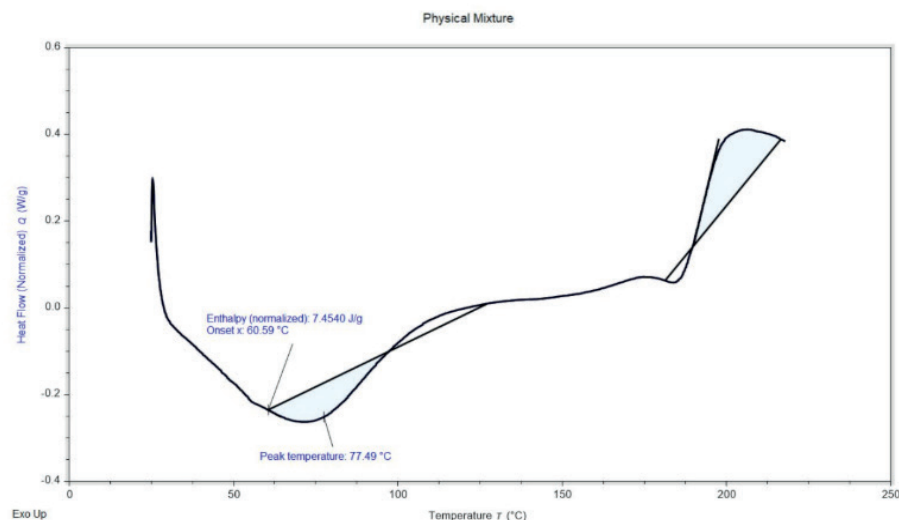
The DSC thermogram of Eudragit S100 displayed a broad endothermic peak at 77.49 °C, as shown in Figure 3B. In the physical mixture of drug and polymer, a single endothermic peak was observed at 195.7 °C, similar to the DSC thermogram of the pure drug, indicating no interaction between the drug and polymer (Figure 3C and 3D). These results suggest that the polymer is compatible with RFN for the design of microspheres. The absence of additional or missing peaks further supports the conclusion that no thermal degradation or interaction occurred, consistent with the findings of Maurya et al. (2012) for RFN-loaded microcapsules.



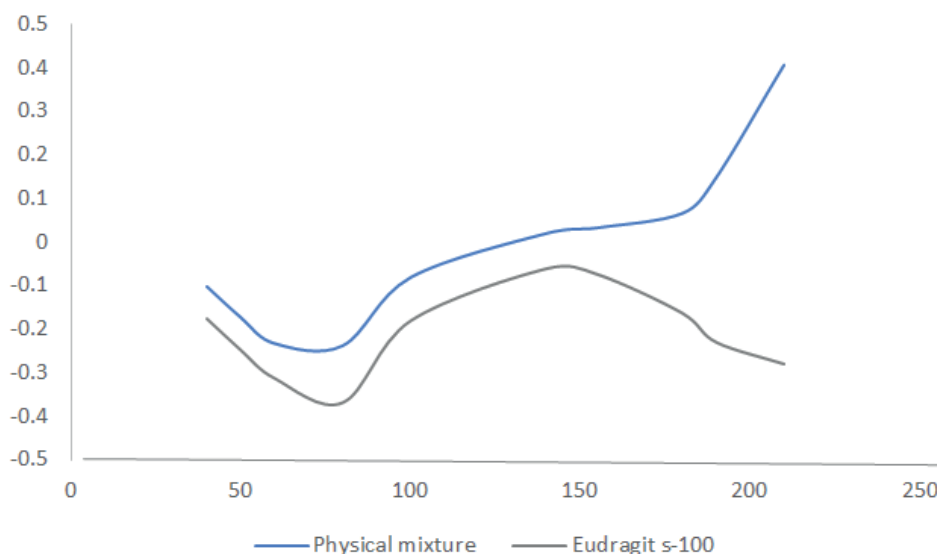
(A)



(B)



(C)



(D)

Figure 3. DSC thermogram of (A) Rifampicin, (B) Eudragit S 100, (C) Physical mixture of Drug and Eudragit S 100, (D) Overlay DSC thermogram of Eudragit S100, and Physical mixture of Drug and Eudragit S100

Formulation of Rifampicin floating microspheres

RFN microspheres were successfully prepared using the solvent evaporation technique by varying formulation variables, such as polymer concentration (Eudragit S100), and process variables, including stirring speed (300, 400, and 500 rpm) and stirring time (1, 1.5, and 2 hours), while keeping the quantity of RFN constant.

Evaluation of rifampicin-loaded floating microspheres

Shape of floating microspheres

All floating microspheres were observed to be spherical solid particles under an optical microscope (Figure 4A) and appeared as free-flowing powder (Figure 4B).



(A) (B)

Figure 4. (A) Microscopic image of RFN floating microspheres (FES4), (B) Final floating microspheres (FES4)

Production yield of microspheres

All floating microsphere formulations exhibited yield percentages ranging from $71.87 \pm 0.04\%$ to $96.1 \pm 0.07\%$, indicating efficient encapsulation using the solvent evaporation technique. Such high yields are favourable for scalability and are comparable to those reported in similar studies on floating microspheres (Bakshi, 2016; Phutane et al., 2010)

Particle size

As the microspheres are administered orally, particle size plays a significant role in achieving prolonged gastric retention of the drug formulation and, consequently, its therapeutic performance. The particle size of the floating microspheres increased from $41.66 \pm 1.77 \mu\text{m}$ to $114.16 \pm 2.88 \mu\text{m}$ as the ratio of Eudragit S100 increased from 1:1 to 1:5 (Figure 5Ai and B), due to the increased viscosity of the medium and resistance to shear during emulsification. At higher viscosities, enhanced interfacial tension and reduced shearing efficiency result in the formation of larger particles (FES1–FES5). This trend is consistent with the findings of Agarwal et al. (2022) and Gupta et

al. (2014), where polymer content significantly influenced microsphere diameter and flotation potential. A statistically significant difference in particle size was observed ($p < 0.05$).

To prevent foaming on the emulsion surface—which could affect the rate of dichloromethane evaporation and, in turn, the formation of floating microspheres—a minimum stirring speed of 300 rpm and a stirring time of one hour were employed. Among the first five formulations, FES4 exhibited the smallest particle size and was selected for further optimization by modifying parameters such as stirring speed and stirring time.

When the propeller rotation speed was increased from 300 to 500 rpm, the average particle size significantly decreased from $80.83 \pm 1.40 \mu\text{m}$ to $41.66 \pm 1.44 \mu\text{m}$ (Figure 5Aii), possibly due to foam formation and breakage of microspheres at high speed. Increasing the stirring time from 1 to 2 hours resulted in a slight decrease in particle size, from $80.83 \pm 1.40 \mu\text{m}$ to $72.73 \pm 1.41 \mu\text{m}$ (Figure 5Aiii), likely due to irregular microsphere formation.

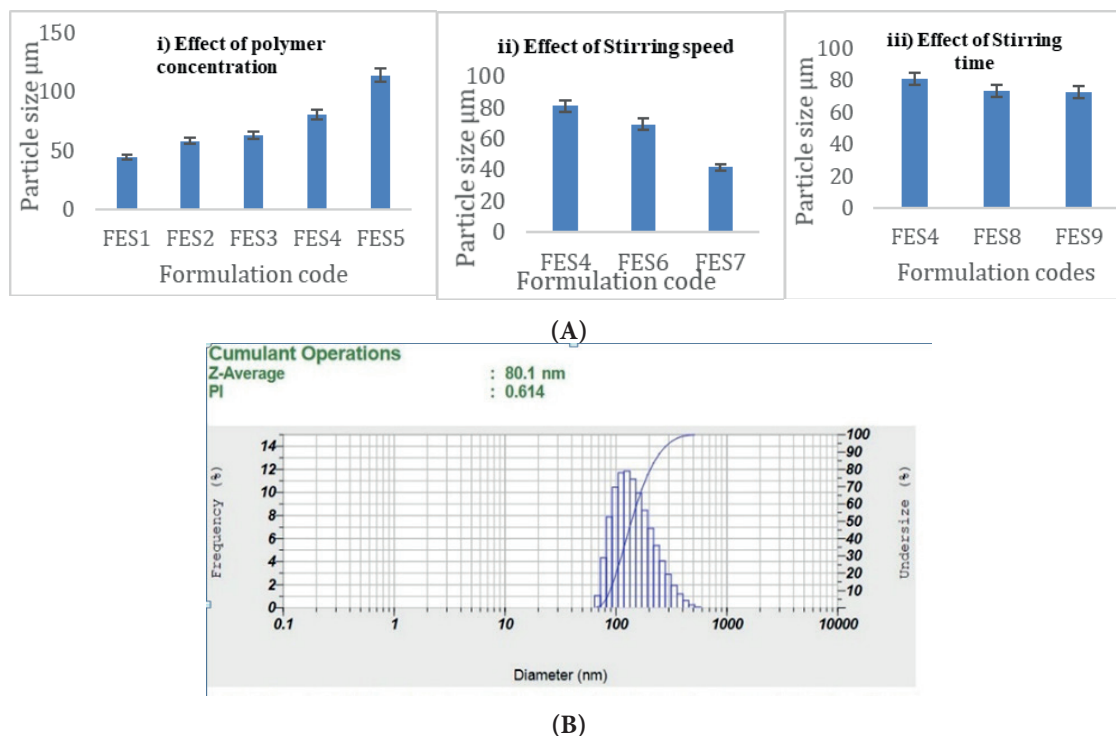


Figure 5. (A) Effect of (i) polymer concentration, (ii) stirring speed, and (iii) stirring time on particle size of RFN floating microspheres ($n = 3$) (B) Particle size analysis of final floating microspheres (FES4)

Drug content and entrapment efficiency

As the polymer ratio increased from 1:1 to 1:5, the drug content increased up to the FES4 formulation and then decreased, likely due to polymer hardening, as reported previously (Nila et al., 2014). When the rotation speed was increased from 300 to 500 rpm, a slight decrease in drug content was observed. A modest increase in entrapment efficiency was noted with increasing polymer ratio, ranging from $88.21 \pm 0.10\%$ to $93.16 \pm 0.45\%$ (Figure 6). The differences between the floating microspheres were statistically significant ($p < 0.05$) based on ANOVA analysis.

Compared with previously reported gastro-retentive microsphere systems, the optimized FES4 formulation demonstrated superior performance in terms of entrapment efficiency, floating behavior, and sustained drug release. Goyal et al. (2011) reported entrapment efficiencies of approximately 85% for rifampicin-loaded microspheres using ethyl cellulose and HPMC, whereas the present study achieved up to 93.16% with Eudragit S100. The improvement in entrapment efficiency can be attributed to increased matrix density, consistent with earlier work by Bhadouriya et al. (2012).

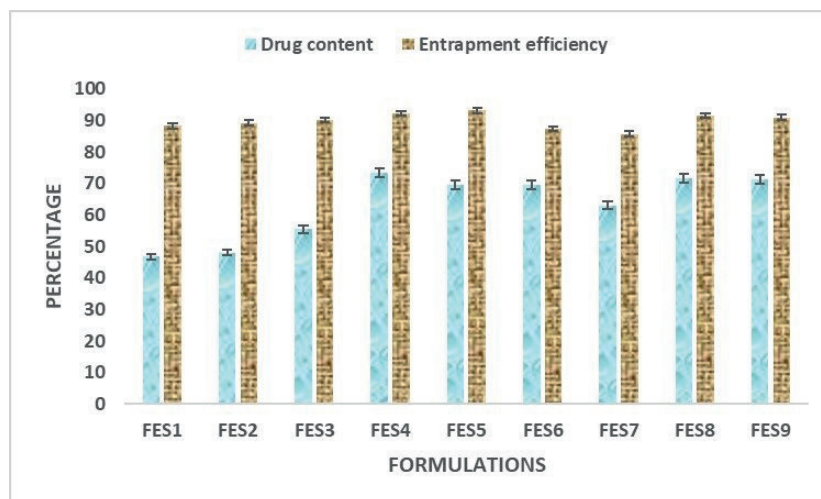


Figure 6. Effect of polymer concentration, stirring speed, and stirring time on drug content and entrapment efficiency of various RFN floating microspheres (n = 3)

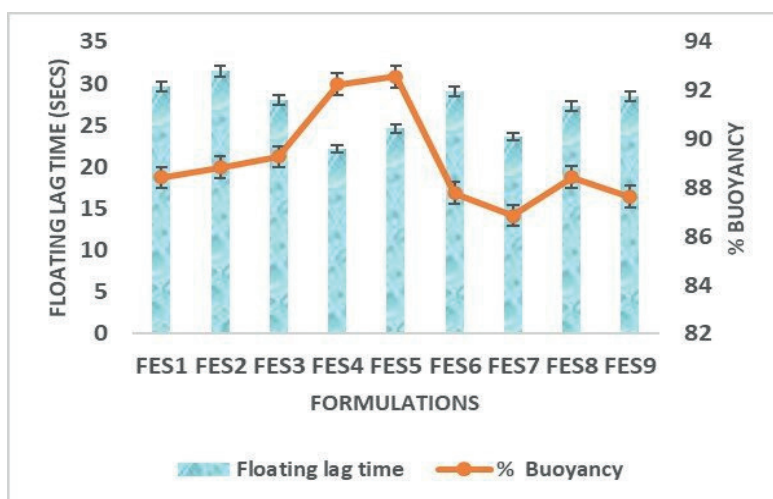
Floating lag time and *in vitro* buoyancy

The improved buoyancy observed with higher polymer concentrations is consistent with the findings of Gupta et al. (2014), who reported a similar trend in Eudragit-based microspheres. A higher percentage of buoyancy is essential for the drug formulation to achieve prolonged gastric residence, as noted in previous studies (Ammar et al., 2016). The *in vitro* floating behavior of all the prepared microspheres was evaluated. According to the literature (Agarwal et al., 2022;

Pingale & Amrutkar, 2021), floating ability increases with particle size. All formulations floated for 24 hours, as shown in Figure 7A. The percent buoyancy for all developed formulations exceeded 86%, with the FES4 formulation exhibiting the highest buoyancy at 92.56% (Figure 7B). Similarly, Gupta et al. (2014) reported Eudragit-based floating microspheres of famotidine with buoyancy exceeding 90%, comparable to the 92.56% buoyancy observed for FES4 in the present study.



(A)



(B)

Figure 7. (A) Photograph showing the floatability of the developed formulation up to 24 hours.

(B) Effect of polymer concentration, stirring speed, and stirring time on floating lag time and % buoyancy of various RFN microspheres (n=3)

Swelling index of microspheres

The swelling index increased from $48 \pm 0.04\%$ to $73 \pm 0.09\%$ with increasing polymer concentration, as the water-absorbing capacity of the microspheres depends on the polymer content. In contrast, variations in rotation speed and stirring time did not pro-

duce any significant changes in the swelling index, as shown in Figure 8. This observation is consistent with previous studies by Kawashima et al. (1992), Mukund et al. (2012), and Pingale & Amrutkar (2021) which identified polymer concentration as a key determinant of swelling behaviour.

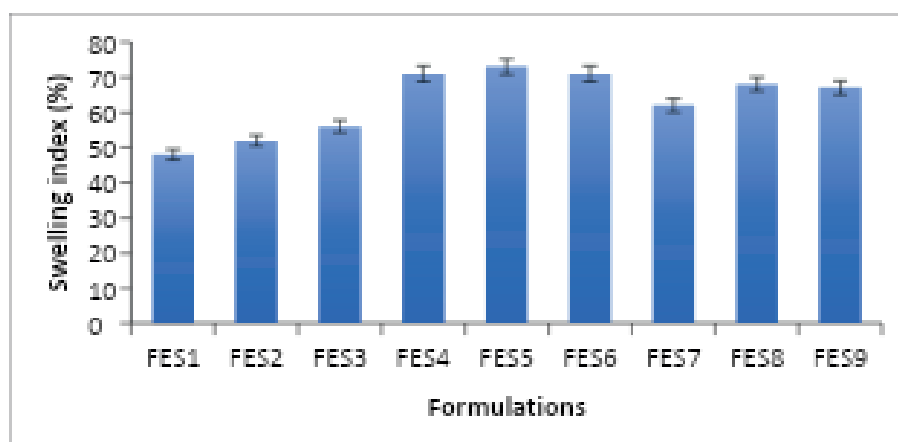


Figure 8. Effect of polymer concentration, stirring speed, and stirring time on swelling index of RFN microspheres (n=3)

Scanning electron microscopy

SEM was used to evaluate the surface characteristics of the floating microspheres, and the results are presented in Figure 9. The microspheres were spherical with smooth surfaces and a hollow inner cavity.

They did not exhibit agglomeration, as evident from the photomicrograph. This structure supports prolonged buoyancy and controlled drug release, consistent with observations reported in floating systems by Bhadouriya et al. (2012) and Kawashima et al. (1992).

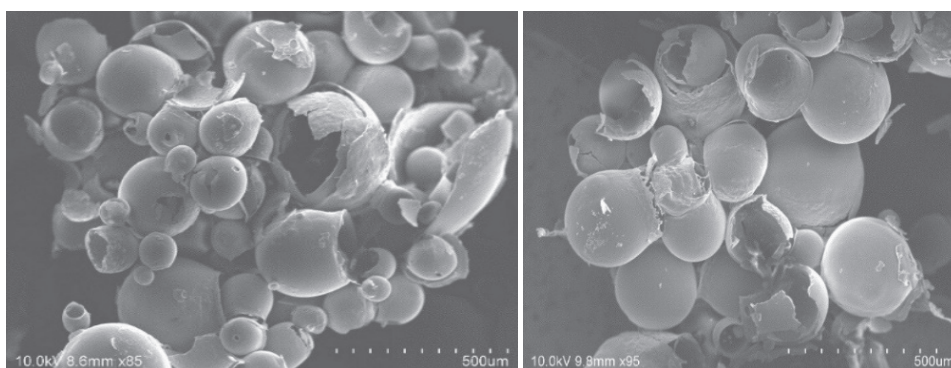


Figure 9. SEM images of RFN FES4 microspheres

In vitro drug release studies

The results suggest that all formulations exhibited a noticeable initial burst release during the first 2 h, followed by a sustained release profile over the next 24 h, as shown in Figure 10, which presents the quantitative dissolution data. The initial burst release may be attributed to the drug present on the surface of the microspheres, a pattern also observed by Kumar, Jat & Kumar (2019), Kumar & Srivastava (2021), and Pingale & Amrutkar (2021).

An increase in rotation speed from 300 to 500 rpm resulted in enhanced drug release, likely due to a reduction in microsphere particle size. The smaller particle size increases the surface area, leading to faster dissolution. Drug release from the formulations at 24 h ranged from $67.8 \pm 2.3\%$ to $91.4 \pm 1.9\%$, with the FES4 formulation exhibiting significantly higher release compared to the others ($p < 0.01$, ANOVA).

The release profile of FES4 is consistent with non-Fickian diffusion behaviour ($n = 0.367$) and follows the Higuchi model ($R^2 = 0.984$), indicating that drug release is governed by both diffusion and polymer matrix erosion.

Similar kinetics have been reported in Eudragit-based sustained-release systems (Devi et al., 2016; Verma et al., 2020). The Higuchi model behaviour of FES4 aligns with findings from Maurya et al. (2012) and Verma et al. (2020), confirming diffusion-controlled release.

Release kinetics studies

The release kinetics of the floating microspheres were analysed and are presented in Table 2. The results indicate that the Higuchi model provided the best fit, exhibiting the highest R^2 value (0.9840). The n value was 0.367, suggesting a non-Fickian transport mechanism.

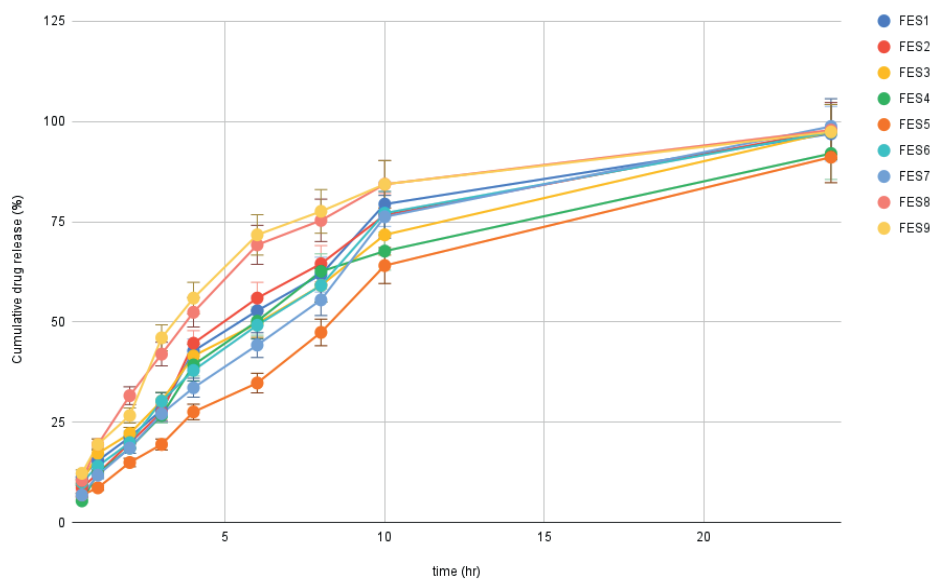


Figure 10. Cumulative percentage drug release from various Eudragit S-100-based floating microspheres

Table 2. Drug release kinetics of FES4 formulation

Formulation code	Zero order	First order	Higuchi	Hixson-crowell	Korsmeyer peppas	Best fit model
R	0.9736	0.9807	0.984	0.8832	0.949	Higuchi
K value	3.595	0.107	21.610	0.031	37.388	

Anti-microbial activity

The antibacterial efficacy of rifampicin (RF-N)-loaded floating microspheres was evaluated against *S. aureus*. A clear zone of inhibition was observed, as shown in Figure 11A and 11B. The mean zone of inhibition measured 21.32 ± 0.21 mm for the pure drug and 15.25 ± 0.10 mm for the optimised formulation (FES4), while no inhibition was observed for the blank microspheres. This confirms the sterility of the formulation and indicates the absence of residual antimicrobial effects from the excipients (Goyal et al., 2011; Jain et al., 2008; Pingale & Amrutkar, 2021).

S. aureus was selected as a model organism for preliminary antimicrobial testing due to its classification as a biosafety level-2 (BSL-2) pathogen, allowing evaluation under standard laboratory conditions. In contrast, *M. tuberculosis*, the primary causative agent of tuberculosis and the main therapeutic target of rifampicin, is a BSL-3 organism that requires specialised high-containment facilities, which were not available for the present study.

The primary objective of this study was to develop and systematically characterise rifampicin-loaded floating microspheres designed for enhanced gastric retention and sustained drug release. Within this framework, *S. aureus* was strategically selected as a safe and practical model organism to serve multiple purposes. It enabled assessment of whether the microencapsulation process effectively preserved the antimicrobial activity of rifampicin, facilitated preliminary evaluation of the formulation’s antibacterial potential, and supported iterative optimization of critical formulation parameters—such as polymer composition, drug entrapment efficiency, and release kinetics. Importantly, the use of *S. aureus* allowed these evaluations to be conducted under standard laboratory conditions, thereby circumventing the stringent biosafety requirements associated with *M. tuberculosis* (a BSL-3 pathogen), thus streamlining early-stage formulation development.

S. aureus is extensively used in pharmaceutical research as a surrogate organism for preliminary screen-

ing of antibacterial activity. Its application in this study enabled rapid and efficient evaluation of drug integrity following encapsulation, as well as verification of the formulation's sterility. This approach aligns with standard practices in formulation research, where initial antimicrobial validation is typically performed using non-mycobacterial strains before advancing to more complex, pathogen-specific assays (Bhise et al., 2010).

Although *S. aureus* is not etiologically linked to tuberculosis, its use in this context is scientifically justified and widely accepted for preliminary antimicrobial evaluation. Further investigations using *M. tuberculosis* or suitable non-pathogenic surrogates such as *Mycobacterium smegmatis* are warranted to directly confirm the anti-tubercular potential of the developed system under appropriate biosafety conditions.

Moreover, rifampicin exhibits broad-spectrum antibacterial activity, including notable efficacy against *S. aureus*, particularly in complex clinical scenarios. Owing to its lipophilic nature, rifampicin can effectively penetrate biofilms and intracellular compartments, making it especially valuable in the treatment

of persistent *S. aureus* infections such as prosthetic joint infections and infective endocarditis. Additionally, rifampicin is commonly employed in combination with other antibiotics to enhance therapeutic outcomes and prevent the rapid emergence of resistance—a significant concern when the drug is used as monotherapy. In clinical practice, rifampicin has also been utilised to eradicate *S. aureus* nasal carriage in healthcare settings to reduce the risk of infection transmission, although its application in this context is limited due to resistance development when used alone.

Evaluating the antibacterial activity of rifampicin-loaded floating microspheres against *S. aureus* is a justified and effective approach for confirming the preservation of drug bioactivity post-encapsulation and for supporting the formulation's antimicrobial potential. While not specific to tuberculosis, the inclusion of *S. aureus* as a model organism provides valuable insights during the early development phase. Future studies are planned to validate the anti-tubercular efficacy of the formulation against *M. tuberculosis* or appropriate mycobacterial surrogate models.

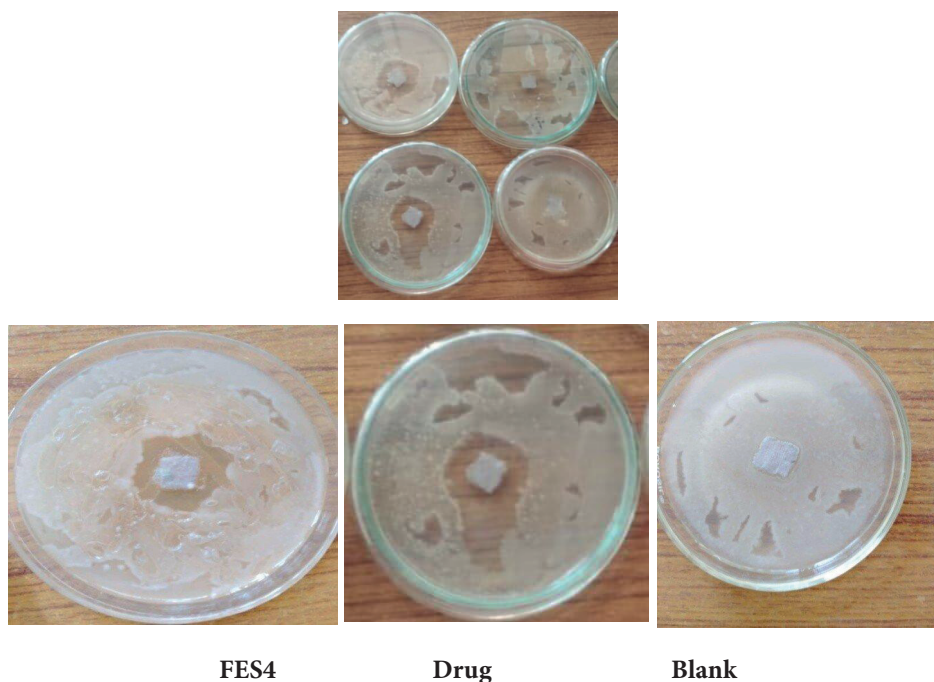


Figure 11. (A) Zone of Inhibition of FES4, formulation, Drug and Blank against *S. aureus* after 18 h incubation at 37°C

Stability studies

The stability evaluation of FES4 was carried out over three months in compliance with ICH guidelines, during which the percentage of buoyancy and drug content was assessed. The findings, as shown in Figure 12, indicate negligible variation in both drug content

and buoyancy. This suggests that the developed formulation maintains its performance during prolonged storage—an essential criterion for regulatory approval and marketability, as highlighted in stability studies by Khan et al. (2020).

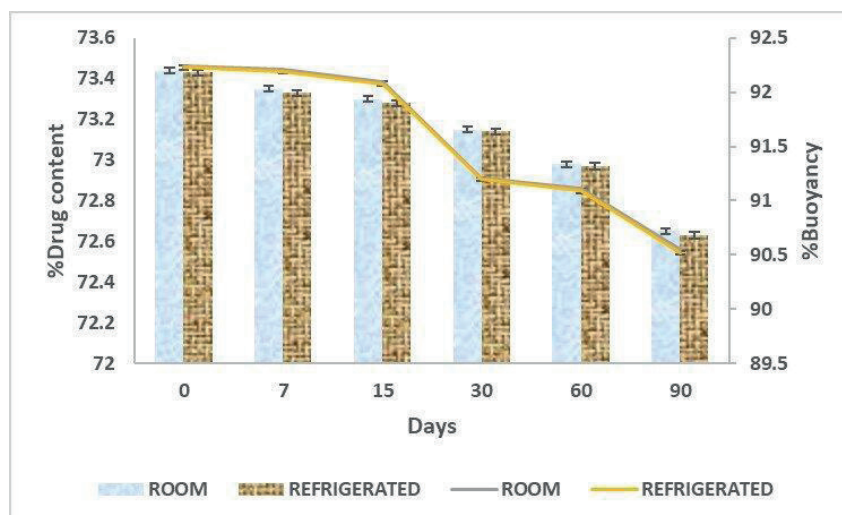


Figure 12. Stability studies of FES4 for 90 days in refrigerated temperature and $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$

CONCLUSION

In conclusion, the development of gastro-retentive floating microspheres containing rifampicin (RFN) yielded promising results as a controlled drug delivery system, effectively addressing the challenges associated with RFN's short half-life and limited gastric residence. Drug–excipient compatibility was confirmed through UV, FTIR, and DSC analyses, indicating no significant interactions between rifampicin and the formulation excipients.

Floating microspheres were successfully prepared using Eudragit S100 via the solvent evaporation technique, and the influence of key formulation parameters—including stirring speed and polymer concentration—was systematically evaluated. These variables significantly influenced entrapment efficiency, buoyancy, and particle size.

Among all batches, formulation FES4 (drug-to-polymer ratio of 1:4) was identified as the optimized

formulation, exhibiting excellent buoyancy (92.24%), high entrapment efficiency (88.21–93.16%), and prolonged drug release. The drug release profile of FES4 followed the Higuchi kinetic model and was governed by non-Fickian diffusion, suggesting a combination of diffusion- and erosion-controlled mechanisms.

Stability studies conducted under ambient and room temperature conditions for 90 days confirmed the robustness of the formulation, showing no significant changes in key parameters and indicating good shelf-life stability.

Compared with previously reported gastro-retentive microsphere systems, the optimized formulation (FES4) demonstrated superior performance in terms of entrapment efficiency, floating behavior, and sustained drug release. Notably, the innovative use of Eudragit S100—a polymer typically reserved for colon-targeted delivery—within a gas-trapping floating system represents a novel approach to enhance gastric

retention. This unconventional application, in combination with solvent evaporation and gas-trapping techniques, resulted in improved performance over systems employing ethyl cellulose, HPMC, or Eudragit RL/RS and Eudragit RLPO (Bhise et al., 2010; Patel et al., 2012; Pingale & Amrutkar, 2021).

These findings collectively highlight the originality, efficacy, and potential clinical relevance of the Collectively, these findings underscore the originality, efficacy, and potential clinical relevance of the developed Eudragit S100-based floating microsphere system for rifampicin delivery.

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AUTHOR CONTRIBUTION STATEMENT

All authors contributed to data collection, processing, writing, revision of the draft, reading, and approval of the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Evaluation of Drug Interactions with Psychotropic Drugs Causing QTc Prolongation – A Study in Palliative Care Patients

Fadain ABISHOV*, Ozgenur GERIDONMEZ**, Yahya SEKIN***,
Kamer TECEN-YUCEL****

Evaluation of Drug Interactions with Psychotropic Drugs Causing QTc Prolongation – A Study in Palliative Care Patients

QTc Uzamasına Neden Olan Psikotrop İlaçlarla İlaç Etkileşimlerinin Değerlendirilmesi – Palyatif Bakım Hastalarında Bir Çalışma

SUMMARY

The use of multiple QTc-prolonging psychotropic drugs or agents affecting their metabolism can lead to significant drug-drug interactions (DDIs). This study aims to compare the severity ratings of potential DDIs (pDDIs) specified in two interaction databases and identify clinically relevant interactions associated with QTc prolongation in palliative care patients. This prospective study was conducted in a palliative care unit of a state hospital between October 2023 and May 2024. Potential DDIs and their severities were assessed according to Micromedex® and Lexicomp® databases. Electrocardiograms (ECGs) were performed on patients flagged as having the risk of QTc prolongation. The Drug Interaction Probability Scale (DIPS) was applied to evaluate the clinical relevance of the detected interactions. A clinical pharmacist provided recommendations based on DIPS assessments. A total of 209 pDDIs involving 77 different drug pairs were identified among 120 patients. Interrater reliability between the two databases was poor for major pDDIs (kappa = -0.034) and overall (kappa = -0.052). A pDDI involving a psychotropic drug was detected in 61.7% of patients. QTc prolongation was observed in 66.7% of patients who underwent ECG (n=58). According to DIPS, 122 clinically relevant DDIs (63 unique pairs) were identified. Physicians accepted 10.5% of the clinical pharmacist's recommendations, leading to modifications in psychotropic drug regimens. Follow-up ECGs revealed no QTc prolongation after the interventions. The findings highlight the frequent exposure of palliative care patients to psychotropic drug interactions that may prolong QTc. Although drug interaction databases provide guidance, discrepancies in severity ratings and clinical relevance necessitate individualized patient evaluation by a multidisciplinary team, including clinical pharmacists, to ensure medication safety.

Keywords: Psychotropic drugs, drug interaction programs, clinically relevant drug interactions, palliative care patients.

ÖZ

QTc uzatan psikotrop ilaçların veya metabolizmalarını etkileyen ajanların birlikte kullanımı, önemli ilaç-ilac etkileşimlerine (İİE) yol açabilmektedir. Bu çalışmanın amacı, iki ilaç etkileşim veri tabanı tarafından tanımlanan potansiyel ilaç-ilac etkileşimlerinin (pİİE'ler) şiddet derecelerini karşılaştırmak ve palyatif bakım hastalarında QTc uzaması ile ilişkili klinik olarak anlamlı etkileşimleri belirlemektir. Bu prospektif çalışma, Ekim 2023 ile Mayıs 2024 tarihleri arasında bir devlet hastanesinin palyatif bakım ünitesinde yürütülmüştür. Potansiyel ilaç etkileşimleri ve şiddetleri, Micromedex® ve Lexicomp® veri tabanları kullanılarak değerlendirilmiştir. QTc uzaması riski taşıdığı belirlenen hastalar için elektrokardiyogram (EKG) çekilmiştir. Saptanan etkileşimlerin klinik önemini değerlendirmek için İlaç Etkileşimi Olasılık Ölçeği (DIPS) uygulanmıştır. Klinik eczacı, DIPS değerlendirmelerine dayanarak hekime öneride bulunmuştur. 120 hasta arasında toplam 77 farklı ilaç etkileşim çiftini içeren 209 pİİE tespit edildi. İki veri tabanı arasında gözlemciler arası güvenilirlik, majör şiddetteki pİİE (katsayı = -0,034) ve genel pİİE şiddeti için (katsayı = -0,052) düşüktü. Psikotrop bir ilaç içeren pİİE, hastaların %61,7'sinde (n=74) tespit edildi. EKG çekilen hastaların %66,7'sinde (n=58) QTc uzaması gözlemlendi. DIPS'e göre, 122 klinik olarak anlamlı ilaç etkileşimi (63 farklı ilaç etkileşim çifti) tespit edildi. Hekimler, klinik eczacının önerilerinin %10,5'ini kabul etti ve bu da psikotrop ilaç tedavisinde değişikliklere yol açtı. Eczacı müdahalelerinden sonra takip edilen EKG'lerde QTc uzaması gözlemlenmedi. Bulgular, palyatif bakım hastalarının QTc'yi uzatabilen psikotrop ilaç etkileşimlerine sık sık maruz kaldığını vurgulamaktadır. İlaç etkileşim veri tabanları rehberlik sağlasa da, şiddet derecelendirmelerindeki ve etkileşimin klinik önemiyle ilgili farklılıklar, ilaç güvenliğini sağlamak için klinik eczacıların dahil olduğu multidisipliner bir ekip tarafından bireyselleştirilmiş hasta değerlendirmesini zorunlu kılmaktadır.

Anahtar Kelimeler: Psikotrop ilaçlar, ilaç etkileşimi programları, klinik olarak önemli ilaç etkileşimleri, palyatif bakım hastaları.

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* ORCID: 0009-0004-1925-2571, Anadolu University, Faculty of Pharmacy, Department of Clinical Pharmacy, 26470, Eskişehir, Türkiye

** ORCID: 0000-0002-8223-2723, Hacettepe University, Faculty of Pharmacy, Department of Clinical Pharmacy, 06100, Ankara, Türkiye & Anadolu University, Faculty of Pharmacy, Department of Clinical Pharmacy, 26470, Eskişehir, Türkiye

*** ORCID: 0009-0002-8670-5126, Yunus Emre State Hospital, Palliative Care Service, Eskişehir, Türkiye

**** ORCID: 0000-0002-5381-0950, Anadolu University, Faculty of Pharmacy, Department of Clinical Pharmacy, 26470, Eskişehir, Türkiye

° Corresponding Author; Kamer Tecen-Yucel
E-mail: kamertecen@anadolu.edu.tr

INTRODUCTION

Palliative care services focused on comfort and symptom control are vital for those with terminal illnesses. Current estimates indicate that around 25-42% of individuals nearing the end of life could gain from palliative care. The increasing population of older individuals and the rising incidence of chronic illnesses in various nations suggest that a greater number of individuals may require palliative care in the future (Etkind et al., 2017).

Behavioral symptoms, such as anxiety, restlessness, depression, and delirium, are common in the end stages of life and can be extremely distressing for both patients and caregivers. Psychotropic drugs, including antipsychotics and benzodiazepines, are commonly prescribed in palliative care for the management of both behavioral and physical symptoms, such as nausea (Gerlach et al., 2021). The prescription of psychotropic drugs in elderly patients poses challenges due to prevalent comorbidities and polypharmacy, which frequently lead to numerous drug interactions. The interaction of these two factors, along with the changes in pharmacokinetics and pharmacodynamics that come with getting older, as well as the higher risk of tolerance and dependence-related conditions, leads to a higher risk of side effects, especially corrected QT (QTc) prolongation (Mangoni & Jackson, 2004).

The guidelines established by the American Heart Association (AHA) and the American College of Cardiology Foundation (ACCF) indicate that a QTc interval exceeding 470 ms in adult males and 480 ms in adult females is considered abnormal. The risk of developing torsades de pointes (TdP) increases when the QTc interval is ≥ 500 ms in patients (Drew et al., 2010). According to research, the risk of heart problems in people with long QT syndrome (LQTS) goes up by about 5% for every 10 ms increase in the QTc interval (Khatib et al., 2021).

The influence of psychotropic drugs on cardiac repolarization is becoming increasingly recognized.

Numerous antipsychotic and antidepressant drugs are associated with QT prolongation (Funk & Bostwick, 2013). Haloperidol presents an increased risk of QTc prolongation when administered intravenously (Beach, Celano, Noseworthy, Januzzi & Huffman, 2013). While atypical antipsychotics are associated with QTc prolongation, they pose a lower risk compared to typical antipsychotics (Beach et al., 2013). Antidepressants like Citalopram, Escitalopram, and Sertraline are known to drastically elevate QTc intervals (Funk & Bostwick, 2013). Citalopram has been linked to numerous instances of TdP, resulting in the establishment of dosage restrictions by the FDA (Åström-Lilja, Odeberg, Ekman & Hägg, 2008).

Evidence indicates that the simultaneous administration of various QTc-prolonging drugs, or other treatments that may alter the metabolism of a QTc-prolonging agent, can lead to detrimental drug-drug interactions (DDIs) (Wysowski, Corken, Gallo-Torres, Talarico & Rodriguez, 2001). For a more accurate assessment of QTc prolongation risk, it is important to look at how pharmaceutical agents and patient-specific factors interact with each other. Patient-specific risk factors encompass sex, age, cardiac conditions, dyselectrolytemia, and the administration of diuretics and antiarrhythmics.

Drug interaction programs are regarded as essential tools for alerting physicians to identify DDIs. Previous studies have identified significant discrepancies in the outcomes generated by drug interaction programs concerning potential drug-drug interactions (pDDI) (Tecen-Yucel et al., 2020). Numerous pDDIs can be identified using drug interaction programs; however, limited research has assessed their clinical relevance.

This study aimed to characterize the differences in severity levels of pDDIs identified by two drug interaction programs for psychotropic drugs, as well as to identify clinically relevant DDIs in palliative care patients receiving psychotropic drugs.

METHOD

Study design, setting and participants

The study was conducted prospectively in the palliative care unit of a state hospital, where an average of 110 patients are followed up annually. The participants completed a written informed consent form. This study aimed to identify potential and clinically relevant drug interactions between two psychotropic drugs or between psychotropic drugs and non-psychotropic drugs used in palliative care patients by using the Micromedex® and Lexicomp® drug interaction programs and to assess the risk of these interactions causing QTc prolongation.

According to the sample size calculation (Raosoft sample size calculator), a minimum of 86 patients were planned to participate in the study with a 95% confidence interval and a 5% margin of error.

Patients who were hospitalized in the palliative care unit between October 2023 and May 2024 who were over 18 years old, used at least one psychotropic drug, and had one or more electrocardiograms (ECGs) performed during their initial and follow-up visits were included in the study. Patients who did not meet these criteria were excluded from the study.

Study procedure

A clinical pharmacist participated in clinic visits with physicians and examined patient demographics (age, gender, number of prescribed medications per patient, and diagnosis), drug treatments, and ECG results from the preceding month.

Initially, pDDIs were observed with psychotropic drugs that cause QTc prolongation, and their severity levels were detected from a patient's treatment schedule utilizing Micromedex® and Lexicomp® drug interaction programs. These two drug interaction programs are frequently utilized by physicians in hospitals. The Lexicomp® database (by Wolters Kluwer) and the Micromedex® database (by IBM) necessitate subscriptions for access to medication interaction information (Wolters Kluwer (n.d.), IBM, (n.d.)).

Table 1 was categorized pDDIs into five classifications for analysis: severe (contraindicated), major, moderate, minor, and none. When a drug interaction combination was categorized into multiple classifications by the programs, the most severe category was chosen for comparison between the Micromedex® and Lexicomp® programs. If the possible drug interaction was classified as severe/contraindicated, major, moderate, or minor to produce QTc prolongation in any program, an ECG was performed. The calculated QTc was derived using the Bazett Formula ($QTc = QT/(RR)^{0.5}$). QTc intervals ranging from 450 to 500 ms in males and from 470 to 500 ms in females were deemed borderline prolonged. $QTc \geq 500$ ms or a variation over 60 ms from baseline was classified as prolonged. The cutoffs were selected based on the research in the scientific literature indicating an increased risk of sudden cardiac death or arrhythmias (Das, Ramasubbu, Agnihotri, Kumar & Rawat, 2021).

Upon determining that a patient's clinical status aligned with the effects (related signs and symptoms) of a pDDI, the Drug Interaction Probability Scale (DIPS) criteria were employed for a causal assessment of the specific interaction. A clinical pharmacist ultimately intervened to address identified clinically relevant drug interactions using the DIPS, a tool including 10 questions that evaluate the likelihood of a causal association between an observed occurrence and the effects of a drug interaction. The assessment of DDI encompasses the following criteria: (1) prior reliable reports; consistency with the established characteristics of (2) precipitant or (3) object drug; (4) temporal progression; (5) dechallenge; (6) rechallenge; (7) alternative explanations; (8) concentration of the object drug in blood or other fluids; (9) additional objective evidence aside from drug concentration; and (10) alteration in the interaction with the precipitant drug dosage modification. Each question may be responded to with "yes," "no," or "unknown/not applicable," accompanied by a designated numeric score for each inquiry. The final score corresponds to a qualitative

scale that indicates the likelihood of the reaction being a medication interaction. The likelihood of drug interaction was classified as uncertain (<2), possible (2-4), probably (5-8), or highly probable (>8) (Horn, Hansten & Chan, 2007).

Table 1. Classification of potential drug-drug interactions based on severity as indicated by drug interaction programs

Severity of Interaction (Category)	Micromedex	Lexicomp
Severe (5)	Contraindicated	(X) Avoid combination
Major (4)	Major	(D) Consider therapy modification
Moderate (3)	Moderate	(C) Monitor therapy
Minor (2)	Minor	(B) No action needed
None (1)	Unknown	(A) No known interaction

Statistical analysis

Data were analyzed using descriptive statistics (including mean, standard deviation, median, interquartile range, frequency, and percentages). The statistical analysis of the study was performed using SPSS version 23 (IBM, Armonk, NY). The normality of the data was determined using Shapiro-Wilk tests.

The Fleiss’ kappa statistic was used to summarize the agreement in the category of pDDI provided by 2 drug interaction programs. The Fleiss’ kappa is a measure of interrater reliability that removes agreement expected by chance and is suitable for 2 or more raters. A kappa value varies between –1 and 1, with 1 indicating perfect agreement, –1 indicating perfect disagreement, and 0 indicating agreement expected by chance (Fleiss, 1971). The interpretation of Fleiss’ kappa values is based on guidelines established by Landis and Koch, such that <0.0 is poor agreement; 0.0-0.2 is slight agreement; 0.21-0.40 is fair agreement; 0.41-0.60 is moderate agreement; 0.61-0.80 is substantial agreement; and 0.81-1.00 is almost perfect agreement (Landis & Koch, 1977). P values were calculated for the kappa, with a P<0.05 meaning that rater agree-

ment was unlikely to be because of chance. A high level of concordance across evaluators does not necessarily imply that the response is accurate, nor does a lack of agreement guarantee its inaccuracy. All Fleiss’ kappa values were computed utilizing the R software package “irr,” version 0.84.1 (Gamer, Lemon, Fellows & Singh, 2019).

Ethics committee approval

This study was approved by the Anadolu University Scientific Research and Publication Ethics Committee (Protocol number: 744611).

RESULTS AND DISCUSSION

A total of 120 patients were included in the study; 62.5% (n=75) were women, and the mean age was 77.7±12.7 years. The most common diseases were hypertension (58.3%), dementia (46.7%), and diabetes (36.7%). Polypharmacy, defined as the regular use of 5 or more medications at the same time (Varghese, Ishida, Patel & Haseer Koya, 2024), was observed in 94.2% of the patients, and the most commonly prescribed psychotropic drugs were quetiapine (60.0%), haloperidol (24.2%), and donepezil (24.2%) (Table 2).

Table 2. Demographic characteristics of the patients (n=120)

Variables	Participants (n=120)
Age (Mean±SD)	77.7±12.7
Gender	
Female	75 (62.5%)
Male	45 (37.5%)
No. of drugs prescribed per patient	
≤5	7 (5.8%)
6-9	41 (34.2%)
10-14	50 (41.7%)
≥15	22 (18.3%)
Diagnosis	
Hypertension	70 (58.3%)
Dementia	56 (46.7%)
Diabetes Mellitus	44 (36.7%)
Cerebrovascular Accident	26 (21.7%)
Kidney Disease	20 (16.7%)
Heart Disease	20 (16.7%)
Cancer	16 (12.3%)
Asthma & COPD	14 (11.7%)
Pneumonia	12 (10.0%)
General Condition Disorder	12 (10.0%)
Parkinson's Disease	8 (6.7%)
Respiratory Failure	7 (5.8%)
Thyroid Disease	5 (4.2%)
Epilepsy	4 (3.3%)
Dyspnea	4 (3.3%)
ALS	3 (2.5%)
Others*	18 (15.0%)
Psychotropic drugs	
Quetiapine	72 (60.0%)
Haloperidol	29 (24.2%)
Donepezil	29 (24.2%)
Memantine	24 (20.0%)
Levetiracetam	24 (20.0%)
Tramadol	19 (15.8%)
Escitalopram	12 (10.0%)
Levodopa/Carbidopa/Entacapone	11 (9.2%)
Olanzapine	8 (6.7%)
Citalopram	6 (5.0%)
Sertraline	6 (5.0%)
Mirtazapine	5 (4.2%)
Modafinil	5 (4.2%)
Diazepam	5 (4.2%)
Valproic Acid	4 (3.3%)
Alprazolam	4 (3.3%)
Aripiprazole	4 (3.3%)
Trazodone	3 (2.5%)
Others**	15 (12.5%)

*Other (diagnosis): subarachnoid hemorrhage (n=2), prostate (n=2), intracranial hemorrhage (n=1), history of myocardial infarction (n=1), rheumatoid arthritis (n=1), atrial fibrillation (n=1), bipolar disorder (n=1), nutritional disorder (n=1), pulmonary embolism (n=1), multiple trauma (n=1), bradycardia (n=1), schizophrenia (n=1), cholecystitis (n=1), pleural effusion (n=1), anemia (n=1), vertigo(n=1)

**Other (psychotropic drugs): chlorpromazine (n=2), rasagiline (n=2), phenytoin (n=2), carbamazepine (n=2), gabapentin (n=2), duloxetine/paroxetine (n=2), amantadine (n=1), levodopa/benserazide (n=1), rivastigmine (n=1)

Two drug interaction programs detected 209 pDDIs, including 77 different drug interaction pairs that may cause QTc prolongation in patients. While 52 pDDIs (20 different interaction pairs) were observed only with the Micromedex® drug interaction program and 48 pDDIs (16 different interaction pairs) were observed only with the Lexicomp® drug interaction program, 109 pDDIs (40 different drug interaction pairs) were common to both programs (Figure 1). The interrater reliability was poor ($\kappa = -0.034$) for major pDDI; in addition, the overall interrater reliability was only poor ($\kappa = -0.052$). Table 3 shows the number of pDDIs by severity level in Micromedex® and Lexicomp® programs.

The most common pDDI pair seen was haloperidol-quetiapine ($n=22$ patients, 18.3%). Of those 22, 16 patients could get an ECG, and QTc prolongation was seen in 12 (75.0%) of them. Along with other common possible interactions, donepezil-quetiapine was seen in 12 patients (10.0%), and QTc prolongation was found in 8 of 9 patients (88.0%) for whom an ECG could be done. A potential interaction between levetiracetam and quetiapine was observed in 11 patients (9.2%), and QTc prolongation was detected in 6 (60.0%) of 10 patients for whom an ECG could be conducted. Table 4 shows the drug interaction pairs observed with drug interaction programs in each of the 120 patients, as well as the ECG results performed on the patients in whom these interactions were observed.

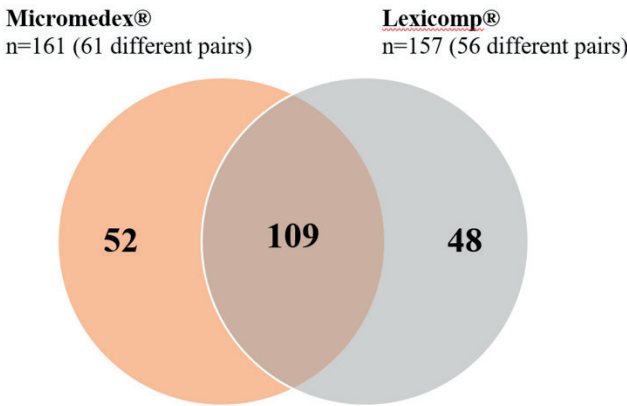


Figure 1. Overlap of pDDIs pairs between Micromedex® and Lexicomp® drug interaction programs

Table 3. The number of pDDIs observed with psychotropic drugs by the drug interaction programs

	Minor	Moderate	Major	Severe	Total number of pDDIs, n
Lexicomp®	30 (16 different pairs)	77 (28 different pairs)	43 (6 different pairs)	7 (6 different pairs)	157 (56 different pairs)
Micromedex®	0	0	161 (61 different pairs)	0	161 (61 different pairs)

pDDI= potential drug-drug interaction

Table 4. QTc prolongation on ECG according to drug interaction pairs (n=120)

DDIs pairs (number of patients, %)	Severity in Lexicomp®	Severity in Micromedex®	QTc prolongation in ECG		
			Yes	No	No ECG was taken
Haloperidol-Quetiapine (n=22, 18.3%)	D	Major	12	4	6
Donepezil-Quetiapine (n=12, 10.0%)	-	Major	8	1	3
Levetiracetam-Quetiapine (n=11, 9.1%)	C	-	6	4	1
Quetiapine-Ondansetron (n=9, 8.0%)	D	Major	5	2	1
Quetiapine-Salbutamol (n=7, 5.8%)	C	-	4	2	1
Quetiapine-Memantine+ Donepezil (n=7, 5.8%)	-	Major	4	1	2
Haloperidol-Ipratropium+ Salbutamol (n=6, 5.0%)	C	-	1	3	2
Haloperidol-Olanzapine (n=5, 4.1%)	C	Major	3	1	1
Donepezil-Escitalopram (n=5, 4.1%)	B	Major	2	2	1
Escitalopram-Quetiapine (n=5, 4.1%)	D	Major	4	1	-
Olanzapine-Quetiapine (n=5, 4.1%)	D	Major	4	1	-
Haloperidol-Memantine+ Donepezil (n=5, 4.1%)	C	Major	2	1	2
Aripiprazol-Memantine+ Donepezil (n=4, 3.3%)	-	Major	-	1	3
Donepezil-Haloperidol (n=4, 3.3%)	C	Major	2	-	2
Escitalopram-Ipratropium+ Salbutamol (n=4, 3.3%)	B	-	2	-	2
Mirtazapine-Quetiapine (n=4, 3.3%)	C	Major	2	-	2
Quetiapine-Sertraline (n=3, 2.5%)	C	Major	3	-	-
Aripiprazole-Haloperidol (n=3, 2.5%)	-	Major	-	1	2
Aripiprazole-Quetiapine (n=3, 2.5%)	-	Major	-	1	2
Donepezil-Ondansetron (n=3, 2.5%)	B	Major	2	1	-
Donepezil-Sertraline (n=3, 2.5%)	-	Major	3	-	-
Haloperidol-Levetiracetam (n=3, 2.5%)	C	-	2	1	-
Haloperidol-Ondansetron (n=3, 2.5%)	-	Major	2	1	-
Levetiracetam-Domperidone (n=3, 2.5%)	B	-	2	1	-
Citalopram-Haloperidol (n=3, 2.5%)	C	Major	2	1	-
Citalopram-Quetiapine (n=3, 2.5%)	C	Major	2	1	-
Escitalopram-Levetiracetam (n=2, 1.6%)	B	-	1	1	-
Escitalopram-Olanzapine (n=2, 1.6%)	C	Major	1	-	1
Escitalopram-Trazodone (n=2, 1.6%)	-	Major	1	-	1
Haloperidol-Solifenacin (n=2, 1.6%)	C	Major	1	-	1
Olanzapine-Salbutamol (n=2, 1.6%)	B	-	1	-	1
Olanzapine-Solifenacin (n=2, 1.6%)	-	Major	1	-	1
Sertraline-Ondansetron (n=2, 1.6%)	-	Major	1	1	
Haloperidol-Salbutamol (n=2, 1.6%)	C	-	1	-	1
Haloperidol-Mirtazapine (n=2, 1.6%)	C	Major	-	-	2
Haloperidol-Moxifloxacin (n=2, 1.6%)	C	Major	-	-	2
Quetiapine-Metronidazole (n=2, 1.6%)	C	Major	-	-	2
Quetiapine-Moxifloxacin (n=2, 1.6%)	X	Major	1	-	1
Citalopram-Ipratropium+ Salbutamol (n=2, 1.6%)	B	-	1	1	-
Dexmedetomidine-Haloperidol (n=1, 0.8%)	-	Major	-	1	-
Dexmedetomidine-Ondansetron (n=1, 0.8%)	B	Major	-	1	-

Dexmedetomidine-Quetiapine (n=1, 0.8%)	-	Major	-	1	-
Donepezil +Memantine- Famotidin (n=1, 0.8%)	-	Major	1	-	-
Donepezil+ Memantine-Solifenacin (n=1, 0.8%)	-	Major	1	-	-
Escitalopram-Haloperidol (n=1, 0.8%)	C	Major	-	-	1
Escitalopram- Domperidone (n=1, 0.8%)	X	Major	-	1	-
Levetiracetam-Amiodaron (n=1, 0.8%)	C	-	1	-	-
Olanzapine-Memantine +Donepezil (n=1, 0.8%)	-	Major	1	-	-
Quetiapine-Amiodaron (n=1, 0.8%)	X	Major	1	-	-
Quetiapine-Ciprofloxacin (n=1, 0.8%)	C	Major	-	1	-
Quetiapine-Trazodone (n=1, 0.8%)	C	Major	1	-	-
Quetiapine-Solifenacin (n=1, 0.8%)	C	Major	1	-	-
Quetiapine-Domperidone (n=1, 0.8%)	X	Major	-	1	-
Citalopram-Dexmedetomidine (n=1, 0.8%)	B	Major	-	1	-
Citalopram-Ondansetron (n=1, 0.8%)	C	Major	-	1	-
Escitalopram-Moxifloxacin (n=1, 0.8%)	C	Major	-	-	1
Mirtazapine-Olanzapine (n=1, 0.8%)	-	Major	-	-	1
Mirtazapine-Solifenacin (n=1, 0.8%)	-	Major	-	-	1
Mirtazapine-Moxifloxacin (n=1, 0.8%)	B	Major	-	-	1
Escitalopram-Solifenacin (n=1, 0.8%)	B	Major	-	-	1
Olanzapine-Moxifloxacin (n=1, 0.8%)	C	Major	-	-	1
Donepezil-Trazodone (n=1, 0.8%)	-	Major	-	-	1
Donepezil-Mirtazapine (n=1, 0.8%)	-	Major	-	1	-
Quetiapine-Levofloxacin (n=1, 0.8%)	D	Major	-	1	-
Escitalopram-Memantine+ Donepezil (n=1, 0.8%)	B	Major	1	-	-
Escitalopram-Salbutamol (n=1, 0.8%)	B	-	1	-	-
Mirtazapine-Memantine+ Donepezil (n=1, 0.8%)	-	Major	1	-	-
Citalopram-Amiodaron (n=1, 0.8%)	X	Major	-	1	-
Citalopram-Sertraline (n=1, 0.8%)	-	Major	-	1	-
Amiodaron-Sertraline (n=1, 0.8%)	C	Major	-	1	-
Escitalopram-Ondansetron (n=1, 0.8%)	C	Major	-	1	-
Quetiapine-Chlorpromazine (n=1, 0.8%)	X	Major	1	-	-
Haloperidol- Chlorpromazine (n=1, 0.8%)	D	Major	1	-	-
Levetiracetam- Chlorpromazine (n=1, 0.8%)	C	-	1	-	-
Levetiracetam-Ondansetron (n=1, 0.8%)	B	-	1	-	-
Levetiracetam-Citalopram (n=1, 0.8%)	B	-	1	-	-
Rivastigmine-Domperidone (n=1, 0.8%)	B	-	1	-	-

74 (61.7%) of 120 palliative care patients had pDDIs with psychotropic drugs that could cause QTc prolongation (Table 5). 57 of 74 patients (77.0%) un-

derwent ECG. Despite having a pDDI, 17 patients could not undergo an ECG due to discharge, death, or ward change.

Table 5. Prevalence of potential QTc prolonging drug-drug interactions in palliative care patients

Type of prevalence	Patients (n)	%
Overall prevalence	74	61.7
QT-DDI per patient		
1-2	42	35.0%
3-4	17	14.2%
>4	15	12.5%
Gender-wise prevalence		
Male	25	20.8%
Female	49	40.9%
Age-wise prevalence		
<60	4	3.3%
60-69	9	7.5%
70-79	16	13.3%
80-89	37	30.8%
>90	8	6.8%

QT-DDI: QT prolonging drug-drug interactions

Of the 57 patients with ECG, 38 (66.7%) showed QTc prolongation. Table 6 presents the QTc prolongation and DIPS scores according to drug interaction pairs in 57 patients who underwent ECG.

In order to evaluate the clinical significance of potential drug interactions in patients with ECG, DIPS scoring was implemented. Accordingly, 8 (4.9%) of 161 potential drug-drug interactions detected in Micromedex® were classified as “probable,” and 77 (47.8%) were classified as “possible” with a score between 2-8 in DIPS. Of the 157 potential drug-drug interactions in Lexicomp®, 11 (7.0%) were classified as “probable” and 76 (48.4%) as “possible” according to DIPS. A total of 122 (58.3%) out of 209 potential drug interactions were clinically relevant. Micromedex® and Lexicomp® drug interaction programs identified 52.7% and 55.4% of the potential drug

interactions as clinically relevant, respectively.

A total of 38 patients were recommended for medication change due to suspicion of potential drug interaction-induced QT prolongation. Some of these recommendations were rejected due to patient discharge status, service change, hospital drug shortages, and patient inapplicability. In only 4 patients (10.5%), the clinical pharmacist’s recommendations were accepted by the physician, and in all of these patients, no QT prolongation was observed in ECGs taken after drug discontinuation or switching. Two of these patients stopped taking haloperidol, and three of them started taking alprazolam instead of quetiapine. Additionally, in one patient, memantine was switched to rivastigmine, and in another patient, duloxetine was switched to escitalopram.

Table 6. Potential drug-drug interactions observed with psychotropic drugs by the drug interaction programs and the drug interaction probability scale (n=57)

DDIs pairs (number of patients, %)	Number of patients with QT prolongation	DIPS (number of patients)			
		Highly Probable	Probable	Possible	Doubtful
Haloperidol-Quetiapine (n=16, 28.1%)	12 (75.0%)	0	2	14	0
Donepezil-Quetiapine (n=9, 15.8%)	8 (66.7%)	0	1	7	0
Levetiracetam-Quetiapine (n=10, 17.5%)	6 (60.0%)	0	1	5	0
Quetiapine-Ondansetron (n=7, 12.3%)	5 (71.2%)	0	0	5	0
Quetiapine-Salbutamol (n=6, 10.5%)	4 (66.7%)	0	0	4	0
Quetiapine-Memantine+Donepezil (n=5, 8.8%)	4 (80.0%)	0	0	4	0
Haloperidol-Ipratropium+ Salbutamol (n=4, 7.0%)	1 (25.0%)	0	1	0	0
Haloperidol-Olanzapine (n=4, 7.0%)	3 (75.0%)	0	1	2	0
Donepezil-Escitalopram (n=4, 7.0%)	2 (50.0%)	0	0	2	0
Escitalopram-Quetiapine (n=5, 8.8%)	4 (80.0%)	0	1	3	0
Olanzapine-Quetiapine (n=5, 8.8%)	4 (80.0%)	0	2	2	0
Haloperidol-Memantine+ Donepezil (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Aripiprazol-Memantine+ Donepezil (n=1, 1.8%)	0 (0%)	0	0	0	0
Donepezil-Haloperidol (n=2, 3.5%)	2 (100%)	0	1	1	0
Escitalopram-Ipratropium+ Salbutamol (n=2, 3.5%)	2 (100%)	0	1	1	0
Mirtazapine-Quetiapine (n=2, 3.5%)	2 (100%)	0	0	2	0
Quetiapine-Sertraline (n=3, 5.3%)	3 (100%)	0	0	3	0
Aripiprazole-Haloperidol (n=1, 1.8%)	0 (0%)	0	0	0	0
Aripiprazole-Quetiapine (n=1, 1.8%)	0 (0%)	0	0	0	0
Donepezil-Ondansetron (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Donepezil-Sertraline (n=3, 5.3%)	3 (100%)	0	0	3	0
Haloperidol-Levetiracetam (n=3, 5.3%)	2 (66.7%)	0	1	1	0
Haloperidol-Ondansetron (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Levetiracetam-Domperidone (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Citalopram-Haloperidol (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Citalopram-Quetiapine (n=3, 5.3%)	2 (66.7%)	0	0	2	0
Escitalopram-Levetiracetam (n=2, 3.5%)	1 (50%)	0	0	1	0
Escitalopram-Olanzapine (n=1, 1.8%)	1 (100%)	0	1	0	0
Escitalopram-Trazodone (n=1, 1.8%)	1 (100%)	0	0	1	0
Haloperidol-Solifenacin (n=1, 1.8%)	1 (100%)	0	0	1	0
Olanzapine-Salbutamol (n=1, 1.8%)	1 (100%)	0	0	1	0
Olanzapine-Solifenacin (n=1, 1.8%)	1 (100%)	0	0	1	0
Sertraline-Ondansetron (n=2, 3.5%)	1 (50%)	0	0	1	0
Haloperidol-Salbutamol (n=1, 1.8%)	1 (100%)	0	0	1	0
Quetiapine-Moxifloxacin (n=1, 1.8%)	1 (100%)	0	0	1	0
Citalopram-Ipratropium+ Salbutamol (n=2, 3.5%)	1 (50%)	0	0	1	0
Dexmedetomidine-Haloperidol (n=1, 1.8%)	0 (0%)	0	0	1	0
Dexmedetomidine-Ondansetron (n=1, 1.8%)	0 (0%)	0	0	1	0
Dexmedetomidine-Quetiapine (n=1, 1.8%)	0 (0%)	0	0	1	0
Donepezil +Memantine- Famotidin (n=1, 1.8%)	1 (100%)	0	0	1	0

Donepezil+ Memantine-Solifenacin (n=1, 1.8%)	1 (100%)	0	0	1	0
Escitalopram- Domperidone (n=1, 1.8%)	0 (0%)	0	0	1	0
Levetiracetam-Amiodaron (n=1, 1.8%)	1 (100%)	0	0	1	0
Olanzapine-Memantine +Donepezil (n=1, 1.8%)	1 (100%)	0	0	1	0
Quetiapine-Amiodaron (n=1, 1.8%)	1 (100%)	0	0	1	0
Quetiapine-Ciprofloxacin (n=1, 1.8%)	0 (0%)	0	0	1	0
Quetiapine-Trazodone (n=1, 1.8%)	1 (100%)	0	0	1	0
Quetiapine-Solifenacin (n=1, 1.8%)	1 (100%)	0	0	1	0
Quetiapine-Domperidone (n=1, 1.8%)	0 (0%)	0	0	1	0
Citalopram-Dexmedetomidine (n=1, 1.8%)	0 (0%)	0	0	1	0
Citalopram-Ondansetron (n=1, 1.8%)	0 (0%)	0	0	1	0
Donepezil-Mirtazapine (n=1, 1.8%)	0 (0%)	0	0	1	0
Quetiapine-Levofloxacin (n=1, 1.8%)	0 (0%)	0	0	1	0
Escitalopram-Memantine+ Donepezil (n=1, 1.8%)	1 (100%)	0	0	1	0
Escitalopram-Salbutamol (n=1, 1.8%)	1 (100%)	0	0	1	0
Mirtazapine-Memantine+ Donepezil (n=1, 1.8%)	1 (100%)	0	0	1	0
Citalopram-Amiodaron (n=1, 1.8%)	0 (0%)	0	0	1	0
Citalopram-Sertraline (n=1, 1.8%)	0 (0%)	0	0	1	0
Amiodaron-Sertraline (n=1, 1.8%)	0 (0%)	0	0	1	0
Escitalopram-Ondansetron (n=1, 1.8%)	0 (0%)	0	0	1	0
Quetiapine-Chlorpromazine (n=1, 1.8%)	1 (100%)	0	0	1	0
Haloperidol- Chlorpromazine (n=1, 1.8%)	1 (100%)	0	0	1	0
Levetiracetam- Chlorpromazine (n=1, 1.8%)	1 (100%)	0	0	1	0
Levetiracetam-Ondansetron (n=1, 1.8%)	1 (100%)	0	0	1	0
Levetiracetam-Citalopram (n=1, 1.8%)	1 (100%)	0	0	1	0
Rivastigmine-Domperidone (n=1, 1.8%)	1 (100%)	0	1	0	0

Our findings reveal common prescriptions of drug interactions involving psychotropic drugs associated with known risks of QTc prolongation in patients receiving palliative care. A significant proportion of palliative care patients experienced both minor and major polypharmacy, which included agents known to induce high-risk QTc prolongation. In the study, polypharmacy was observed in 94.2% of the patients. Similar to our study, McNeil et al. found 94% polypharmacy in patients near the end of life (McNeil, Kamal, Kutner, Ritchie & Abernethy, 2016). A meta-analysis involving patients aged 65 years and older (2005–2020) indicated that the frequency of polypharmacy varied from 4% to 96.5%, depending on the healthcare setting and region (Pazan & Wehling, 2021).

In the present study, the most frequently prescribed psychotropic drugs were quetiapine, hal-

operidol, and donepezil, respectively. Azab et al. (2024) indicated that antipsychotics were the most frequently prescribed psychotropic drugs, which is consistent with our finding (Azab, Novella, Ianes & Pasina, 2024). Since the implementation of Medicare's documentation requirements for medications in hospice care, an evaluation conducted in 2016 identified lorazepam, morphine, and haloperidol as the most commonly prescribed medications (Mohamed, Uvais, Moideen, Cp & Saif, 2024). Unlike our finding, Santos-Peres et al. (2021) found that anxiolytics/hypnotics were the most frequently prescribed psychotropic drugs (Santos-Pérez, Salgueiro-Vázquez, Sáinz-Gil & Martín-Arias, 2021).

This study assessed drug interactions with psychotropic drugs using two commonly utilized drug interaction programs. The findings indicated signifi-

cant differences among these programs regarding the identified number of pDDIs. The interrater reliability was poor ($\kappa = -0.034$) for major pDDI; in addition, the overall interrater reliability was only poor ($\kappa = -0.052$). Health care professionals must recognize the variety of potential pDDIs identified by various drug interaction programs.

Drug interaction programs compile information from scientific literature and categorize it for health-care professionals. However, there is growing concern among these professionals regarding the variability in quality and effectiveness of the information provided by different programs. Also, these programs can't take into account the specific needs of each patient, which means they can't adjust doses for each person and don't provide specific safety instructions for health-care professionals (Hammar, Hamqvist, Zetterholm, Jokela & Ferati, 2021).

In clinical practice, physicians receive numerous pDDI alerts, many of which can be rapidly disregarded. Excessive alerts may obscure the significance of a specific DDI. Consequently, pharmacists' vigilant oversight of palliative care patients can facilitate the identification and prevention of drug-drug interactions, potentially enhancing patient health outcomes (Russ-Jara et al., 2023). Research conducted by Robleck et al. (2016) demonstrated that pharmacist intervention significantly reduces the number of patients with clinically significant DDIs (Robleck et al., 2016).

The interaction of psychotropic drugs with each other or with other drugs increases the risk of QTc prolongation. This study identified numerous QTc-prolonging drug-drug interactions involving combinations of antipsychotics with antipsychotics, antipsychotics with antidepressants, and antidepressants with antidepressants. Combining antipsychotic and antidepressant drugs in polypharmacy has been shown to cause torsades de pointes (TdP) and a significant prolongation of the QTc. The concurrent administration of antipsychotic and antidepressant medications leads to a cumulative effect on the QTc interval.

The arrhythmogenic potential of antipsychotics varies significantly (Das et al., 2021).

In the current study, quetiapine, haloperidol, and olanzapine were the antipsychotics most frequently associated with QTc-prolonging drug-drug interactions in the palliative care patients. In this study involving geriatric patients, escitalopram and citalopram were the antidepressants most frequently associated with QTc-prolonging drug-drug interactions. The most frequently observed pDDIs pairs in the study were haloperidol-quetiapine and donepezil-quetiapine, respectively. Similar to our study, Wang et al. indicated that more than 10% of patients receiving quetiapine and haloperidol therapy developed QTc prolongation (Wang et al., 2024). The study by Vogel et al. found that adding 20 mg of donepezil to the treatment of a person who was taking quetiapine made the QT interval longer, which is also consistent with our finding (Vogel, Mican & Smith, 2019).

Consequently, drug interactions with psychotropic drugs must be thoroughly evaluated and subsequently confirmed by healthcare professionals in palliative care patients by utilizing suitable and validated instruments such as DIPS to ensure the treatment's efficacy. The Naranjo algorithm, another tool utilized in the literature, evaluates the likelihood of adverse drug reactions instead of drug interactions (Naranjo et al., 1981). The DIPS algorithm facilitates objective, reliable, and transparent evaluation of causation in clinically relevant DDIs.

In the study, a pDDI with psychotropic drugs that could cause QTc prolongation was detected in 74 out of 120 palliative care patients (61.7%). QTc prolongation was observed in 66.7% of the patients ($n=38$) who had an ECG performed ($n=58$).

The Micromedex® program provided 161 of the identified pDDIs with psychotropic drugs that could cause QTc prolongation, and the Lexicomp® program provided 157. Clinically relevant pDDIs constituted 52.7%, and 55.4% of these totals, respectively. In this study, 122 clinically relevant DDIs (63 different pairs) were identified, which were also recognized in prior research (Das et al., 2021, Wang et al., 2024).

In only 4 (10.5%) out of 38 patients, the recommendations made by the clinical pharmacist were accepted by the physician. The accepted recommendations were the discontinuation of haloperidol, which had no indication, starting alprazolam instead of quetiapine, switching from rivastigmine to memantine, and from escitalopram to duloxetine. The patients underwent another ECG one week later, showing no signs of QTc prolongation.

This study has some limitations. At the beginning of the study, we did not know the patients' ECG (baseline) results. In addition, other risk factors of the patients, apart from laboratory results, were frequently not systematically documented. In instances where patient risk factor documentation was insufficient, we engaged our clinical collaborators to obtain this information. Unfortunately, our recommendations to the attending physician have often been rejected due to the risk of QTc prolongation resulting from drug interactions. The main reason the physician rejected the pharmacist's recommendations was that the patients were terminally ill, limiting the available drug alternatives. However, it is still observed that QTc prolongation is resolved in patients who accept the clinical pharmacist's recommendations. A further limitation is the comparison of categories related solely to severity (severe, major, moderate, minor, and none) of pDDIs. The investigation did not encompass additional features, functions, or user-friendliness of the drug interaction programs. Furthermore, in certain instances, it was challenging to ascertain whether the observed adverse reaction resulted from a drug-drug interaction or from a single drug alone. This limitation was addressed through a consensus decision regarding the certainty of DDI-related adverse reactions with the treating physician.

CONCLUSION

This study's results demonstrate that a considerable number of palliative care patients receive drug combinations that may result in drug interactions with psychotropic drugs, which are linked to a risk of

QTc prolongation. In palliative care services, requests for ECG and other pertinent laboratory tests are typically not made. Current international guidelines are not implemented in clinical practice, and there is an absence of guidelines specific to Turkey. This situation necessitates the implementation of ECG monitoring protocols and associated laboratory investigations. The AHA and the ACCF recommend conducting an ECG before initiating a QTc-prolonging medication, 8-12 hours following dose escalation of such medications, or in instances of overdose involving a QT-prolonging drug. Reliable evidence-based online drug information resources, including the AzCERT/CredibleMeds Drug Lists, Lexicomp® drug interaction program, and Micromedex® interaction program, can aid clinical professionals in the selection of medications for psychiatric patients. The probability of identifying clinically relevant DDIs remains low, regardless of the chosen interaction program by a health institution. Consequently, patient monitoring must be conducted by a multidisciplinary healthcare team including a clinical pharmacist.

AUTHOR CONTRIBUTION STATEMENT

Concept: FA., K.T.Y.; Design: FA., Ö.G., Y.S., K.T.Y.; Supervision: K.T.Y., Y.S.; Data Collection and/or Processing: FA., Ö.G.; Analysis and/or Interpretation: Ö.G., Y.S., K.T.Y.; Literature Review: FA., K.T.Y.; Writing the Article: FA., Ö.G., K.T.Y.; Critical Review: Y.S., K.T.Y.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Development of Mirtazapine Loaded Transdermal Drug Delivery System

Özlem KARABUDAK*, Fatma Gülgün YENER**, Aykut KUL***, Ece Merve YILMAZ****

Development of Mirtazapine Loaded Transdermal Drug Delivery System

Mirtazapin İçeren Transdermal Taşıyıcı Sistemin Geliştirilmesi

SUMMARY

Mirtazapine, alpha-2 receptor antagonist is an atypical antidepressant drug used in the treatment of major depression in adults; commercially available in oral tablet, disintegrating tablet and oral solution forms. Transdermal drug delivery systems can deliver the active drug substance subcutaneously into the systemic circulation, which provides a higher bioavailability by avoiding hepatic first-pass metabolism in the oral route and maintains a constant plasma level. The oral bioavailability of Mirtazapine is limited to 50%. The objective of this study is to design and formulate Mirtazapine into a transdermal drug delivery system, conduct the in-vitro drug product performance tests and introduce a suitable transdermal film formulation. In this study, transdermal film formulations of Mirtazapine were designed and generated. Films have a matrix type structure in which HPMC, HEC and PVP K30 are used as polymers and PEG 400 as a plasticizer. The prepared films have been proven to be stable by physical tests and FT-IR analysis. It has been experimentally obtained that in the best formulation, Mirtazapine can be released for more than 24 hours, and 14.41% of the drug in the dosage form permeates cumulatively through the artificial membrane within 24 hours. With the addition of oleic acid to the formulation, an 11.53% increase in permeation through the artificial membrane was achieved ($p < 0.05$). This study implies that Mirtazapine can be stably formulated into a transdermal film, providing sustained release and permeating through transdermal diffusion membrane, which presents a promising point of reference for research pursuits and commercial medicine development.

Keywords: Mirtazapine, transdermal film, transdermal patch, antidepressant, oleic acid..

ÖZ

Mirtazapin, yetişkinlerde majör depresyon tedavisinde kullanılan, alfa-2 reseptör antagonisti atipik bir antidepresan ilaçtır. İlacın, oral tablet, dağılan tablet ve oral solüsyon formları ticari olarak mevcuttur. Transdermal ilaç salım sistemleri, ilaç etkin maddesini deri altından sistemik dolaşıma verebilen; böylece oral yoldan ilaç alımındaki hepatik ilk geçiş etkisinden kaçınarak daha iyi bir biyoyararlanım ve plazma seviyesi sürekliliği sağlayan, dozaj formlarıdır. Mirtazapinin oral biyoyararlanımı %50 ile sınırlıdır. Bu çalışmanın amacı, Mirtazapin içeren transdermal ilaç salım sisteminin tasarlanması ve formüle edilmesi, in-vitro performans testlerinin gerçekleştirilmesi ve uygun bir transdermal film formülasyonunun ortaya konmasıdır. Bu çalışmada, Mirtazapin'in transdermal film formülasyonları tasarlanmış ve ortaya konmuştur. Polimer olarak HPMC, HEC ve PVP K30, plastifiyan olarak PEG 400'ün kullanıldığı filmler matriks yapıdadır. Hazırlanan filmlerin, yapılan fiziksel testler ve FT-IR analizi ile stabil olduğu kanıtlanmıştır. Ortaya konan en iyi formülasyondan, 24 saatten daha fazla süre boyunca Mirtazapin salınabildiği ve dozaj formundaki ilacın % 14,41'nin 24 saat içinde yapay membrandan kümülatif olarak geçtiği deneysel olarak elde edilmiştir. Formülasyona oleik asit ilavesi ile, yapay membrandan permeasyonda %11,53 artış elde edilmiştir ($p < 0,05$). Bu çalışma, Mirtazapin'in sürekli salım sağlayabilen ve stabil transdermal filmlerinin formüle edilebileceğini, transdermal difüzyon membranından geçebileceğini ortaya koymakta; ileri araştırmalar ve ticari ilaç ürünleri geliştirilmesi için gelecek vaadeden bir referans oluşturmaktadır.

Anahtar Kelimeler: Mirtazapin, transdermal film, transdermal yama, antidepresan, oleik asit.

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* ORCID: 0009-0003-4615-4398, Department of Pharmaceutical Technology, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye

** ORCID: 0000-0002-7234-0034, Department of Pharmaceutical Technology, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye

*** ORCID: 0000-0002-1195-0939, Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye

**** ORCID: 0000-0001-8300-3327, Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye

INTRODUCTION

Mirtazapine is an alpha-2 receptor antagonist atypical antidepressant drug, which is named as 1,2,3,4,10,14b-hexahydro-2-methylpyrazino (2,1-a) prido (2,3-c) benzazepine. It is generally prescribed for the treatment of major depressive disorder in adults. Panic disorder, insomnia, post-traumatic stress disorder and social anxiety are the other indications Mirtazapine is prescribed for (Croom et al., 2009; Watanabe et al., 2011). The drug is also often off-label prescribed for the geriatric patients to treat the sleep disorders (Eronen et al., 2024; Nguyen et al., 2025). Currently, Mirtazapine is commercially available in tablet, disintegrating tablet and oral solution dosage forms (RX Media Pharma, 2023).

Transdermal drug delivery systems are advanced dosage forms that enable drug delivery across the skin barrier into the systemic circulation. Although it is a non-invasive application method, since permeation through the skin barrier is limited by various factors, this has made the transdermal delivery systems one of the focal points in drug research studies and commercial product development in recent years (Buzia et al., 2023; Jeong et al., 2021; Tiwary & Sapra, 2007; Ramadon et al., 2022). Nowadays, painkillers, hormonal therapy drugs and some drugs used in the treatment of central nervous system and cardiovascular system diseases are prosperously administered via transdermal dosage forms (Bird & Ravindra, 2020).

Drug administration via the transdermal delivery systems has several advantages over the oral route. Through the passage of the drug from the skin barrier into the systemic circulation, high systemic bioavailability is obtained, avoiding hepatic first-pass metabolism. More consistent plasma levels can be attained since the transdermal systems maintain plasma concentrations in the therapeutic window for prolonged periods, possibly allowing reduction in dosing frequency, eliminating dose fluctuations of oral medication (Carat, 2025; Escobar-Chavez et al., 2012). Trans-

dermal systems avoid unpredictable absorption issues caused by food intake and other gastrointestinal (GI) factors, providing more reliable results (Laurn & Ar-ean, 2024). Transdermal drug delivery systems typically provide controlled and sustained drug release, which results in an extended duration of therapeutic effect, half-life, compared to oral administration. Since the transdermal systems assure gradual and continuous drug input into the systemic circulation rather than a rapid bolus, improved half-life is attained. Subsequently, plasma concentrations decline more slowly, maintaining therapeutic levels for a longer period (Berner & John, 1994; Guy & Hadgraft, 1985). The ability to identify the applied medicine, stop the administration of medication and the side effects at any time is another substantial advantage of the transdermal systems, which offers safety in case of emergencies (Pastore et al., 2015; Patel et al., 2012; Tanwar & Sachdeva, 2016). Transdermal drug delivery systems ensure an alternative drug delivery route for the patients, especially elderly patients, with swallowing difficulties or cognitive challenges and those who receive multiple medications (Harnett et al., 2023). Besides improving patient compliance, transdermal technology generates possibilities for product line extensions for pharmaceutical companies, which provides strategic benefits in competitive therapeutic categories.

There are mainly four types of transdermal patches: matrix-type, membrane, drug-in-adhesive and micro-reservoir systems (Wong et al., 2023). Matrix-type transdermal system comprises a drug layer with a semi-solid matrix that holds a dispersed drug solution or drug suspension with a polymeric part directly in contact with the skin. The adhesive layer surrounds the drug layer and keeps the system adhered to the skin during dosing. In matrix-type systems, drug release is controlled by loading dose, solubility of drug in the polymer and the diffusion behavior of the drug in the polymer matrix (Ping, 2011; Siepmann & Pappas, 2011). A membrane-controlled transdermal sys-

tem contains a drug reservoir and a rate-controlling polymeric membrane. The drug molecules are released only through this membrane, which consists of semipermeable materials that provide the regulated passage of the drug (Bathe & Kapoor, 2015; Zhan et al., 2015). Drug-in-adhesive systems consist of an adhesive layer containing the drug. Following the application, the adhesive layer adheres onto the skin and the drug is gradually released during the dosing period (Pastore et al., 2015; Wolff, 2000). The micro-reservoir system comprises a matrix dispersion system and a reservoir. The drug is suspended in an aqueous solution of water-soluble liquid polymer, then this mixture is evenly dispersed in lipophilic polymer, producing a large number of microscopic drug reservoirs (Hanbali et al., 2019; Waghulde et al., 2013).

Despite the potential benefits of transdermal drug delivery, currently there is no commercial product available for transdermal Mirtazapine treatment in the field of human medicine. Moreover, the scientific literature on Mirtazapine delivery via transdermal systems is highly limited. There are a few studies covering a transdermal Mirtazapine system, and those studies are all published very recently, which indicates a growing interest in the topic. In one of these studies, therapeutic deep eutectic solvents, which are utilized to improve the solubility of drugs, were combined with Mirtazapine and medium-chained fatty acids to create a transdermal drug delivery system. The study performed *in vivo* studies as well and concluded that the relative bioavailability of Mirtazapine was improved 6.4-fold by including medium-chain fatty acids and provided 24-hour sustained release characteristics (He et al., 2025). In a very recent study, drug-in-adhesive formulations were generated by incorporating a pressure-sensitive adhesive system along with oleic acid as a permeation enhancer. The performed *ex vivo* and *in vivo* studies showed sustained drug release, enhanced skin permeation, and improved pharmacokinetics outperforming the oral route (Satpute et al., 2025). Another study developed a drug-in-adhesive transdermal patch using a pressure-sensitive adhesive

and a chemical penetration enhancer. *In vivo* experiments were performed to investigate the pharmacokinetics of the optimized formulation, and the results demonstrated that a long-acting patch was developed (Liu et al., 2025).

Although there is no commercial product and very limited literature regarding the transdermal Mirtazapine system for human medicine, a noteworthy veterinary product, FDA-approved Mirtazapine transdermal ointment is commercially available (Mirataz®) in the United States (mirataz.com/pi). The product was designed and produced to benefit the off-label indication of Mirtazapine, appetite stimulation. It is currently recommended for application in cats only. Few prior related studies to this product were conducted and published. In these studies, Mirtazapine transdermal ointment formulation was fabricated and applied to owned healthy young cats in veterinary clinical conditions. The drug was applied to the skin of the inner ear pinna. The evaluation was made as per the analyzed serum Mirtazapine levels and the increase in appetite behaviors in cats. Single and repeated transdermal doses of Mirtazapine transdermal ointment attained measurable plasma concentration in cats (Benson et al., 2017; Buhles et al., 2018; Poole et al., 2019).

There are certain drug properties that should be considered while developing a transdermal drug delivery system. Drugs with lower molecular weight (<500 g/mol) can be absorbed through the skin via passive diffusion (Brown et al., 2006). The partition coefficient (Log P) is one of the key determinants of permeability, and should be between 1.0 and 4.0 (Chandrashekar & Shobha Rani, 2008). It is commonly accepted that an optimal drug candidate for transdermal delivery should possess a low melting point (< 200°C) (Velasco-Aguirre & Sintov, 2019). Mirtazapine has a molecular weight of 265.35 g/mol, a melting of 114-116°C (British Pharmacopoeia 2023), and a log P value of 2.9 (Ezealisiji et al., 2015). For this study, Mirtazapine was selected as the ideal drug

candidate to formulate into a transdermal film due to its appropriate molecular properties for transdermal drug delivery. Among the different types of transdermal patches, the matrix type patch was selected essentially due to the simple design and relative ease of fabrication. The conventional manufacturing process is also cost-effective.

This study centers on the design, formulation, and optimization of a novel matrix-type transdermal drug delivery system. By incorporating oleic acid as a penetration enhancer, the study optimizes the drug content and flux.

MATERIALS AND METHODS

Materials

Mirtazapine hemihydrate was purchased from Zhejiang Liaoyuan Pharmaceuticals Co, Ltd. (China). Hydroxypropyl methylcellulose (HPMC) (Benecel TM K4M) and hydroxyethyl cellulose (HEC) (Natrosol TM 250 HHX) were obtained from Ashland Global (USA) as gift samples. Polyvinylpyrrolidone (PVP K30) was received as a gift sample from BOAI NKY Pharmaceuticals (China). Polyethylene glycol 400 (PEG 400) and oleic acid were procured from BASF (Germany). All other solvents and chemicals were used during this study were analytical grade and purchased from Merck (Germany).

Preparation method for transdermal film formulations

The preparation method was developed based on the solvent-casting method (Cherukuri et al., 2017; Yener et al., 2010). The following process steps were consecutively applied for this purpose. HPMC, HEC and distilled water were weighed into a beaker. The beaker was airtightly covered with parafilm and left at room temperature for 24 hours to create the aqueous gel. The resulting gel was mixed with a magnetic stirrer at 90 rpm for 30 minutes at 35 °C (Beaker A). The temperature was lowered to 25°C just after this step,

and the process was proceeded.

The 5% (w/v) PVP K30 solution that provides matrix formation, was prepared in a separate beaker, mixed with a magnetic stirrer at 180 rpm for 1 hour (Beaker B). The PVP K30 solution in Beaker B was added dropwise to the gel mixture in Beaker A. It was mixed in a magnetic stirrer at 150 rpm for 1 hour.

The 4.5% (w/v) Mirtazapine/ ethanol solution was prepared in another beaker (Beaker C). Mirtazapine-ethanol solution in Beaker C was added dropwise to the contents of Beaker (A+B) and mixed at 200 rpm for 1 hour. The weighted amount of PEG 400 was added dropwise to this mixture and mixed with a magnetic stirrer at 250 rpm for 30 minutes. The resulting gel was kept in an ultrasonic water bath for 1 hour. 20 grams of the final gel mixture were placed in a petri dish. To remove the last air bubbles in the gel, the petri dishes were left at room temperature for 1 hour. It was dried at 38°C for 18 hours.

The basis of formulations, quantification, and the ratio of the selected polymers were created after a detailed review of the previous studies (Cherukuri et al., 2017; Kriplani et al., 2020; Parhi et al., 2022; Saidin et al., 2018; Singh & Bali, 2016; Vlad et al., 2025; Yener et al., 2010). Several films were fabricated with this method, simple physical characterization was carried out (was checked for homogeneous appearance, ease of peeling from the petri dish, being durable and flexible), and 20 formulations were determined to be suitable for carrying out the study. The formulations are given in Table 1. The transdermal films containing Mirtazapine were prepared with the method explained. After determining the formulation that had the highest penetration rate, that formulation was prepared with incorporated oleic acid at a formulation in 4% concentration, to investigate the penetration enhancement effect of oleic acid on the formulated film.

Table 1. Formulation of Mirtazapine transdermal films

Formulation ID	Quantity							
	HPMC		HEC		PVP K30		PEG 400	
	(mg)	(%)*	(mg)	(%)*	(mg)	(%)*	(mg)	(%)*
F1	250	0.8	250	0.8	250	0.8	210	0.7
F2	250	0.8	150	0.5	250	0.8	210	0.7
F3	200	0.6	250	0.8	250	0.8	210	0.7
F4	350	1.1	150	0.5	250	0.8	210	0.7
F5	300	1.0	350	1.1	250	0.8	210	0.7
F6	350	1.1	100	0.3	250	0.8	210	0.7
F7	350	1.1	200	0.6	250	0.8	210	0.7
F8	350	1.1	250	0.8	250	0.8	210	0.7
F9	400	1.3	150	0.5	250	0.8	210	0.7
F10	400	1.3	200	0.6	250	0.8	210	0.7
F11	300	1.0	100	0.3	250	0.8	210	0.7
F12	200	0.6	200	0.6	250	0.8	210	0.7
F13	400	1.3	100	0.3	250	0.8	210	0.7
F14	450	1.4	200	0.6	250	0.8	210	0.7
F15	450	1.4	100	0.3	250	0.8	210	0.7
F16	450	1.4	150	0.5	250	0.8	210	0.7
F17	500	1.6	250	0.8	250	0.8	210	0.7
F18	250	0.8	200	0.6	250	0.8	210	0.7
F19	300	1.8	250	0.8	250	0.8	210	0.7
F20	400	1.3	300	1.0	250	0.8	210	0.7

* % amount of the component in the total weight (25 grams) of the gel prepared to form transdermal films.

Physicochemical evaluation of the transdermal films

Weight variation

1 cm² sections were cut out from 10 different points of each prepared film. The sections were weighed one at a time on a precision scale, and the measurements were noted. The mean and SD values of the obtained measurements were calculated (Nandi & Mondal, 2022; Prabhu et al., 2011).

Thickness

1 cm² sections were cut out from 10 different points of each film. The thickness of the sections was measured with a digital caliper. The mean and SD values of the measurements obtained were calculated and recorded (Hanbali et al., 2019; Parmar et al., 2023).

Content uniformity test

1 cm² sections were cut out from 6 different points of each transdermal film containing Mirtazapine. Each

section was placed in a 25 mL volumetric flask, and 10 mL of ethanol was added. It was kept in an ultrasonic water bath to dissolve for 24 hours. The obtained solution was filtered through a membrane filter with a 0.45 µm pore size. The filtrate was then quantitatively analyzed with HPLC to determine the drug content. The mean and SD values of the measurements obtained were calculated and noted (Shivalingam et al., 2021).

Folding endurance test

2 x 2 cm were cut out from each prepared film. The section was folded repeatedly from a precise point until the crack formation was observed on the film surface. The folding endurance value is determined as the number of times the film can be folded at the same place without creating any crack line on the film surface. The test was performed with three films from each formulation (Idrees et al., 2014).

Stability studies

Mirtazapine transdermal films were tested for their physicochemical stability. The films were placed in stability chambers in closed glass petri dishes. They were kept in stability chambers under conditions of $25 \pm 2^\circ\text{C}$, $40 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity. Quantification was performed on samples taken at 1, 3, and 6th months. FTIR examination was also performed on the samples taken in the 6th month.

In vitro drug product performance tests

In vitro dissolution test

Dissolution studies were carried out to obtain dissolution profiles of the six film formulations selected as per their physicochemical properties. The Disk Assembly method was used from the “Dissolution Test for Transdermal Patches” section in the Guide on the Quality of Transdermal Patches in the European Pharmacopoeia (EMA/CHMP/QWP/608924/2014, 2014). For this purpose, a 2x2 cm section was cut out from the film and fixed between the watch glass and a 125 μm mesh stainless steel net. In the study, pH 7.4 phosphate buffer solution (PBS) was used as the dissolution medium. 900 mL of pH 7.4 PBS was taken into the vessel of the dissolution device. The temperature was set at $32 \pm 0.5^\circ\text{C}$. The stirring speed was set at 50 rpm. A sample of 1 mL was taken from the solvent medium at 15 minutes, 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours. Following each sample collection, 1 mL of pH 7.4 PBS was immediately added to the vessel. The collected samples were filtered through 0.45 μm pore size PTFE syringe filters. The test was repeated 6 times for each film formulation.

In vitro permeation test

To test the permeation of Mirtazapine from the prepared films through the synthetic skin membrane, a method was created by following the recommendations in the relevant guide of the European Medicines Agency (EMA) (EMA/CHMP/QWP/608924/2014, 2014). The experiment was performed using the Franz diffusion cell system. The Franz cell system used has a heated water jacket. The receptor compartment

volume is 12 mL, and the orifice area is 1.77 cm^2 . In studies conducted with Franz diffusion cells, the synthetic skin membrane Strat M[®], which can simulate human skin, was used. pH 7.4 phosphate buffer was chosen as the dissolution medium, and the study was carried out at $32 \pm 0.5^\circ\text{C}$ (Neupane et al., 2020). Before starting the experimental timing, it was ensured that the temperature of both the pH 7.4 phosphate buffer in the receptor chamber and the Strat M[®] membrane on top of it was allowed to reach 32°C . Experimental timing was initiated by placing the test film section on the membrane. Samples were taken at 15 and 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours. Following sample collection, the receptor from the dissolution medium was added to the receptor chamber. Air bubbles that formed in the receptor chamber during sample collection were removed. The samples taken were filtered through a PTFE syringe tip filter with a 0.45 μm pore opening and analyzed by the HPLC quantification method. The experiment was repeated 6 times for each formulation.

Data treatment and statistics

Statistical evaluations of the data were made by using one-way analysis of variance (ANOVA) test in order to determine their differences. The level of significance was set to $\alpha = 0.05$.

RESULTS AND DISCUSSION

In this study, transdermal film formulations of Mirtazapine were successfully prepared using the solvent-casting method. The physicochemical properties of the Mirtazapine transdermal films were investigated, and test results are shown in Table 2.

It was observed that incorporating a higher concentration of HEC into the formulations made the transdermal films remarkably more elastic and durable. This correlates with the existing literature (Birsan et al., 2018; Tudoroiu, 2025).

The weight of different films per cm^2 varied between $9.10 \text{ mg} \pm 0.352$ and $14.14 \text{ mg} \pm 0.401$. The thickness of different formulations ranged from $0.101 \text{ mm} \pm 0.018$ to $0.193 \text{ mm} \pm 0.038$. These SD values as-

sured that the preparation method was capable of developing patches with the least intra-batch variability.

Mirtazapine quantity in different formulations varied from $0.47 \text{ mg/cm}^2 \pm 0.044$ to $0.56 \text{ mg/cm}^2 \pm 0.058$. Minimum SD values in the film drug content variation ensured the uniformity of the prepared films using the solvent casting method.

For all of the formulations, the folding endurance value was greater than 100, which proved that the prepared transdermal film formulations were flexible enough to withstand mechanical force and would be able to keep their physical integrity following the film application onto the skin.

Table 2. The physicochemical properties of Mirtazapine transdermal films

Film ID	Thickness (mm)	SD $\pm$	Film weight (mg/cm^2)	SD $\pm$	Mirtazapine quantity (mg/cm^2)	SD $\pm$	Folding endurance
F1	0.122	0.028	11.48	0.941	0.50	0.031	> 100
F2	0.114	0.019	9.75	0.579	0.51	0.032	> 100
F3	0.126	0.015	9.10	0.352	0.50	0.037	> 100
F4	0.0116	0.020	11.48	1.099	0.53	0.062	> 100
F5	0.193	0.038	13.45	0.793	0.52	0.057	> 100
F6	0.115	0.016	10.47	0.662	0.53	0.045	> 100
F7	0.143	0.029	13.28	0.554	0.53	0.042	> 100
F8	0.123	0.018	10.5	0.714	0.59	0.023	> 100
F9	0.127	0.013	10.85	0.632	0.52	0.068	> 100
F10	0.132	0.024	13.84	0.471	0.54	0.068	> 100
F11	0.101	0.018	11.91	0.395	0.50	0.026	> 100
F12	0.123	0.017	10.91	0.457	0.53	0.050	> 100
F13	0.105	0.018	10.62	0.703	0.56	0.058	> 100
F14	0.146	0.022	14.14	0.401	0.51	0.058	> 100
F15	0.113	0.018	9.84	0.674	0.53	0.059	> 100
F16	0.128	0.026	11.84	0.740	0.48	0.054	> 100
F17	0.187	0.031	12.74	0.696	0.51	0.024	> 100
F18	0.111	0.017	10.48	0.620	0.48	0.054	> 100
F19	0.111	0.009	9.70	0.635	0.47	0.044	> 100
F20	0.159	0.025	12.93	0.827	0.52	0.032	> 100

The stability studies proved that there is no significant change in drug content and the physical characteristics of the prepared film formulations. The results were given in Table 3.

An FT-IR spectrophotometer was used, and IR spectra were obtained by direct analysis with the ATR unit. The device and software are activated in transmission mode. Measurements were made in the wavelength range of $4000\text{--}400 \text{ cm}^{-1}$ with a spectral resolu-

tion of 4 cm^{-1} . The FT-IR spectra were obtained on day zero and day 180 of the stability studies. Both the overlapping Mirtazapine spectra are given in Figure 1.

Peaks observed in the FT-IR spectrum of Mirtazapine λ (cm^{-1}): 2981-2964: Aliphatic C-H stretch band, 1585-1566: C=N- stretch band, 1442-1494: Aromatic C=C stretch band, 3016-3057: Aromatic C-H stretch band, 763-786: 1,2 disubstituted benzene out-of-plane deformation band.

Table 3. Stability study of Mirtazapine transdermal films

Formulation ID	25 ± 2°C and 75 ± 5% RH				40 ± 2°C and 75 ± 5% RH			
	% Drug content				% Drug content			
	initial	30 days	90 days	180 days	initial	30 days	90 days	180 days
F1	99.97	99.46	99.37	98.94	99.97	99.38	99.28	98.75
F2	100.06	99.93	99.87	99.80	100.06	99.74	99.70	99.60
F3	100.07	99.99	99.95	99.92	100.07	99.80	99.61	99.19
F4	99.99	99.97	99.91	99.84	99.99	99.70	99.52	99.17
F5	99.73	99.61	99.54	98.92	99.73	99.25	98.89	98.24
F6	99.75	99.68	99.60	98.87	99.75	99.44	99.19	98.22
F7	99.49	99.33	99.21	98.75	99.49	99.12	98.78	98.23
F8	99.62	99.59	99.56	99.47	99.62	99.36	99.11	98.92
F9	99.86	99.78	99.73	99.63	99.86	99.46	99.16	98.79
F10	99.34	99.20	98.92	97.93	99.34	98.78	98.24	97.62
F11	99.55	99.49	99.42	99.32	99.55	99.18	98.95	98.37
F12	99.49	99.22	98.97	98.55	99.49	98.88	98.59	98.16
F13	99.86	99.81	99.72	99.61	99.86	99.57	99.26	98.86
F14	99.97	99.94	99.86	99.76	99.97	99.73	99.42	99.06
F15	99.29	99.19	99.06	98.94	99.29	98.72	98.51	98.13
F16	99.92	99.82	99.46	98.98	99.92	99.46	99.15	98.66
F17	99.27	99.05	98.82	98.20	99.27	98.66	98.22	97.79
F18	99.37	99.18	98.95	98.66	99.37	98.73	98.46	98.17
F19	98.95	98.65	97.95	96.89	98.95	98.40	97.80	97.56
F20	99.59	99.21	98.83	98.12	99.59	99.04	98.56	97.79

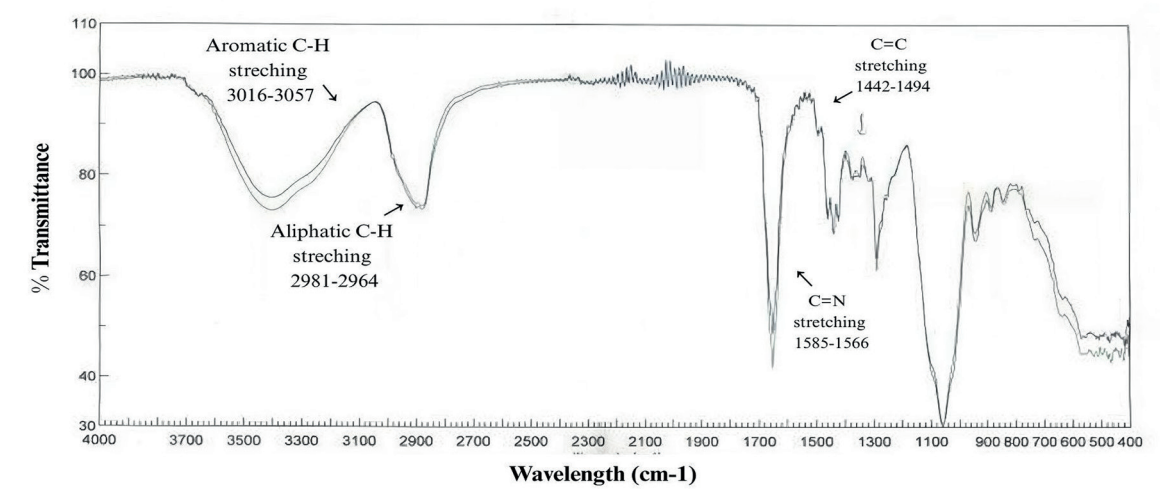


Figure 1. Overlapping image of the Mirtazapine spectra on the initial and day 180

It was determined that the formulations maintained their physical integrity and chemical stability at both 25 ± 2°C and 40 ± 2°C temperatures for 6 months. In line with these findings, it was concluded that transdermal films containing Mirtazapine can be

stored at room temperature.

Six of the prepared formulations were tested for their *in vitro* performance. After studying Mirtazapine solubility in different dissolution media, pH 7.4 phos-

phate buffer was selected as the dissolution medium since it effectively simulates physiological environments and assures the Sink condition. The dissolution test was carried out in the Sotax AT-7 dissolution system. The quantitative analysis of the samples was

made by using a validated HPLC method. The results were given in Table 4.

The dissolution profiles of the formulations were formed by plotting the % released drug vs. time and are given in Figure 2.

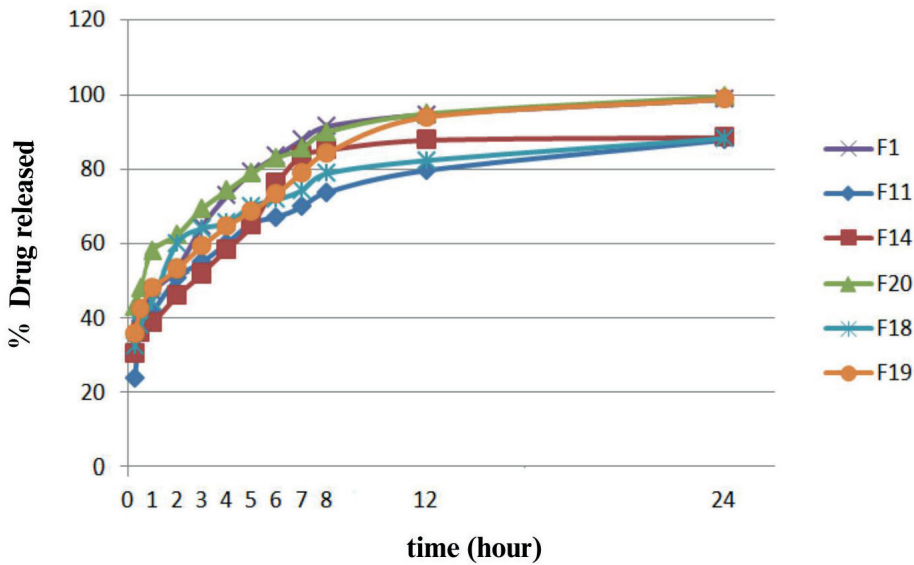


Figure 2. Dissolution profiles of the Mirtazapine transdermal films

Table 4. Dissolution results of Mirtazapine transdermal films

Time (hour)	F11		F14		F20		F1		F18		F19	
	% released drug	SD (±)	% released drug	SD (±)	% released drug	SD (±)	% released drug	SD (±)	% released drug	SD (±)	% released drug	SD (±)
0.25	23.88	0.070	30.62	0.07	43.07	0.048	30.69	0.063	32.41	0.049	35.92	0.059
0.5	39.01	0.036	36.33	0.069	48.10	0.043	39.29	0.062	38.33	0.053	42.52	0.105
1	41.83	0.082	38.67	0.039	57.87	0.069	47.13	0.062	44.66	0.075	48.1	0.090
2	50.78	0.081	46.03	0.092	62.20	0.065	53.19	0.051	60.00	0.054	53.12	0.088
3	54.84	0.135	51.95	0.049	69.22	0.070	64.54	0.086	63.92	0.051	59.17	0.079
4	60.00	0.151	58.28	0.165	74.11	0.064	72.73	0.092	65.37	0.193	64.47	0.088
5	65.37	0.127	64.82	0.107	78.85	0.040	79.06	0.085	69.98	0.191	68.67	0.082
6	67.02	0.082	76.10	0.182	82.98	0.028	83.46	0.075	71.70	0.169	73.28	0.083
7	69.70	0.097	83.67	0.202	85.39	0.024	87.8	0.062	74.17	0.171	78.99	0.055
8	73.69	0.103	85.05	0.169	89.93	0.031	91.44	0.072	78.78	0.159	84.29	0.040
12	79.61	0.044	87.73	0.077	94.89	0.019	94.68	0.076	82.29	0.14	93.99	0.057
24	87.80	0.053	88.49	0.038	99.50	0.017	98.74	0.075	88.28	0.066	98.81	0.039

The data obtained from the dissolution study were plotted in several kinetic models to determine the release kinetics of the transdermal films. The plotted data are presented in Table 5.

Table 5. Release kinetics of Mirtazapine from transdermal film formulations.

		F11	F14	F20	F1	F18	F19
Zero-order kinetics	k0	2.3104	2.5569	2.2454	2.6989	2.0971	2.6311
	r2	0.6971	0.6161	0.6724	0.6064	0.6270	0.7590
1 st order kinetics	k1	0.0733	0.0864	0.1977	0.1734	0.0717	0.1717
	r2	0.9072	0.7092	0.9982	0.9523	0.9523	0.9880
Higuchi	D	14.0759	15.9044	13.8475	17.0668	13.1450	15.6225
	r2	0.9076	0.8362	0.8970	0.8505	0.8642	0.9387
Hixson Crowell	kH	0.0754	0.0869	0.1287	0.1301	0.0720	0.1280
	r2	0.8437	0.6794	0.9302	0.8393	0.7714	0.9426
Korsmeyer peppas	n	0.2963	0.2192	0.1857	0.2799	0.2799	0.1995
	r2	0.9212	0.9717	0.9751	0.9834	0.9839	0.9906

It was determined that when applied to kinetic models, the dissolution profile of the tested films conforms to the Korsmeyer-Peppas, which gave the highest determination coefficient. Depending on the “n” value, the profile fits different drug release mechanisms. The release of the prepared formulations is explained by diffusion in accordance with Fick’s law, since the “n” value was obtained as ≤ 0.45 . The transdermal films prepared in the study were designed to have a matrix structure. The drug release rate is not constant in matrix structured films; it is related to the concentration gradient, diffusion distance, and swelling degree, and most of the time the release occurs by the Fick diffusion mechanism (Fu & Kao, 2010; Siepmann & Siepmann, 2012).

It is well acknowledged that the quantification of the samples taken within the first hour of the dissolution studies of matrix transdermal films provides more distinctive data for evaluating the dissolution behavior (EMA/CHMP/QWP/608924/2014, 2014).

The dissolution studies were applied to the 6 formulations. The drug release rate wasn’t constant, and the drug release didn’t follow zero-order kinetics. It showed a distinctive initial burst and subsequently slowed. Particularly, F20, F11 and F19 ($R^2 \approx 0.99$) showed first-order release, meaning a concentration-dependent release rate existed. Korsmeyer Pep-

pas was the best fit for all the formulations, explaining the concentration-dependent and diffusion-controlled kinetics. This substantiates that the release is mainly controlled by diffusion through the polymer matrix, but not by polymer erosion or relaxation.

An evaluation was made in line with the results of the dissolution studies, and 3 formulations were selected for the *in vitro* permeation studies. In the F11, F14 and F18 formulations, less than 45% of the drug amount in the formulation was released in the first hour, and it was observed that the drug release from these formulations continued more than 24 hours. In the designed transdermal film formulation, F11, F14 and F18 were selected to continue with *in vitro* permeation studies, as the preferred feature is that the release extends over a longer time. When the dissolution data is evaluated, release occurs faster in formulations such as F1 and F20 containing higher concentrations of HEC than the transdermal films containing the same amount of HPMC. Therefore, it was concluded that HEC has an increasing effect on the release rate. With an increase in the ratio of HEC, drug release increased, which can be explained by the high water solubility of the polymer due to the multiple hydroxyl groups in its structure. Increased porosity of the polymer matrix led to the rapid dissolution of the drug (Tudoroiu et al., 2025).

In *in vitro* permeation studies, a synthetic skin membrane that can simulate human skin was used. The Merck Strat-M® product, which correlates with working with human skin and is sensitive to penetration enhancers, was preferred (Haq et al., 2018). The *in vitro* permeation study of transdermal film formulations containing Mirtazapine was conducted in the Franz diffusion cell system using pH 7.4 phosphate buffer. The permeation study results were given in Table 6 and Figure 3.

Among the transdermal films tested, at the end of 24 hours, the highest Mirtazapine penetration through the Strat-M® membrane was determined in F14, the formulation containing no penetration

enhancer, with a cumulative penetrated amount of 14.41% ($p < 0.05$). F18 and F11 provided cumulative permeated amounts of 11.15% and 9.25%, respectively ($p < 0.05$). Since the permeation performance was the highest in F14, oleic acid was incorporated into the formulation, and the F14OA formulation was created to study the permeation enhancement effect of oleic acid. The cumulative permeated Mirtazapine amount was 16.07% in F14OA (Table 6). Among the permeation tested formulations, optimal permeation performance was realized by the formulation that comprises the highest ratio of HPMC. This can be attributed to the likely formation of hydrophilic pores within the film structure and the following swelling of the polymer (Aho et al., 2017; Yener et al., 2010).

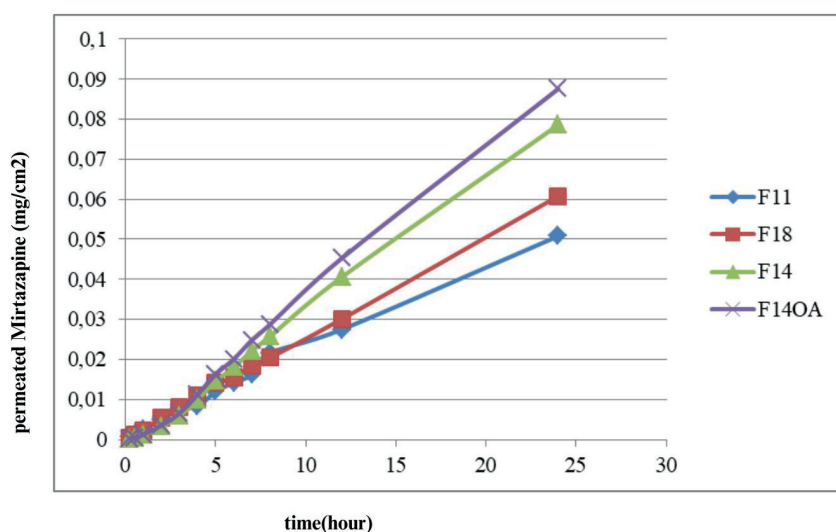


Figure 3. Permeation profiles of Mirtazapine from the different formulations through the Strat-M® membrane

The permeation studies were applied to the 3 formulations, and before reaching the steady-state, all of them had a short lag time of about 2 hours. Between the 4th and 12th hours, for all formulations the permeation increased linearly, showing steady-state flux. This type of kinetics, steady-state following a lag time, suggests Fickian diffusion.

The used non-animal-based, synthetic transdermal diffusion testing membrane was sensitive to the penetration enhancers and simulated Stratum corneum behavior to a comparable degree. Oleic acid incorporation into the F14OA formulation was resulted

in improved permeation consistent with the previous studies (Kichou et al., 2023; Rowat et al., 2006; Satpute et al., 2025; Virani et al., 2024).

For all the formulations (F11, F18, F14, F14OA), the flux was calculated and given in Table 7. F14OA had the finest flux, 3.254 $\mu\text{g}/\text{cm}^2\cdot\text{hour}$ permeation. Recognizing the mandatory *in vivo* and clinical studies requirement, if the bioavailability is reachable by the transdermal drug delivery, it was calculated that 52.972 mg Mirtazapine containing transdermal film in a size of 85.61 cm^2 could present the equivalent treatment as a 15 mg oral tablet administration in a day.

Table 6. Mirtazapine quantity permeated through the Strat-M® membrane.

	F18			F14			F11			F14OA		
time (hour)	permeated cumulative Mirtazapine quantity (mg/ cm ²)	% permeated cumulative	SD (±)	permeated cumulative Mirtazapine quantity (mg/ cm ²)	% permeated cumulative	SD (±)	permeated cumulative Mirtazapine quantity (mg/ cm ²)	% permeated cumulative	SD (±)	permeated cumulative Mirtazapine quantity (mg/ cm ²)	% permeated cumulative	SD (±)
0.25	0.0005	0.09	0.005	0.0002	0.04	0.005	0.0007	0.13	0.019	0.0002	0.04	0.003
0.50	0.0011	0.20	0.009	0.0003	0.05	0.010	0.0011	0.19	0.014	0.0003	0.05	0.003
1	0.0023	0.42	0.015	0.0011	0.21	0.013	0.0026	0.48	0.022	0.0013	0.23	0.008
2	0.0053	0.98	0.014	0.0032	0.58	0.010	0.0049	0.90	0.015	0.0036	0.65	0.016
3	0.0081	1.49	0.034	0.0059	1.09	0.022	0.0076	1.38	0.063	0.0066	1.21	0.021
4	0.0110	2.03	0.020	0.0099	1.81	0.023	0.0083	1.84	0.024	0.0113	2.07	0.024
5	0.0141	2.58	0.017	0.0146	2.67	0.020	0.0120	2.19	0.018	0.0163	2.98	0.027
6	0.0154	2.82	0.020	0.0180	3.29	0.029	0.0142	2.60	0.015	0.0201	3.68	0.045
7	0.0183	3.36	0.023	0.0222	4.06	0.021	0.0162	2.96	0.017	0.0248	4.54	0.046
8	0.0204	3.75	0.041	0.0257	4.71	0.029	0.0216	3.97	0.014	0.0287	5.26	0.068
12	0.0301	5.52	0.036	0.0406	7.45	0.018	0.0275	5.04	0.026	0.0453	8.31	0.030
24	0.0608	11.15	0.051	0.0786	14.41	0.017	0.0508	9.25	0.025	0.0876	16.07	0.080

Table 7. Cumulative permeated Mirtazapine concentration at Steady-state, flux and permeability coefficient (n=6) (5th hour)

Formulation	Concentration (%)	SD±	Jss (µg/cm ² .hour)	SD±	P (cm/minute)	SD±
F11	2.19	0.018	2.386	0.011	0.0004	0.00001
F18	2.58	0.017	2.816	0.016	0.0005	0.00001
F14	2.67	0.020	2.913	0.021	0.0005	0.00001
F14OA	2.98	0.027	3.254	0.010	0.0006	0.00001

CONCLUSION

In the presented research study, stable Mirtazapine matrix-type transdermal film formulations were obtained with HMPC, HEC and PVP K30 as polymers and PEG 400 as the plasticizer. The prepared films proved to be of desired pharmaceutical quality and stability by physicochemical tests and FT-IR scanning.

It was found that the developed formulations released Mirtazapine for more than 24 hours. The best permeation profile was obtained in the F14 formulation, which cumulatively penetrated 14.41% ($p < 0.05$) through the synthetic membrane during 24 hours. F14OA, the oleic acid incorporated version of F14, has successfully increased the Mirtazapine permeation by 11.51% through the synthetic membrane during 24 hours.

As a result, Mirtazapine transdermal film formulations were designed and prepared in desired pharmaceutical quality, with an even improved permeation profile by incorporating oleic acid into the formulation as a penetration enhancer. Despite the promising results, certain limitations need to be noted. The study was conducted using an *in vitro* model to test the pharmaceutical quality of the formulations, and further *in vivo* tests are required to confirm the efficacy of the formulation as a human medicine. In light of these considerations, Mirtazapine transdermal film formulations are suggested to be able to improve patient compliance by creating an alternative to the oral use of Mirtazapine, which has limited oral bioavailability.

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AUTHOR CONTRIBUTION STATEMENT

Initial literature survey, experimental design (ÖK, FGY, AK, EMY), sourcing for materials, laboratory work (ÖK), data acquisition and analysis, interpretation of result, writing and revision of the manuscript (ÖK, FGY, AK, EMY).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Ibuprofen Loaded Sodium Alginate Sponges for Acute Wound Treatment

Kadir AYKAÇ[°], Ceyda KÖROĞLU ÖZMEN^{**}

Ibuprofen Loaded Sodium Alginate Sponges for Acute Wound Treatment

SUMMARY

In this study, the potential of ibuprofen-loaded sodium alginate sponges for wound treatment was investigated through in vitro and cell culture cytotoxicity assays. Sodium alginate solutions at two different concentrations were dried using a lyophilizer to obtain drug delivery systems in the form of sponges containing various concentrations of ibuprofen. The prepared formulations were characterized for critical properties including water-holding capacity, molecular interactions (FTIR), tissue elasticity (texture analysis), drug content, drug release kinetics, and cytotoxicity. The results showed that the sponges could absorb water up to 5 times their own weight and dissolved completely within 2 hours, with a faster drug release observed compared to pure ibuprofen. Drug release kinetics revealed that Formulation A followed a first-order kinetic model, while Formulation B conformed to the Hopfenberg model. Cytotoxicity tests indicated that pure ibuprofen exhibits dose-dependent toxicity, however this was significantly reduced when incorporated into the sodium alginate formulation. Overall, the findings suggest that ibuprofen-loaded sodium alginate sponges have a high potential for use in wound treatment.

Keywords: Ibuprofen, sponges, wound treatment.

Yara Tedavisi İçin İbuprofen Yüklü Sodyum Aljinat Süngerler

ÖZ

Bu çalışmada, ibuprofen yüklü sodyum aljinat süngerlerinin yara tedavisinde kullanım potansiyeli in vitro ve hücre kültürü-sitotoksiste çalışmaları ile araştırılmıştır. Bu amaçla, iki farklı derişimde hazırlanan sodyum aljinat çözeltileri, liyofilizatör aracılığıyla kurutularak, çeşitli derişimlerde ibuprofen içeren sünger formunda ilaç taşıyıcı sistemler elde edilmiştir. Hazırlanan formülasyonların su tutma kapasitesi, moleküler etkileşimleri (FTIR), doku esnekliği (tekstür analizleri), etkin madde miktarı, ilaç salım kinetiği ve sitotoksitesi gibi kritik özellikleri incelenmiştir. Çalışma sonucunda, süngerlerin kendi ağırlığının 5 katına kadar su tutabildiği ve 2 saat içinde tamamen çözündüğü gözlenmiştir. Ayrıca, saf ibuprofene kıyasla etkin madde salımının daha hızlı olduğu tespit edilmiştir. İlaç salım kinetiği incelendiğinde, A formülasyonlarının birinci derece kinetik modele, B formülasyonlarının ise Hopfenberg modeline uyduğu belirlenmiştir. Sitotoksiste testleri, saf ibuprofenin doza bağlı toksite gösterdiğini, ancak sodyum aljinat formülasyonuna katılmasıyla bu toksitenin azaldığını ortaya koymuştur. Elde edilen bulgular, ibuprofen yüklü sodyum aljinat süngerlerin yara tedavisinde kullanım potansiyelinin yüksek olduğunu göstermektedir.

Anahtar Kelimeler: İbuprofen, sünger, yara tedavisi.

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[°] ORCID: 0000-0001-7426-1332, Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskişehir, Türkiye.

^{**} ORCID: 0000-0001-5326-7495, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Anadolu University, Eskişehir, Türkiye.

INTRODUCTION

Dermal injuries are wounds on the skin, often caused by cuts, incisions, or bruises. Wound healing outcomes are significantly influenced by numerous factors, including tissue perfusion, oxygen saturation, infectious agents, edema, local inflammation, systemic stress, metabolic status, nutritional deficiencies, and coexisting morbidities (Tottoli et al., 2020). Wound healing is a complex physiological process that restores skin integrity and regenerates damaged tissues, involving various cell populations, the extracellular matrix, and soluble mediators such as growth factors and cytokines (Velnar et al., 2009). The wound healing process occurs in four consecutive yet overlapping phases. This progression involves hemostasis for cessation of hemorrhage, inflammation for the recruitment of necessary immune cells, proliferation for new tissue synthesis, and re-epithelialization to ultimately restore the functional skin barrier (Guo & DiPietro, 2010; Peña & Martin, 2024). Due to its vital role as a barrier, cutaneous wound healing is an indispensable survival mechanism that facilitates to wound closure and tissue restoration. The process is physiologically complex, relying on the coordinated action of diverse cell populations and molecular mediators. These elements engage in an intricate, temporally sequenced cascade to ensure the structural and functional re-establishment of the dermis (Pazyar et al., 2014; Sorg et al., 2017).

The inflammatory phase, which succeeds the initial injury, encompasses both hemostasis and cutaneous neurogenic inflammation (Schultz et al., 2011). The inflammatory phase, which begins immediately after injury and lasts around an hour, is marked by blood clotting and the activation of the innate immune system, thereby setting the stage for healing. A rapid influx of neutrophils then occurs within 24 hours to eliminate pathogens and clear the wound. These cells gradually decrease over the next week as the healing process moves forward (Cañedo-Dorantes & Cañedo-Ayala, 2019). Hemostasis is initially

established through the formation of a platelet plug, subsequently stabilized by the development of a fibrin matrix that provides a provisional scaffold for cellular infiltration and tissue regeneration (Yang et al., 2021). The proliferative phase begins 2-10 days after injury, focusing on new tissue formation. This involves cell proliferation and migration, leading to the formation of highly vascularized granulation tissue in the dermis. Simultaneously, re-epithelialization begins as keratinocytes migrate from the wound edges to restore the skin's structural and functional integrity (Tottoli et al., 2020).

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to manage tissue injury by providing analgesic, anti-inflammatory, and antipyretic effects which improve patient comfort and recovery (Chen & Drago, 2013). While the long-term use of NSAIDs for chronic wounds is debated due to concerns that they may impair healing, a growing body of evidence supports their beneficial short-term use. Administering of NSAIDs for a brief period can reduce excessive inflammation, enhance patient comfort, and foster a more favorable environment for wound healing to progress (Anderson & Hamm, 2012). Ibuprofen (2-(4-isobutylphenyl)propanoic acid) is a widely used NSAID, employed to treat a variety of conditions, including fever, pain, and inflammation. In wound healing, ibuprofen's ability to modulate inflammation provides both symptomatic relief and regulation of the tissue repair process. The analgesic and anti-inflammatory effects of ibuprofen arise from the inhibition of Cyclooxygenase (COX) enzymes, thereby reducing prostanoïd synthesis and mitigating pain and inflammation (Rainsford, 2009). While the application of NSAIDs for wound healing is a contentious issue, studies on formulations containing ibuprofen are frequently reported in the literature. Furthermore, ibuprofen has been utilized within the context of wound healing, where its capacity to modulate inflammation contributes not only to symptomatic relief but also to the regulation of the tissue repair process (Anderson

& Hamm, 2012; Andrgie et al., 2020a; Jan-Roblero & Cruz-Maya, 2023; Khalil et al., 2025; Morgado et al., 2017; Price et al., 2007).

Over the past decades, a diverse range of advanced wound dressing materials (e.g., microneedles, hydrocolloids, hydrogels, nanofibers, foams, and films) has been investigated, with the collective purpose of optimizing the wound healing microenvironment by regulating moisture balance, reducing microbial invasion, and providing the structural scaffolding necessary for tissue regeneration (Long et al., 2022; Sadeghi-Aghbash et al., 2023; Tenorová et al., 2022; Wang et al., 2022). Sponges are effective wound dressings primarily due to their porosity, high absorption capacity, and air permeability, properties that allow them to promote rapid hemostasis. 3D porous structure of sponges also allows them to act as carriers for bioactive agents (like anti-inflammatory or antibacterial substances), which enhances their therapeutic potential in wound healing (Long et al., 2022).

Alginates are linear, water-soluble, and highly swellable anionic polysaccharides naturally derived from the cell walls of brown algae (Shaikh et al., 2022). Alginate biopolymers are composed of 1,4-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers, which are arranged in block-like sequences that can be either heterogeneous (MG) or homogeneous (poly-M, poly-G). Owing to their inherent hydrophilic properties, alginates can be processed into sponge-like forms, making them suitable for application on wounds, where they promote moisture retention and facilitate the healing cascade (Barbu et al., 2021). A study reported the development of ibuprofen-loaded nanofibers. The resulting controlled-release system demonstrated sustained drug release that extended for 14 days (Mohiti-Asli et al., 2017). In another study, thermosensitive hydrogel systems were developed, and nearly 70% ibuprofen release was observed after 3 days (Andrgie et al., 2020b). Alginates are well known for accelerating the healing of wounds in humans. After lyophilization, which creates the porous structure, the resulting sponge is

used as a topical application matrix (Dai et al., 2009). Sodium alginate has become a preferred material for wound dressings due to its biocompatibility, low immunogenicity, water retention and degradability (Lv et al., 2022). Also, it is highly hydrophilic, enabling it to absorb wound exudate, maintaining a moist microenvironment. Combined with its biodegradability and antibacterial activity, sodium alginate's utilization in creating a 3D porous, high-swelling sponge is a highly promising strategy for managing wounds and burns (Hu et al., 2018; Naghshineh et al., 2019). Most studies focused on wound healing are directed toward controlled and sustained release (Djekic et al., 2020; Romanelli et al., 2009). While many studies focus on controlled and long-term drug delivery, the release of ibuprofen was conversely aimed to be accelerated and its relative solubility enhanced in this study. To achieve this, ibuprofen-loaded, water-soluble sodium alginate sponges were developed, and lyophilization was utilized in their fabrication to ensure a porous and stable structure.

MATERIALS AND METHODS

Materials

Ibuprofen was kindly gifted from Drogosan, Turkey. Sodium alginate was purchased from Sigma Aldrich, Germany. Acetonitrile was provided Carlo Erba, Italy. All other chemicals used were in analytical grade.

Preparation of sponges

Sodium alginate solutions were prepared at 2% and 5% (w/w) concentrations by dissolving the polymer in distilled water based on prior studies with modifications (Wang et al., 2023). To these solutions, different ratios of ibuprofen in ethanol (as detailed in Table 1.) were added and mixed on a magnetic stirrer for 10 minutes to ensure homogeneity. The resulting formulations were then frozen at -20°C and subsequently lyophilized to produce porous sponges. Freeze-drying process facilitated the removal of water by sublimation, which is crucial for creating the desired spongy structure.

Table 1. Composition of sponges

Formulation	Sodium alginate (mg)	Distilled water (g)	Ibuprofen (mg)	Ethanol (mL)
A0	500	25	-	1
A1	500	25	165	1
A2	500	25	330	1
B0	1250	25	-	1
B1	1250	25	165	1
B2	1250	25	330	1

Ibuprofen is a chemically stable drug with a typical shelf life of at least two years under standard storage conditions (Farmer et al., 2002). Consequently, preservative-free solution formulations of ibuprofen are available on the market (Üstünes, 2025). In the preparation of ibuprofen sponges, stability testing was omitted from the scope of the study because the manufacturing process does not involve exposure to temperature and heat that threaten stability, and because the formulations prepared via lyophilization result in a solid matrix form.

HPLC conditions

A method was used to determine the amount of ibuprofen in drug content and release studies using High-Performance Liquid Chromatography (HPLC). The analysis was performed on a C18 column (250 x 4.6 mm, 5 µm), with a mobile phase consisting of acetonitrile, distilled water, and o-phosphoric acid (70:30:1). A photodiode array detector was set to a wavelength of 225 nm. The system operated at a flow rate of 1 mL/min, and the injection volume was 20 µL (Namazov, 2022) chronic or acute pain, as well as as an antipyretic in both children and adults. The negative effects of NSAIDs on the duodenum and stomach in the gastrointestinal tract (GIS). The HPLC method was validated for the study, considering linearity, precision, accuracy, selectivity, and stability parameters. The linear equation was obtained as $y = 37579x + 10380$ ($r^2=0.9999$).

Water absorption capacity and porosity tests

100 mg sponges were weighed then placed in 10 mL phosphate buffer solution (PBS). After 5 minutes

sponges were weighed again. The percentage of the amount of water absorbed to the sponge weight was determined ($n=3$) (Petchsomrit et al., 2017).

Porosity was determined using the ethanol displacement method. An initial ethanol volume (V_1) was measured ($n=3$). A certain sponge sample was immersed, and the resulting volume (V_2) was recorded. After 5 minutes, the sample was removed, the final ethanol volume (V_3) was measured, and porosity was calculated from these volumes according to equation (1) (Naghshineh et al., 2019):

$$\frac{V_1 - V_3}{V_2 - V_3} \times 100 \quad (1)$$

Fourier transform infrared analyses

To determine the structural properties, Fourier transform infrared spectroscopy (FTIR; IRAffinity-1S Shimadzu, Japan) was performed. The analyses were conducted in the 4000 to 500 cm^{-1} range with a high-sensitivity DLATGS detector and a Germanium-coated KBr beam splitter. The spectra of pure ibuprofen and sodium alginate were used as reference materials for comparison.

Morphological analyses

The morphology and porosity of the sponges were determined using Scanning Electron Microscopy (SEM). Analysis was conducted on a Carl Zeiss SUPRA 50VP SEM (Oberkochen, Germany) at a constant voltage of 15 kV, various magnifications, and a temperature of 25 ± 2 °C.

Texture analyses

The mechanical strength of the sponges was assessed using a texture analyser (TA.XT plus, Stable

Micro Systems, UK). Compression mode was chosen to evaluate the flexibility of the sponges. For analyses, the force required for the probe to advance 4 mm into the sponges was measured ($n=3$).

Drug content and loading capacity

The amount of ibuprofen in the sponges was determined using HPLC. A sponge sample (5 mg) was initially dissolved in 10 mL of a 1:1(v/v) mixture of ethanol and distilled water. The resulting solution was then filtered (0.45 μm) before being analyzed by the HPLC system. In pharmaceutical analyses, the drug content is determined by calculating the proportion of the active drug component within the formulation and expressing this as a percentage. To calculate the drug loading capacity, the drug content is divided by the total amount of ibuprofen used, and the result is expressed as a percentage (Khoukhi et al., 2016).

In vitro drug release and release kinetics

In vitro drug release studies were conducted using the dialysis bag method (Yurtdaş-Kırımlioğlu et al., 2018). Drug release was evaluated using sponge samples loaded with 2 mg of ibuprofen. Furthermore, to achieve sink conditions, the study was completed using a sponge formulation containing one-fifth of the saturated concentration of ibuprofen. The experiment was conducted in a 50 mL release medium of pH 7.4 PBS, with the temperature strictly maintained at 37 ± 1 °C to simulate physiological conditions (Mohandas et al., 2015) we have developed a bi-layered sponge that promotes fibrin clot stability and prevents secondary bacterial wound infections. Using the technique of freeze-drying, a bi-layer matrix consisting of hyaluronic acid (HA). To assess the *in vitro* release profile, samples were withdrawn from the release medium at predetermined time points (0.5, 1, 1.5, 2, 3, 4, 5, and 7 hours). An equal volume of fresh medium was simultaneously replaced (Stoyanova et al., 2016) characterized by dissolution-limited oral bioavailability. One approach to improve its water solubility and bioavailability is by solubilizing it in micellar surfactant solu-

tions. Here we investigate the effect of the surfactant type and the mechanism of solubility enhancement of Ibuprofen in surfactant solutions. The equilibrium Ibuprofen solubility in solutions of six surfactants was determined by HPLC. The nonionic surfactant polysorbate 80 (Tween 80). The amount of ibuprofen in each sample was quantified via HPLC ($n=3$). The data was analyzed using DDSolver to model the drug release kinetics (Zhang et al., 2010).

Cytotoxicity study

The cytotoxicity of the drug formulations was evaluated using the MTT assay on human umbilical vein endothelial cells (HUVEC) (Romani et al., 2024). This protocol was adapted from a similar MTT assay previously described (Yalçinkaya et al., 2025). HUVEC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. For the assay, cells were seeded into 96-well plates at a density of 2×10^4 cells and incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2 . After the initial incubation, cells were treated with various concentrations of the formulations and ibuprofen (20-300 $\mu\text{g/mL}$). The formulation and active substance were dissolved in fresh medium. All procedures were carried out aseptically in a Laminar Flow Cabinet (Kojair, Finland). Following 24 and 48 hours of treatment, the medium was removed and 20 μL of MTT solution (5 mg/mL in PBS) was added to each well. The plates were then incubated for another 3 hours to allow formazan crystal formation. Subsequently, the MTT solution was carefully removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the resulting formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Cytation 5, BioTek, UK), and the resulting values were used to calculate cell viability. Viability was expressed as a percentage relative to the untreated control cells, which were considered to have 100% viability. All experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Preparation of sponges

During preparation, Formula B's sodium alginate solutions were more viscous. The addition of ibuprofen made the solutions opaque, as the drug precipitated due to its insolubility in the alginate solutions (Garzón & Martínez, 2004). To ensure a homogeneous distribution of ibuprofen within the sponge, the solutions were stirred on a magnetic stirrer at 750 rpm for 30 minutes before being placed in the refrigerator. After lyophilization, the porous morphology of the sponges was visually apparent. Organoleptic controls revealed that the sponges from Formula A exhibited a softer and more flexible texture, which was attributed to their lower sodium alginate content.

Water absorption capacity and porosity test

Table 2 presents the water absorption capacity and porosity results. The sodium alginate sponges demon-

strated the ability to absorb 2.9 to 5 times their weight in water within a mere 5-minute period. Despite the higher sodium alginate content in Formula B delaying the process, the high hydrophilicity of all sponges led to rapid dissolution and a loss of structural stability within 1–2 hours. To improve structural integrity, sodium alginate is commonly cross-linked with calcium ions. However, the ions present in PBS can disrupt this structure by interacting with the calcium (Chiaoparakobkij et al., 2011). In Formula A, an increase in ibuprofen content led to a decrease in water absorption capacity due to a higher ibuprofen-to-sodium alginate ratio. Conversely, no direct relationship was found between this ratio and water retention in Formula B. Porosity analysis indicated that the formulations exhibited varying degrees of porosity. However, no direct correlation was established between the ibuprofen concentration and the resulting porosity. The results confirmed the porous nature of the sponges.

Table 2. Results of water absorption capacity and porosity tests (n=3; mean $\pm$ SE)

Code	Absorption of Water %	Porosity %
A0	358.1978 $\pm$ 62.0942	75.07 $\pm$ 7.77
A1	328.3034 $\pm$ 29.3729	38.07 $\pm$ 3.77
A2	291.8699 $\pm$ 31.4725	73.24 $\pm$ 1.29
B0	449.4580 $\pm$ 75.5654	59.10 $\pm$ 3.97
B1	460.0879 $\pm$ 25.8998	64.20 $\pm$ 1.72
B2	500.5849 $\pm$ 67.9352	60.40 $\pm$ 2.82

Fourier transform infrared analyses

FTIR spectroscopy is a powerful, high-resolution technique that measures the vibrational frequencies of chemical bonds to identify functional groups and characterize chemical structures, including surface components and drug-polymer interactions (Liu et al., 2018). FTIR analysis results are shown in Figure 1. The FTIR spectrum of ibuprofen reveals its molecular structure through several characteristic absorption bands. A strong band at 2953 cm^{-1} is assigned to the CH_3 asymmetric stretching vibration, while a very strong peak at 1712 cm^{-1} is a clear indicator of the C=O stretching motion. Aromatic C=C bonds are

observed in the region of 1460–1550 cm^{-1} and a band at 1417 cm^{-1} is attributed to the C–C stretching vibration in aromatic ring. Additionally, a very strong peak at 1230 cm^{-1} for C–C stretching and a strong band at 779 cm^{-1} for CH_2 vibrations serve as the molecule's unique fingerprint. Other observed features include CH_3 rocking (strong intensity) at 935 cm^{-1} and CH_2 asymmetric stretching at 2920 cm^{-1} . The medium intensity O–H...O valence stretching vibrations at 2727 cm^{-1} and 2630 cm^{-1} confirm the presence of a hydrogen-bonded dimer. In addition, various in-plane and out-of-plane C–H and O–H deformations were noted at multiple frequencies, with all assigned bands align-

ing with the known functional groups of ibuprofen (Kamari & Ghiaci, 2016; Namazi et al., 2019; Ramukutty & Ramachandran, 2012).

The carboxylate anion form (COO^-) is observed at 1597 cm^{-1} . The band at 821 cm^{-1} is characteristic of mannuronic acid residues within the sodium alginate structure. The bands at 1076 cm^{-1} and 1024 cm^{-1} are assigned to the C-O and C-C stretching vibrations

of the pyranose ring, as well as the C-O-C glycosidic bonds. The band at 1297 cm^{-1} is assigned to skeletal vibration. Additionally, the band at 1406 cm^{-1} is attributed to the C-OH deformation vibration and the symmetric O-C-O stretching vibration of the carboxylate group. Finally, a weak signal around 2956 cm^{-1} is due to C-H stretching vibrations (Huang et al., 2017; Lawrie et al., 2007).

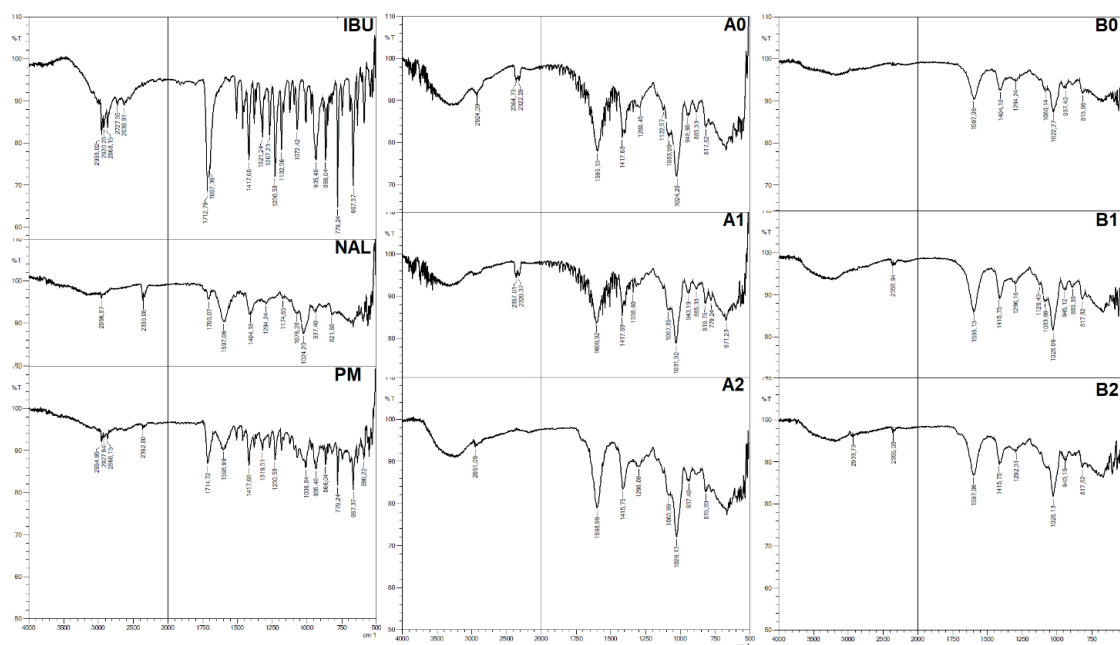


Figure 1. FTIR results of formulations; IBU: Ibuprofen, NAL: Sodium Alginate, PM: Physical mixture of sodium alginate and ibuprofen

Characteristic bands for sodium alginate were detected in both A0 and B0 formulations, with minor shifts in the peaks at 817, 1024, 1083, 1288, 1597, and 2364 cm^{-1} . The FTIR spectrum of the physical mixture revealed characteristic peaks of both ibuprofen and sodium alginate. The prominent bands at 2954, 1714, 1417, 1319, 1230, 935, 866, 779, and 667 cm^{-1} are attributed to ibuprofen, while the peak at 1598 cm^{-1} is assigned to the carboxylate anion of sodium alginate. Analysis of the A1 spectrum revealed distinct peaks corresponding to both ibuprofen and sodium alginate. Specifically, ibuprofen is represented by peaks at 671, 779, and 1417 cm^{-1} , while characteristic sodium alginate peaks appear at 1031, 1087, 2357, and 1600 cm^{-1} .

The spectrum further showed a lack of peaks at 2727 and 2630 cm^{-1} , which implies the absence of hydrogen bonding within the A1 formula (Kumar et al., 2014; Ramukutty & Ramachandran, 2012).

The FTIR spectrum of the A2 formula exhibits characteristic peaks for sodium alginate at 937, 1026, 1083, and 1598 cm^{-1} , and a peak for ibuprofen at 1415 cm^{-1} . A significant finding is the disappearance of the characteristic ibuprofen peaks that were present in the physical mixture spectrum, which was observed in both A1 and A2 formulations. This spectral change is likely caused by the electrostatic interaction between sodium alginate and ibuprofen (Liu et al., 2019) including the zein final concentration, the mass ratio of

zein to SC, and the mass ratio of SC to SA influence the physicochemical properties of the nanoparticles. The fluorescence spectrum confirmed that curcumin was encapsulated in the composite nanoparticles with an increase in the mass ratio of curcumin to zein, and the encapsulation efficiency of curcumin decreased. When coated with SA, there was a significantly improved encapsulation efficiency. Fourier transform infrared spectroscopy (FTIR). The FTIR spectrum of the B1 formula showed that only the ibuprofen peak at 1415 cm^{-1} (C–C stretching vibration in aromatic ring) remained. Furthermore, slight displacements were noted in the peaks assigned to sodium alginate. This trend is also replicated in the B2 formula. The FTIR results show that ibuprofen maintains its stability in sodium alginate sponges.

Morphological analyses

SEM images (Figure 2.) confirmed the porous structure of the sponges. Pores were observed to be irregularly distributed throughout the sponge matrix. Formula B, prepared with a higher sodium alginate percentage, exhibited distinct porosity compared to Formula A.

Texture analysis

The texture analysis results are presented in Figure 3. Formula B0, requires a force of 50 N to be compressed by 4 mm, whereas for A0, this force is 6 N. The sponge texture becomes increasingly soft as the concentration of ibuprofen rises in the B formulations. Approximately 15 N is required to compress Formula B1 by 4 mm, while this value is 10 N for Formula B2. Conversely, Formula A1 was determined to have the softest sponge texture. For Formula A2, the required force increased significantly as the distance increased. This suggests that the sponge is nearing its incompressibility point. In a study conducted by Boateng et al., sodium alginate wafers were prepared at five different concentrations (1% to 5%), utilizing paracetamol as a model drug. According to the texture analysis data obtained from the study, the maximum force required to compress a 2% concentration wafer by 2 mm was recorded as 20.6 N, while this value was observed to be 93.7 N for the 5% concentration. In comparison, it was determined that the ibuprofen-loaded sponge formulations possessed a softer texture, despite being subjected to similar preparation processes (Boateng et al., 2010).

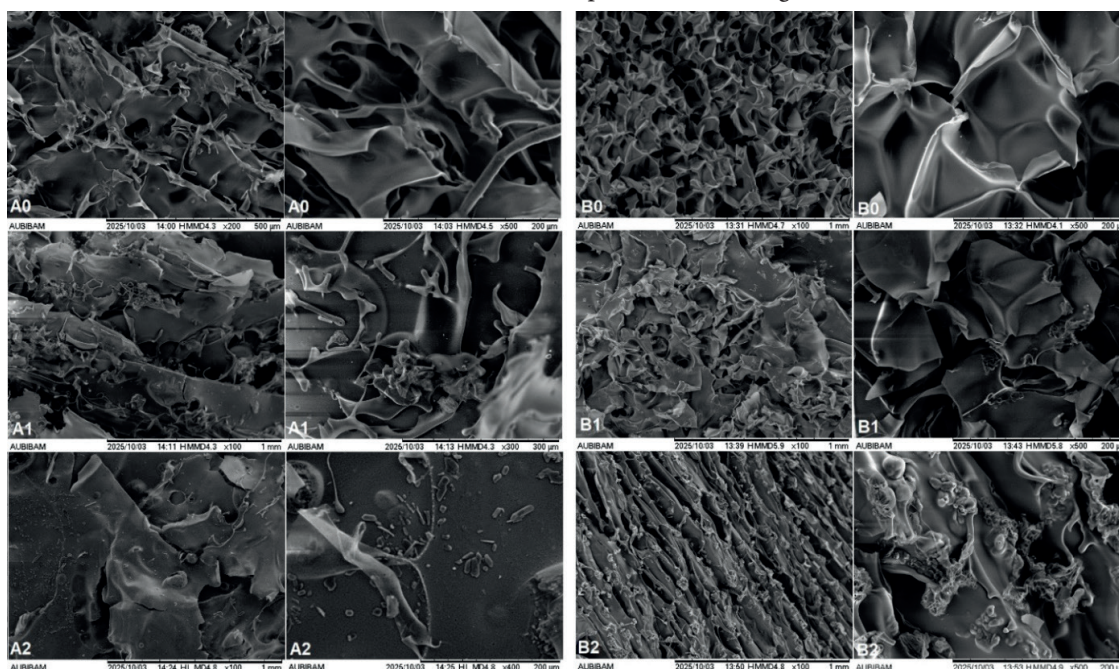


Figure 2. SEM imaging results of formulations

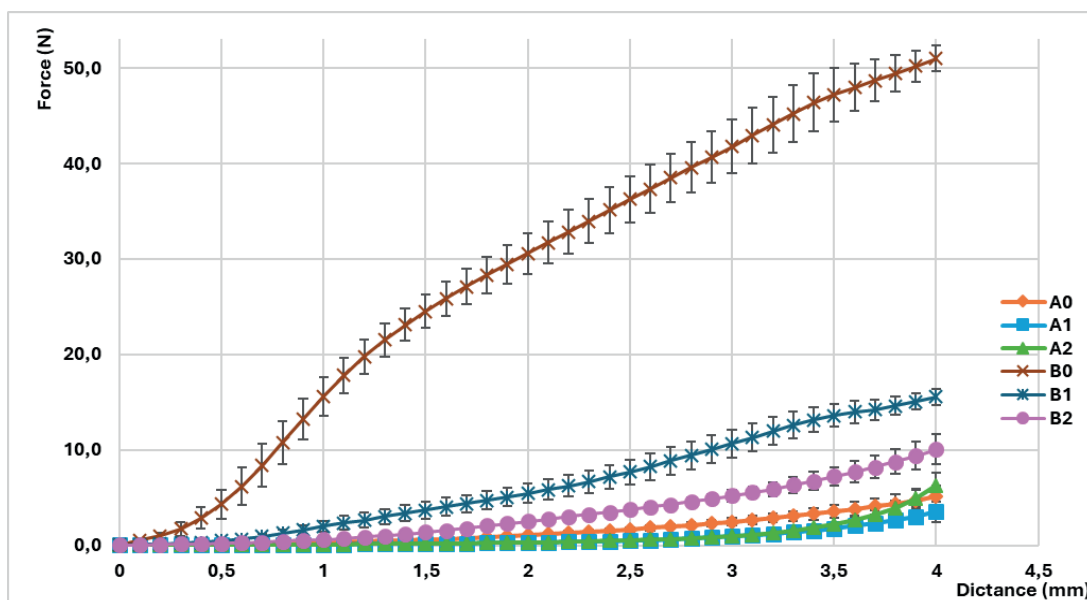


Figure 3. Texture analysis result of sponges (n=3, mean $\pm$ SE)

Drug content and loading capacity

The amount of ibuprofen in the sponges is given in Table 3. Formula B1 demonstrated the highest loading capacity, closely followed by B2, while formula A1 exhibited the lowest. This loss of active substance occurred during the formulation preparation, specifically high-vacuum drying process (Li & Yang, 2015). Furthermore, the inherent hygroscopicity of sodium alginate led to a mass increase, caused by the entrapment of distilled water during the preparation of the solution, which consequently reduced the loading capacity (Xie et al., 2022; Zhang et al., 2024).

Table 3. Drug content of sponges (n=3; mean $\pm$ SE)

Formulation	Drug Content (% w/w)	Loading Capacity (w/w)
A1	10.31 $\pm$ 0.43	41.56 $\pm$ 1.73
A2	24.85 $\pm$ 0.84	62.56 $\pm$ 2.11
B1	9.40 $\pm$ 0.80	80.58 $\pm$ 3.96
B2	15.21 $\pm$ 1.23	72.93 $\pm$ 5.91

In vitro drug release and release kinetics

Based on data from *in vitro* release studies, formula B2 was identified as the fastest-releasing formulation. Conversely, formula A2, while being the slowest-releasing formulation, still achieved about 100% release

within 4 hours (Figure 4.). The prepared formulations enhanced the dissolution rate of ibuprofen (Kaynak et al., 2024). This is particularly notable given that ibuprofen's solubility is pH-dependent, with studies consistently demonstrating an increase in solubility as the pH rises (Higton, 2015; Janus et al., 2020). The pH of sodium alginate solutions typically ranges from 8 to 10 (Latifah et al., 2022). Micro-environmental pH changes and physical interactions with the sodium alginate matrix accelerate ibuprofen solubility (Arica et al., 2005). Immediate release of ibuprofen was also observed in another study that utilized cross-linked sodium alginate beads (Li et al., 2021). In addition to ibuprofen's high solubility in basic environments due to its acidic nature (pKa: 5.2), the polymer/dissolution medium coefficient is also a significant factor influencing the dissolution rate. The inclusion of sodium alginate has been shown to increase the ibuprofen release rate. This is attributed to the excipient's ability to generate osmotic forces capable of disrupting the membranous barrier, which facilitates a higher rate of drug release (Majid Khan & Bi Zhu, 1998). These findings align with and confirm the release results observed in our study.

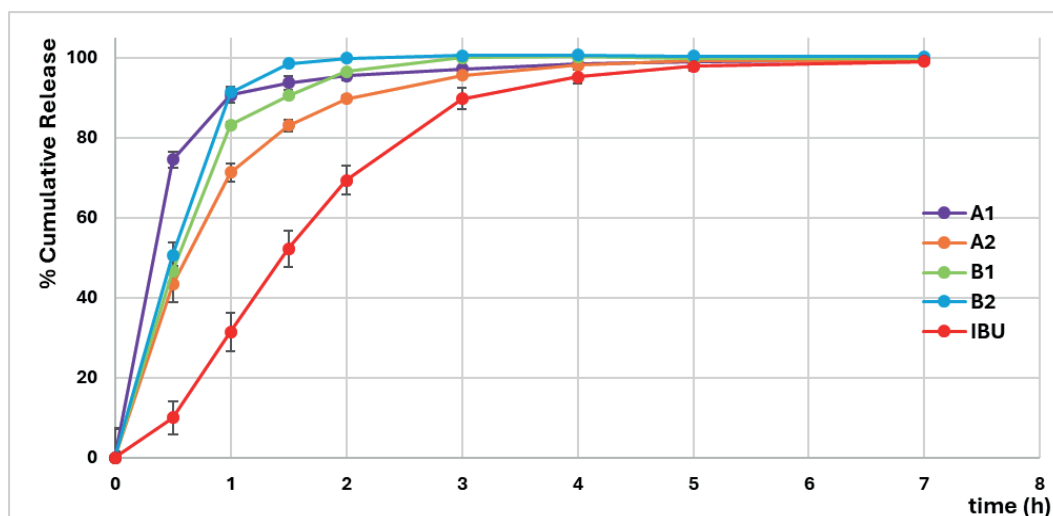


Figure 4. *In vitro* drug release results (n=3, mean $\pm$ SE)

The prepared sponge formulations conformed to different release kinetics, as shown in Table 4. The data were analyzed using DDSolver (Zhang et al., 2010). The lowest Akaike Information Criterion (AIC), highest Model Selection Criterion (MSC), and coefficient of determination (r^2_{adj}) values were used to select the appropriate kinetic model (Zuo et al., 2014). The release kinetics of formulations A1 and A2 were first-order, while the Hopfenberg model was suitable for B1 and B2. In contrast, pure ibuprofen conformed to the Hixson-Crowell model.

Under first-order release kinetics, the drug release rate is directly proportional to the amount of drug remaining in the matrix. Consequently, as the drug concentration decreases, the release rate slows down proportionally. This principle is consistent with the Noyes-Whitney equation (Dokoumetzidis & Macheras, 2006). Consequently, this model is particularly well-suited for porous matrices that contain water-soluble components, where the dissolution and diffusion processes are the primary factors controlling the drug release profile (Askarizadeh et al., 2023).

The Hopfenberg model describes drug release from surface-eroding polymers with a constant surface area. In this model, matrix erosion and time are the limiting factors, influenced by internal and external diffusional resistance (Gupta et al., 2020). This model is relevant for surface-eroding polymers, where the drug release rate is directly influenced by the rate of polymer matrix degradation from the surface (Paarakh et al., 2018). The Hopfenberg model, which assumes matrix erosion is the rate-limiting step for drug release, was a good fit for cylindrical (n=2) and spherical (n=3) systems, respectively, as it accounts for the formulation's geometry (Vidart et al., 2018). For the B1 formula, which exhibited Hopfenberg kinetics, the release exponent (n value) was found to be 4.125. In contrast, the B2 formula showed an n value of 3, consistent with release from a spherical structure (Katzhendler et al., 1997). Drug transport and release from pharmaceutical systems is a complex process driven by a combination of physical and chemical phenomena, making it challenging to develop an accurate mathematical model (Costa & Sousa Lobo, 2001).

Table 4. Kinetic models of formulations

Code	Parameters	Release Models					
		Zero Order	First Order	Higuchi	Korsmeyer-Peppas	Hixon-Crowell	Hopfenberg
A1	k	21.940	2.380	51.780	86.312	0.250	0.250
	r_{2adj}^2	-39.414	0.872	-11.870	0.733	-9.576	-6.827
	AIC	80.927	34.894	71.772	41.533	70.202	68.561
	MSC	-3.949	1.805	-2.805	0.975	-2.609	-2.403
	n	-	-	-	0.096	-	3.000
A2	k	21.307	1.114	49.075	65.312	0.237	0.277
	r_{adj}^2	-3.582	0.991	-0.008	0.725	0.483	0.804
	AIC	77.218	27.342	65.103	55.475	59.765	52.753
	MSC	-1.772	4.462	-0.258	0.946	0.410	1.286
	n	-	-	-	0.290	-	5.250
B1	k	21.931	1.750	51.055	71.270	0.249	0.311
	r_{adj}^2	-5.523	0.940	-0.736	0.557	-0.193	0.980
	AIC	79.161	41.590	68.570	58.410	65.568	33.759
	MSC	-2.125	2.571	-0.802	0.469	-0.426	3.550
	n	-	-	-	0.240	-	4.125
B2	k	22.285	3.075	52.284	39.042	0.240	0.480
	r_{adj}^2	-7.632	0.622	-1.577	-4.322	-0.613	0.966
	AIC	80.443	55.414	70.774	77.340	67.026	36.812
	MSC	-2.406	0.723	-1.197	-2.018	-0.728	3.048
	n	-	-	-	0.503	-	3.000
IBU	k	19.597	0.528	42.823	42.356	0.135	0.135
	r_{adj}^2	0.467	0.940	0.855	0.735	0.961	0.954
	AIC	68.890	51.380	58.486	64.082	48.029	50.029
	MSC	0.380	2.569	1.680	0.981	2.987	2.737
	n	-	-	-	0.423	-	3.000

An analysis of the models suggests that the drug release mechanism in the B formulations involves a combination of swelling, matrix erosion, and mixed diffusion and chain relaxation mechanisms. Considering the porous structure of the sponges and the water solubility of sodium alginate, the release kinetics of formulations A1 and A2 were found to be first-order, which is a result consistent with the literature. The elevated sodium alginate concentration in Formula B, as opposed to Formula A, led to a modification of the release kinetics. This difference is characterized by ibuprofen release being primarily driven by the structural erosion of the sponge matrix. While the precise mechanism for the release of ibuprofen from the B1

formulation has not been elucidated in the existing literature, evidence suggests that the process proceeds via super-case II transport (Martín-Illana et al., 2022).

The difference factor (f_1) and similarity factor (f_2) are key metrics for assessing the difference and similarity between two drug release profiles. An f_1 value of 0–15 and an f_2 value of at least 50 are the established criteria for similarity, as per EMA and FDA guidelines. Both factors are widely used to compare the release rates of drug delivery systems (Muselík et al., 2021; Priese et al., 2023) neither the EMA nor the FDA provides unambiguous instructions for comparing the dissolution curves, except for calculating the similarity factor f_2 . In agreement with the EMA and FDA

strategy for comparing the dissolution profiles, this manuscript provides an overview of suitable statistical methods (CI derivation for f2 based on bootstrap, CI derivation for the difference between reference and test samples, Mahalanobis distance, model-dependent approach and maximum deviation method. The prepared sponge formulations, in this case, have different release profiles than pure ibuprofen, as shown by the results (Table 5.) obtained in this study.

Table 5. Similarity and difference factors of formulations versus pure ibuprofen

Formulation	(f ₁) Difference factor	(f ₂) Similarity factor
A1	037	022
A2	025	032
B1	032	027
B2	036	024

Cytotoxicity study

Due to its biocompatibility and ability to support tissue regeneration, alginate is a favorable polymer for wound healing applications (da Silva et al., 2024). The cytotoxicity of the formulations was evaluated using an MTT assay on human umbilical vein endothelial cells (HUVECs), with the results presented in Figure 5 (Jiang et al., 2021). Additionally, cell cultures in the

multi-well plates were routinely monitored via microscopy throughout the study, and no evidence of contamination was observed.

Based on the MTT assay results, the empty formulation containing 5% alginate exhibited a higher proliferative effect, leading to significantly higher cell viability compared to the 2% alginate empty formulation. These findings are consistent with a study by Jiang et al., which demonstrated the cytoprotective effect of alginate oligosaccharides on endothelial cells under oxidative stress, suggesting their potential antioxidant and antiapoptotic roles (Jiang et al., 2021). Similarly, the cytotoxic effect of Ibuprofen was evaluated against HUVEC cells to validate the assay. A dose of 100 µg/mL ibuprofen demonstrated its toxic potential, reducing cell viability to 44.22% after 24 hours of exposure. This inhibitory effect remained consistent at the 48-hour mark, with cell viability at 46.82%, confirming the cytotoxic nature of the compound at the tested concentration (Eldeeb et al., 2025; Habib et al., 2024) and its metabolites significantly influence the tumor development, and metastatic processes. The design and synthesis of prodrugs intended for non-steroidal anti-inflammatory drugs (NSAIDs).

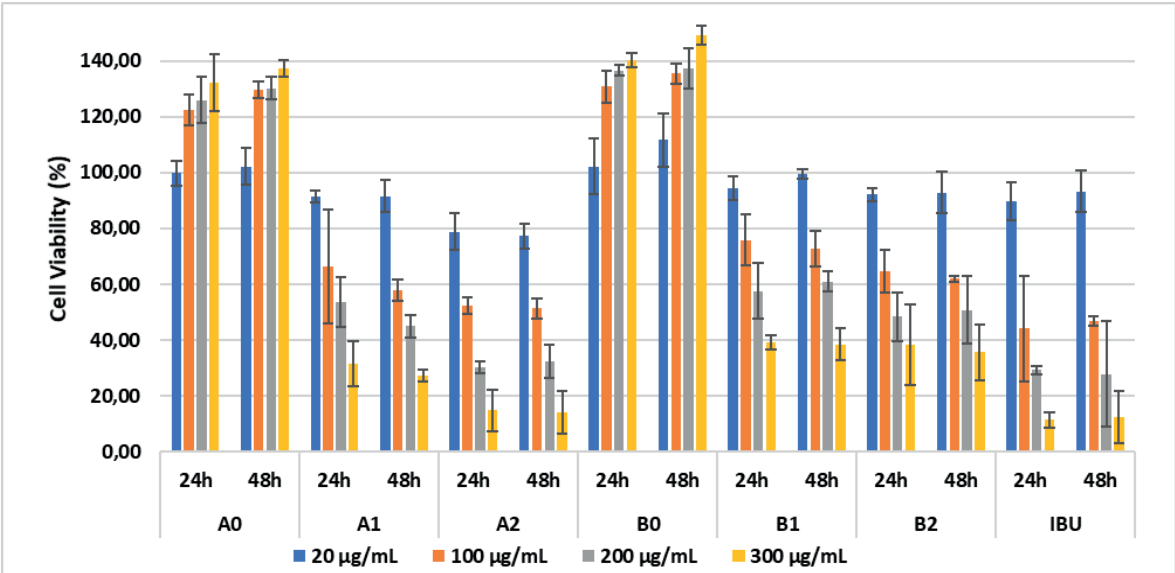


Figure 5. Cytotoxicity results (n = 3; mean ± SD)

Formulations with a lower drug loading capacity were more effective at mitigating the active ingredient's cytotoxicity. This was particularly evident with Formulation B, which contained a higher alginate concentration and provided better protection than Formulation A. An example is the performance of Formulation B1, which maintained cell viability at 75.85% after 24 hours at 100 µg/mL, in contrast to Formulation A1, which resulted in a lower viability of 66.35%. Similar trends were observed in formulations with identical polymer composition but different loading capacities. At a concentration of 100 µg/mL for 24 hours, the lower loading capacity Formulation A1 showed 66.35% cell viability, while Formulation A2 resulted in a lower viability of 52.31%. Our findings suggest a correlation between the total amount of formulation and its ability to inhibit cytotoxicity, a result consistent with literature on other biocompatible polymers. Harrish et al. showed that chitosan, a natural and biodegradable polysaccharide similar to alginate, significantly reduced the toxicity of cadmium sulfide nanoparticles. This observation further highlights the potential of such polymers to improve the safety profile of active compounds and their formulations (Harish et al., 2020).

In conclusion, while the empty formulations were not found to be dose-dependently cytotoxic, the results of the MTT assays demonstrated that ibuprofen exhibited dose-dependent cytotoxicity. Importantly, the study revealed that the prepared formulations effectively mitigated this cytotoxic effect, highlighting the potential of the formulation design to enhance the safety profile of the active ingredient.

CONCLUSION

This study successfully prepared ibuprofen-loaded sodium alginate sponges with properties suitable for wound dressings. The sponges showed high swelling capacity and rapid dissolution. FTIR analysis confirmed molecular interactions that led to a significant

increase in the ibuprofen dissolution rate. The formulations also exhibited different drug release kinetics than pure ibuprofen, with some following the first-order and others the Hopfenberg models. Notably, the sponges were found to reduce the cytotoxicity of pure ibuprofen, highlighting their potential for further *in vivo* investigation as wound dressing applications.

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AUTHOR CONTRIBUTION STATEMENT

KA: Concept, Design, Supervision, Manuscript Writing, Literature Search, Data Collection and Processing CKÖ: Literature Search, Data Collection, and Processing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Formulation and Characterization of Microemulgel Containing *Boesenbergia rotunda* (L.) Mansf. Essential Oil as an Anti-Acne Agent

Sri MULYANINGSIH*, Alifathun Nur WINDA SARI**, Endang DARMAWAN***, Nuri Ari EFIANA****

Formulation and Characterization of Microemulgel Containing *Boesenbergia rotunda* (L.) Mansf. Essential Oil as an Anti-Acne Agent

SUMMARY

This study aimed to formulate and evaluate the physicochemical and antibacterial effects of a microemulgel of *Boesenbergia rotunda* essential oil (BREO) developed as an anti-acne agent. The BREO microemulgel was prepared using Tween 80 as a surfactant, PEG 400 as a co-surfactant, and Viscolam as a gelling agent. The microemulgel was evaluated for homogeneity, pH value, viscosity, spreadability, adhesion test, and stability. The droplet size and zeta potential were measured using a Particle Size Analyzer (PSA). The antibacterial effects were tested using the diffusion method against *Propionibacterium acnes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. BREO microemulsion using Tween 80-PEG 400 (25%:25%) obtained a clear and transparent microemulsion with a transmittance percentage of $98.65 \pm 0.40\%$. BREO microemulgels showed a homogenous system, pH value of 5.95-6.43, viscosity of 6411-36569 cps, spreadability of 5.2-5.5 cm, adhesion 3.62-4.48 seconds, and no visible separation in the stability test. Each formulation exerted an antibacterial effect against bacteria-related acne, with 22.6-24.6 mm inhibition zone diameters. The formulation of F1B had a droplet size of 22 nm, and zeta potential -31.58 ± 0.30 mV. The BREO microemulgel formulation (F1B) met the physical properties, the stability, and the antibacterial activity to be developed as an anti-acne.

Keywords: Acne vulgaris, fingerroot, microemulsion, *Propionibacterium acnes*, surfactant.

Akne Karşıtı Bir Ajan Olarak *Boesenbergia rotunda* (L.) Mansf. Uçucu Yağı İçeren Mikroemüljin Formülasyonu ve Karakterizasyonu

ÖZ

Bu çalışmanın amacı, akne karşıtı bir ajan olarak geliştirilen *Boesenbergia rotunda* esansiyel yağı (BREO) mikroemüljinin fizikokimyasal ve antibakteriyel etkilerini formüle etmek ve değerlendirmektir. BREO mikroemüljeli, yüzey aktif madde olarak Tween 80, yardımcı yüzey aktif madde olarak PEG 400 ve jelleştirici olarak Viscolam kullanılarak hazırlanmıştır. Mikroemüljel homojenlik, pH değeri, viskozite, yayılabilirlik, yapışma testi ve stabilite açısından değerlendirilmiştir. Damlacık boyutu ve zeta potansiyeli bir Parçacık Boyutu Analizörü (PSA) kullanılarak ölçülmüştür. Antibakteriyel etkiler *Propionibacterium acnes*, *Staphylococcus aureus* ve *Staphylococcus epidermidis*’e karşı difüzyon yöntemi kullanılarak test edilmiştir. Tween 80-PEG 400 (%25:25) kullanılan BREO mikroemülsiyonu, $98,65 \pm 0,40$ geçirgenlik yüzdesi ile berrak ve şeffaf bir mikroemülsiyon elde etmiştir. BREO mikroemüljelleri homojen bir sistem, 5,95-6,43 pH değeri, 6411-36569 cps viskozite, 5,2-5,5 cm yayılabilirlik, 3,62-4,48 saniye yapışma ve stabilite testinde gözle görülür bir ayrılma göstermemiştir. Her bir formülasyon, 22,6-24,6 mm inhibisyon zon çapları ile bakterilerle ilişkili aknelere karşı antibakteriyel etki göstermiştir. F1B formülasyonu 22 nm damlacık boyutuna ve $-31,58 \pm 0,30$ mV zeta potansiyeline sahipti. BREO mikroemüljel formülasyonu (F1B), akne karşıtı olarak geliştirilecek fiziksel özellikleri, stabiliteyi ve antibakteriyel aktiviteyi karşılamıştır.

Anahtar Kelimeler: Akne vulgaris, mikroemülsiyon, parmak kökü, *Propionibacterium acnes*, yüzey aktif madde.

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* ORCID: 0000-0001-5414-442X, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia.

** ORCID: 0009-0007-0999-9665, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia.

*** ORCID: 0000-0001-6143-4126, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia.

**** ORCID: 0009-0005-5283-8491, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia.

INTRODUCTION

Acne vulgaris is a prevalent skin disorder among young adults and teenagers. Acne can be caused by various factors, including androgens that activate the sebaceous glands, microbiota imbalances in the pilosebaceous follicle, and innate and cellular immunological responses (O'Neill & Gallo, 2018). Inflammatory papules and pustules are derived from the skin's commensal bacteria, such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*, stimulating the innate and adaptive immune systems (Dréno, 2017). Follicle rupture causes keratin, pro-inflammatory lipids, and germs to be discharged into the dermis, which exacerbates the inflammation (Thiboutot & Zaenglein, 2024).

Antibiotics have long been used to treat acne, leading to a clinical improvement in the condition, but this can lead to bacterial resistance to antibiotics (Dessinioti & Katsambas, 2017). A study in China reported a resistance rate of *P. acnes* to clindamycin, erythromycin, and azithromycin (Zhu et al., 2019). Another study in France showed that *P. acnes* were shown to be resistant to erythromycin (75%) and doxycycline (100%) (Dumont-Wallon et al., 2010). The widespread occurrence of resistance strains in acne patients is a result of the prolonged treatment courses for acne, the usage of topical and/or systemic antibiotics, and the accessibility of over-the-counter antibiotic medications (Dessinioti & Katsambas, 2022). According to this study, choosing non-antibiotic treatments for acne wherever feasible may have major benefits given the rising risk of antibiotic resistance.

Boesenbergia rotunda (L.) Mansf. Essential oil (BREO), also known as the fingerroot essential oil, has the potential to be developed into an anti-acne preparation. It mainly contains camphor, cisocimene, geraniol, and methyl cinnamate, which are known for their antibacterial activity. The previous study reported that BREO had antibacterial activity against acne-causing bacteria (Mulyaningsih et al., 2024). Otherwise, this oil has the potential to be de-

veloped into anti-acne preparations. However, this essential oil provides volatile properties and is easily decomposed by heat, light, and oxygen. In addition, it can cause skin irritation when it is used directly. The development of a convenient formulation is necessary to address this instability and to optimize its activity and efficacy (Kartika, 2018).

Microemulsion gels or also known as microemulgels are used to deliver a variety of medications, including analgesics, anti-inflammatories, antifungals, and anti-acne (Talat et al., 2021). In addition, microemulgel has some benefits, including a long shelf life, being translucent, emollient, thixotropic, greaseless, easily removable, and emollient. Currently, microemulgel is a promising drug delivery system that is widely used to administer hydrophobic compounds (Jain et al., 2019). Microemulgel containing essential oils can increase its antibacterial efficacy because the smaller the particle size of the dispersed phase, the better its bioavailability of antibacterial substances (Karimah & Aryani, 2021). To date, no studies have been conducted on the formulation of BREO in the form of a microemulgel for treating acne. Therefore, this study aimed to investigate the BREO microemulgel formulations with various gelling agents in relation to their physical and antibacterial properties as an anti-acne agent.

MATERIALS AND METHODS

Materials

BREO was distilled from *B. rotunda* rhizomes, and its chemical composition has been verified by gas chromatography-mass spectrometry (GC-MS), *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, and *P. acnes* ATCC 6919.

Optimization of surfactant and co-surfactant of the BREO microemulsion base

Surfactant and co-surfactant were optimized using various concentrations of Tween 80 as surfactant and PEG 400 as co-surfactant (Table 1). Tween 80, PEG 400, and BREO were mixed in a glass beaker and

then added with distilled water to 100 mL. The mixture was stirred with a magnetic stirrer at 300 rpm for 30 minutes. Emulsification of 3% BREO in Tween 80 and PEG 400 was checked by measuring the percentage of transmittance using a spectrophotometer (Shimadzu, Japan) at 650 nm (Islam & Uddin, 2022).

Table 1. Formulation of microemulsion with various percentages of Tween 80 and PEG 400

Formulation	Tween 80 (%)	PEG 400 (%)	BREO (%)
F1	25	25	3
F2	20	30	3
F3	30	20	3

Formulation of BREO microemulgel

The optimum surfactant and co-surfactant combination in BREO microemulsion was used as a microemulgel formulation base with various concentrations of Viscolam as a gelling agent (Table 2). Microemulgel was prepared based on a previous study (Priani et al., 2019). First, Viscolam 8% was prepared by dispersing 8 g of Viscolam in distilled water to a volume of 100

The formulation with the highest percentage transmittance was followed by its microemulsion globule, polydispersity index (PI), and zeta potential determination using a Particle Size Analyzer (PSA) (Malvern Panalytical Ltd.) (Zainol et al., 2015).

mL. Then, it was weighed according to formulas F1A (15 g), F1B (20 g), and F1C (25 g). Tween 80, PEG 400, BREO, and water were mixed and homogenized using a magnetic stirrer (Jieo Tech Japan Inc) at 300 rpm for 30 minutes. Each formulation was mixed with Viscolam, followed by homogenizing using a magnetic stirrer at 300 rpm for 15 minutes. Subsequently, propyl paraben, methyl paraben, and α -tocopherol were added.

Table 2. Formulation of BREO microemulgels

Composition	Concentration (%)		
	F1A	F1B	F1C
BREO	3	3	3
Tween 80	25	25	25
PEG 400	25	25	25
8% Viscolam	15	20	25
Methyl paraben	0.36	0.36	0.36
Propyl Paraben	0.04	0.04	0.04
Tocopherol	0.01	0.01	0.01
Glycerine	0.126	0.126	0.126
Distilled water	until 100 mL	until 100 mL	until 100 mL

Characterization of BREO microemulgel formulations

Organoleptic observations

Organoleptic observations were carried out visually with human senses, including observations of clarity, color, odor, and texture of the microemulgel that was made.

Homogeneity testing

About 1 gram of the microemulgel was uniform-

ly and thinly applied to the transparent glass. If there was no coarseness, the microemulgel was considered homogenous (Dewi et al., 2023).

pH measurement

The pH values were determined by a pH meter (Ohaus Starter ST300). The pH meter was calibrated using a standard solution with a pH value of 4, pH 7, and pH 10 before use. One gram of BREO microemulgel was dispersed in 10 ml of distilled water. After-

wards, the electrode of the pH meter was immersed in the diluted solution until a stable pH measurement was obtained (Rahmania et al., 2020).

Spreadability test

For this test, 0.5 grams of microemulgel were placed in the center of a glass plate. Then, a pre-weighed cover glass was placed on top of the gel. Next, 150-gram weights were added and left for one minute. Spreadability was determined by measuring the average diameter of the spread on several sides (Nurman et al., 2019). This test was replicated three times.

Adhesion test

This test involved placing 0.5 grams of gel on an object glass, covering it with another object glass, and leaving it for five minutes. Then, a 1 kg weight was placed on top for five minutes. Next, both slides were placed on a testing device with an 80-gram load. The time it took for the slides to separate after removing the 80-gram load was recorded (Rahmawati & Setiawan, 2019). This process was repeated three times.

Viscosity measurement

The viscosity of the BREO microemulgel was assessed using a Rheosys Merlin VR rheometer with a 2.0/30 mm cone-plate spindle. The gel was placed on the plate and sealed with the parallel plate. The rheometer was connected to a computer with the Rheosys Micra application installed. The measurement parameters were set as follows: temperature: 25 °C; start speed: 10; end speed: 100; direction: up/down; steps: 12; delay time: 30 seconds; and an integration time of 10 seconds. The viscosity measurement was initiated by pressing "Start," which lasted for a period of time. The viscosity of the three microemulgels was measured using the same treatment and parameters (Sugihartini et al., 2021).

Globule characterization of BREO microemulsion and microemulgel

The produced microemulgel was analyzed for droplet size and polydispersity index (PI), using dynamic light scattering. Zetasizer (Nano ZS, Malvern Panalytical, U.K.) was used to measure the BREO

microemulgel. The prepared sample was put into cuvettes, and scattering intensity was observed at a temperature of 25 °C.

Morphology of Microemulgel

Transmission electron microscopy (TEM) imaging was used to examine the microemulgel's morphology. In short, a droplet of the water-diluted microemulgel suspension was put on a 200-mesh formvar copper grid, allowed to adsorb, and then the excess was wiped off using filter paper. After adding a drop of 2% (w/v) uranyl acetate solution, the sample was allowed to come into contact with it for five minutes. Before the vesicles were photographed using an HR-TEM (Talos F200C G2), the material was dried at room temperature (Sulastrı et al., 2025).

Stability studies of BREO microemulgel

a) Centrifugation assay

Five milliliters of each BREO microemulgel formulation were centrifuged at 3500 rpm for 30 minutes. All formulations were observed for the presence of separation, creaming, or cracking (Kiromah et al., 2023).

b) Heating-cooling cycle

The study was run for three cycles. A cycle involved storage of the BREO microemulgel at 4 °C for 24 hours and then storage at 40 °C for the following 24 hours (Priani et al., 2019). The separation, creaming, or cracking was evaluated for each formulation.

Antibacterial assay of BREO microemulgel

The antibacterial assay used the well diffusion method against bacteria-related acne, including *S. epidermidis*, *S. aureus*, and *P. acnes*. Bacterial suspensions with a concentration of 10^8 CFU/mL were taken with a sterile cotton swab and then spread onto Mueller-Hinton Agar (MHA) media (HiMedia). After that, wells were made on MHA media using a perforator. A total of 100 mg of each formulation, F1A, F1B, and F1C, was placed in the wells and incubated at 37 °C for 24 hours. The same procedure was applied using a microemulgel base as a negative control and 1% clin-

damycin gel as a positive control. After incubation at 37 °C for 24 hours, the diameter of the inhibition zone was measured.

Data analysis

The data on the physical properties of the microemulgel and the inhibition zone diameter were analyzed using GraphPad Prism 8. A data normality test was performed using the Shapiro-Wilk test ($P > 0.05$). The data were analyzed using one-way ANOVA followed by a Tukey post hoc test at a 95% confidence level.

RESULTS AND DISCUSSION

Formulation of BREO microemulgel

The correct ratio of oil phase, water phase, and surfactant plays an important role in the stable microemulsion formation. The optimal surfactant and co-surfactant were selected based on the transmit-

tance percentage. Transmittance values close to 100% are characteristic of high-quality microemulsions that form a clear and transparent microemulsion (Pathan et al., 2012). This indicates that they can disperse light due to their small droplet size. F1 formulation had the highest transmittance percentage compared to F2 and F3 (Table 3). The transmittance percentage of F1 microemulsion was found to be $98.65 \pm 0.40\%$, confirming the good transparent nature of the formulations. The optimum ratio was achieved in F1 with a 25%:25% ratio of Tween 80 and PEG 400 to make a clear and transparent BREO microemulsion. Previous studies have shown that surfactant and co-surfactant solutions with a 1:1 ratio of Tween 80: PEG 400 were thermodynamically stable and transparent. PEG 400, as a cosolvent, is promising for microemulgel formulation stability (Taher et al., 2022).

Table 3. Transmittance percentage of the BREO microemulsion formulation with different percentages of Tween 80 and PEG 400

Formulation	Tween 80 (%)	PEG 400 (%)	BREO (%)	Transmittance (mean $\pm$ SD) (%)
F1	25	25	3	98.65 ± 0.40
F2	20	30	3	12.07 ± 1.11
F3	30	20	3	50.11 ± 10.9

One of the factors responsible for the variation of the transmittance value of the microemulsions is droplet size. Smaller droplets increase transparency (higher transmittance) because they scatter less light (Naganuma & Kagawa, 2002). Measurement of the droplet size of formulation F1 was performed to confirm the microemulsion formation (Table 4). The average droplet size of F1 was 12 nm, which meets the ideal range of 10-200 nm for a microemulsion system. The PI value of formulation F1 was 0.24, between 0.01 and 0.7, and is classified as a monodispersed system (Kiomah et al., 2023). The zeta potential of F1 was -8.235 mV. It is not too negative for skin application due to the possible repulsion. Therefore, it is suitable for topical application to deliver the active ingredient. The Formulation 1 indicated clear or transparent, with a very small droplet size. Production of the microemulsions using a magnetic stirrer yielded small droplet sizes with a relatively low PI. This result is

consistent with those of other studies that prepared microemulsions with a magnetic stirrer, producing droplet sizes of less than 200 nm (Nunes de Godoi et al., 2025; Syapitri et al., 2022).

Table 4. Particle characterization of BREO microemulsion (F1 formulation)

Measurement	Microemulsion (F1)
Droplet size (nm)	12.00 ± 0.07
Polydispersity index (PI)	0.24 ± 0.02
Zeta potential (mV)	-8.235 ± 0.13

Numerous investigations have looked into the physicochemical characteristics of different Tweens. For microemulsion formulations, these references take into account every characteristic and observation that proves Tween to be a convenient cosmetic excipient (Kaur & Mehta, 2017). However, existing guidelines should be followed when using Tween 80 as a surfactant to avoid adverse effects.

Characterization of BREO microemulgel

All formulations of BREO microemulgels were yellowish-white in color and exhibited the characteristic odor of fingerroot, as shown in Table 5. The difference observed among the formulations was in their consistency: F1C indicated the thickest compared to the other formulations. This difference is mainly affected by the amount of the gelling agent Viscolam. The higher the concentration of the gelling agent, the

thicker the gel (Forestryana et al., 2022). During gel formation, the gelling agent absorbs water and remains in the gel, forming a thick liquid. Therefore, the higher the concentration of the gelling agent, the more liquid will be bound and retained, which can lead to an increase in viscosity. Table 5 shows that each microemulgel is homogeneous, with no particles or coarse grains, thus reducing the potential for skin irritation.

Table 5. The organoleptic and homogeneity of BREO microemulgel

Test		F1A	F1B	F1C
Organoleptic	Clarity	Clear	Clear	Clear
	Color	Yellowish white	Yellowish white	Yellowish white
	Odor	Typical aromatic	Typical aromatic	Typical aromatic
	Consistency	Slightly thick	Thick	Thicker
Homogeneity		Homogenous	Homogenous	Homogenous

Ideally, the pH value of the microemulgel is neither too acidic nor too alkaline and matches the natural pH of human skin. The normal skin pH is between 4 and 6 (Lukić et al., 2021; Prakash et al., 2017). Another study reported that the skin preparation for acne should be at a pH between 5.4 and 6 (Kulthanan et al., 2015). If the resulting pH is too acidic, it may irritate, while if the pH obtained is too alkaline, it may cause the skin to dry out and become sensitive (Hwang et al., 2022). Figure 1 presents the pH value of three formulations, which were in the range of 5.95 to 6.43. Thus, the BREO microemulgel can be applied to the facial skin without irritation. Statistical analysis shows that the pH values of the three formulations do not have significant differences ($p>0.05$).

A spreadability test was conducted to evaluate how well the microemulgel spreads on the skin during application. Good spreadability ensures that active ingredients are evenly distributed on the skin. However, if the microemulgel spreads too easily, it can cause discomfort during application. An ideal spreadability test result is between 5 and 7 cm (Nurman et al., 2019). Viscosity is inversely proportional to spreadability. This occurs because higher viscosity results in lower spreadability (Szulc-Musioł et al., 2017). The three BREO microemulgel formulations have a spreadability in the range of 5 to 5.5 cm and no statistically significant difference, meeting the requirements for good spreadability ($p>0.05$).

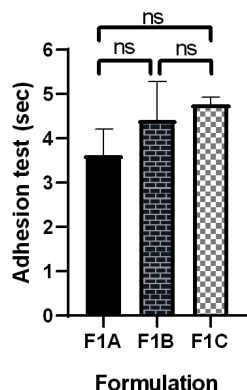


Figure 1. pH value of BREO microemulgel formulations. The asterisk denotes a significant difference ($p < 0.05$), and the letter “ns” denotes not significant ($p > 0.05$).

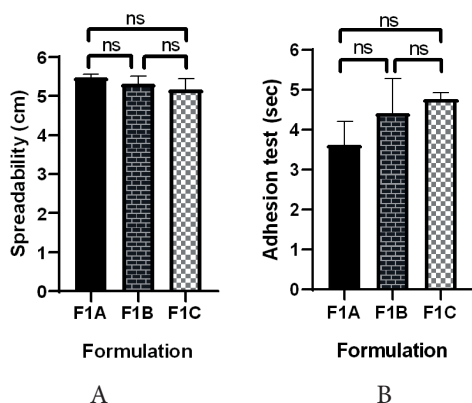


Figure 2. Spreadability (A) and adhesion tests (B) of the BREO microemulgel formulations. The letter “ns” denotes not significant ($p > 0.05$).

Adhesion testing determines a preparation's ability to adhere to the skin. A good adhesion study requires a contact time of more than 4 seconds on the skin surface (Tania et al., 2022). BREO microemulgel formulations that meet this requirement are F1b and F1c, whereas F1a demonstrates an adhesion time of less than four seconds (Figure 2). The adhesion value of F1A is associated with its lower consistency and viscosity, which prevents it from adhering for an extended duration. For topical preparations, such as microemulgels, it is necessary to determine their adhesion time. The amount of gelling agent could affect the adhesivity as well as the viscosity. The higher

the adhesion value, the stronger the contact between the microemulgel and the skin. Good adhesion is associated with better efficacy and absorption of active ingredients (Kurniawan & Aryani, 2024). Thus, good adhesion relates not only to the convenience of use but also to the therapeutic effectiveness of an active ingredient. Nonetheless, the adhesion value and spreadability value are inversely correlated; the higher the spreadability, the lower the adhesion value.

Figure 3 shows that the viscosity of F1A, F1B, and F1C was 6,411.78, 23,361.22, and 36,569.51 cps, respectively. The difference in the results is due to variations in the amount of the gelling agent Viscolam. F1C indicated the highest viscosity due to the most Viscolam added, resulting in a thicker consistency. One-way ANOVA statistical analysis showed a significant difference in the viscosity of the three formulations ($p < 0.05$). This demonstrates that the addition of Viscolam significantly affects the viscosity of BREO microemulgel.

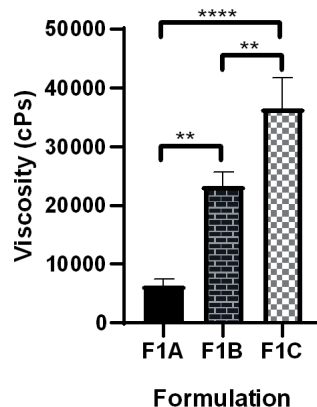


Figure 3. The viscosity of BREO microemulgel formulations. The asterisk denotes a significant difference ($p < 0.05$).

Viscosity influences the distribution of particle sizes, the physical state, and the interactions between drug molecules and excipients, all of which have an impact on drug stability. It is important to avoid phase separation or deterioration while being stored. Viscosity affects the rheological characteristics, influencing the administration route, flow characteristics, and

residence time at the intended location, particularly in gel dosage devices. Finally, viscosity influences the therapeutic outcomes (Kottke, 2023).

The BREO microemulgels flow type was determined by examining the rheogram (Figure 4.) and the results of the linear regression equations between shear stress and shear rate, as well as log shear stress and log shear rate. The log shear stress vs. log shear rate equation for all microemulgels (F1A, F1B, and

F1C) produced an r-value close to one and a slope value greater than one, indicating that they have a pseudoplastic flow type. The viscosity of all microemulgels decreases with increasing shear stress. This is beneficial when the gel is in a package (e.g., a tube) because squeezing the package will cause the gel to flow out. This is also convenient during the filling process because the gel flows easily when given frictional pressure (Calienni et al., 2023).

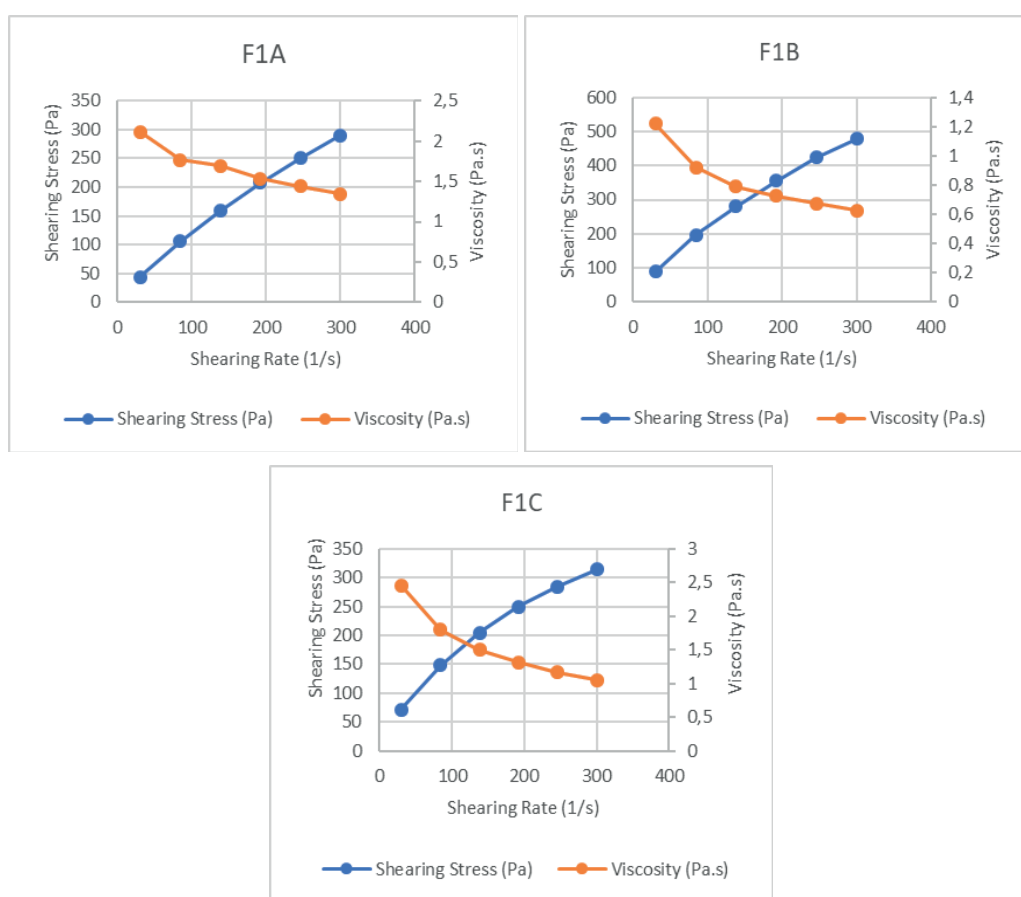


Figure 4. The rheogram of BREO microemulgel formulations

Accelerated stability studies of BREO microemulgel formulations

The accelerated stability studies were performed to determine the physical stability of the microemulgels. Two methods were used: centrifugation and exposure to extreme temperatures. The purpose of the centrifugation study was to ascertain whether or not there was a phase separation brought on by gravity.

The heating-cooling cycle study was conducted to show the microemulgels resistance to extreme temperatures. This stability is important to prevent damage to the preparation due to thermal expansion and contraction, maintain optimal system performance in the long term, and reduce the risk of failure due to repeated thermal stress. All microemulgel formulations produced stable results, characterized by an absence of separation, creaming, or cracking (Table 6).

Table 6. The result of the stability studies of BREO microemulgel formulations

Formulation	Centrifugation method	Heating-cooling cycle
F1A	No separation, no creaming, no cracking	No separation, no creaming, no cracking
F1B	No separation, no creaming, no cracking	No separation, no creaming, no cracking
F1C	No separation, no creaming, no cracking	No separation, no creaming, no cracking

Measurements of microemulgels globule size and zeta potential

The particle size measurement indicated that the average globule size of F1A, F1B, and F1C was about 22-210 nm, placing F1B within the microemulsion size range of 10-200 nm (Seng & Loong, 2019). Based on the PI value obtained as 0.37, the droplet size uniformity level in the F1B microemulgel is close to monodispersion (Mahbubul, 2019). Interestingly, the droplet size of F1A was larger than that of F1B. F1A had a zeta potential value of -19.61 ± 0.29 , which is closer to 0, while F1B had a zeta potential value of -30. The closer the value is to zero, the greater the chance of coagulation between droplets. Additionally, lower viscosity increases the likelihood of aggregation in gel preparations (Namburu et al., 2007).

Table 7. Particle characterization of BREO microemulgel

Measurement	F1 A	F1B	F1C
Droplet size (nm)	86.2 ± 1.63	21.7 ± 0.19	210.2 ± 2.9
Polydispersity index (PI)	0.67 ± 0.13	0.37 ± 0.06	0.38 ± 0.03
Zeta potential (mV)	-19.61 ± 0.29	-31.58 ± 0.30	-31.38 ± 0.50

Zeta potential is one of the parameters used to evaluate the physical stability of materials in a colloidal environment. It is the surface charge of the globules that maintains an optimal distance to prevent coalescence. Due to electrostatic repulsion, zeta potential affects the stability of microemulsion systems. Emulsions with larger zeta potential values are more stable than emulsions with smaller values. The stability of a system can be categorized based on its zeta potential value: good stability (ζ 40-60), somewhat good stability (ζ 30-40), incipient stability (ζ 10-30), or easy coagulation or flocculation (ζ 0-5) (Prakash et al., 2014). The F1B and F1C microemulgels had zeta potential values of -31.58 mV and -31.38 mV, respectively (Table 7). This value indicates that the F1B microemulgel

F1B appears clear or transparent due to its small microemulsion droplet size, stability, homogeneity, and negligible light scattering. The PI measurement results in this study demonstrated that all microemulsion base formulations are monodisperse, resulting in a more homogenous microemulsion that can avoid creaming or cracking. Microemulsion base systems have a consistent particle size distribution and are typically more stable (Kamaria et al., 2015). This implies that Tween 80, functioning as a surfactant, has created micelles by adhering to the oily phase droplets' surface. By lowering interfacial tension, these micelles can produce a microemulsion with tiny particles (Ashara et al., 2016). Furthermore, as a co-surfactant, PEG 400 has a role in preventing the recombined phase from separating.

is stable. The zeta potential of the three BREO microemulgels is approximately two to four times more negative than that of the microemulsions. This difference is probably due to Viscolam®'s components, Sodium Polyacryloyldimethyl Taurate, which releases sodium cations and amide protons when dispersed in water. This results in the accumulation of anions on the surface of the microemulgel droplets, causing them to become negatively charged (negative zeta potential).

Figure 5 shows the TEM image of the spherical microemulgel droplets had smooth surfaces, and were fairly uniform in size. TEM analysis (F1A) revealed droplet sizes ranging from 50 to 100 nm. This result was consistent with the particle measurement results obtained using PSA, which showed droplet sizes of 80 nm.

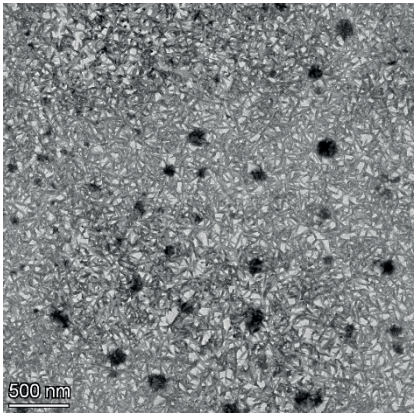


Figure 5. TEM analysis of BREO microemulgel morphology

Antibacterial activity of BREO microemulgel formulations

Figure 6 shows that BREO microemulgel can inhibit the growth of *S. epidermidis*, *S. aureus*, and *P. acnes* bacteria. The negative control, which contained only the gel base and no BREO, did not inhibit all three bacteria. Based on the diameter of the inhibition zone, F1B exhibited the greatest antibacterial activity against all three bacteria, especially against *P. acnes*. The three formulations exhibited similar antibacterial activity against

S. aureus and *S. epidermidis*. All three formulations contain BREO at the same concentration; the difference lies in the amount of Viscolam added. The amount of Viscolam 8% added does not affect the antibacterial activity of the BREO microemulgels. The significant variation of antibacterial activity is likely due to differences in particle size among the three microemulgels. F1B had the smallest particle size, so it more easily penetrated the cytoplasmic membranes of microorganisms, increasing antimicrobial activity (Monteiro et al., 2023).

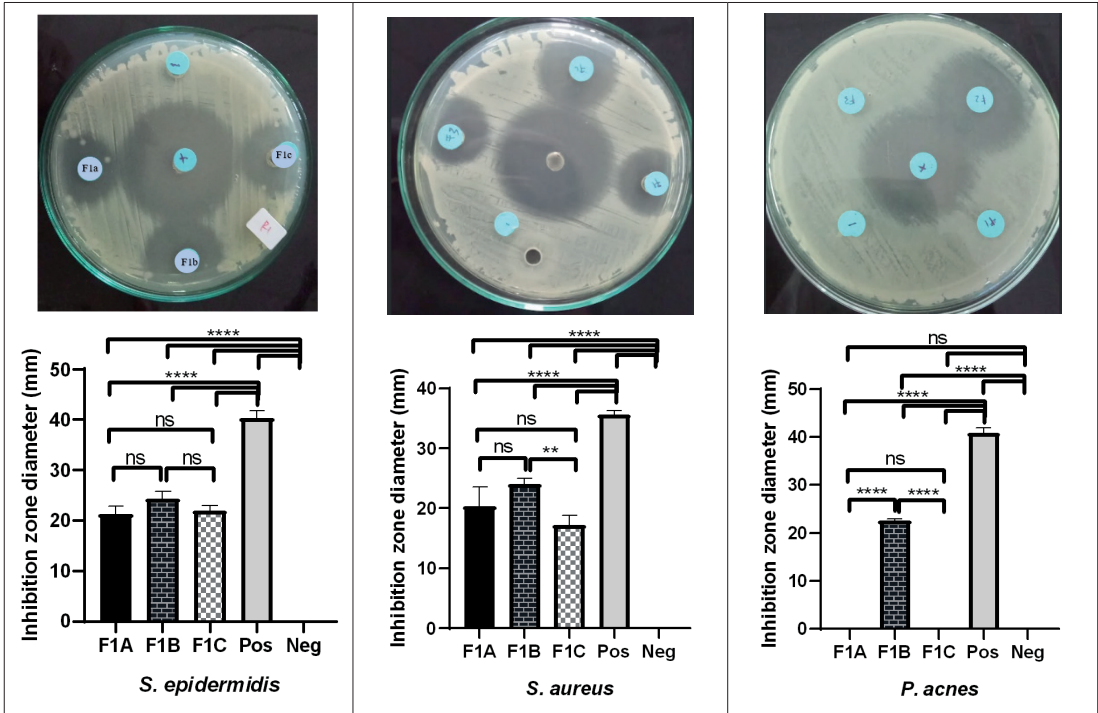


Figure 6. Antibacterial activity of BREO microemulgel against bacteria-related acne (*means significantly different. The asterisk denotes a significant difference ($p < 0.05$), the letter “ns” denotes not significant ($p > 0.05$)).

CONCLUSION

The BREO microemulgel can be prepared with 3% BREO, 8% Viscolam, and Tween 80-PEG 400 (25%:25%). Organoleptic characteristics, pH, spreadability, adhesion test, viscosity, physical stability, globule size, polydispersity index, and zeta potential were all found to be best suited for Formula F1B. It was also effective against *P. acnes*, *S. aureus*, and *S. epidermidis*. BREO microemulgel has the potential to be developed as an anti-acne product.

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AUTHOR CONTRIBUTION STATEMENT

SM: Study concept and design, supervision, manuscript drafting and review. ANWS: Data collection, statistical analysis and manuscript review. ED: Technical support and manuscript review. NAE: Literature collection and manuscript review.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Synthesis of Some Benzothiazolone Derivatives and Their Effects on Quorum Sensing

Yasemin DÜNDAR^{**}, Meryem Burcu KÜLAHCI^{**}, Özge KUYRUKÇU ÖZTÜRK^{***}, Ebru BEYZİ^{****}

Synthesis of Some Benzothiazolone Derivatives and Their Effects on Quorum Sensing

Bazı Benzotiyazolon Türevlerinin Sentezi ve Bunların Quorum Sensing Üzerindeki Etkileri

SUMMARY

Quorum sensing (QS), which enables bacteria to communicate by sensing their population density and, in particular, to regulate biofilm formation and virulence factor production in pathogenic bacteria, has emerged as a novel target for the control of bacterial infections. In this study, the QS inhibitory effects of benzothiazolone and its nitrogen-substituted derivatives were investigated. For this purpose, the effects of the synthesized compounds on violacein production by *Chromobacterium violaceum*, inhibition and eradication of biofilm formed by *Pseudomonas aeruginosa*, inhibition of proteolytic activity, and their influence on bacterial swimming and swarming motilities were examined. Among the synthesized compounds, benzothiazolone (Q1) and N-acetylbenzothiazolone (Q8) were determined to be the most active QS inhibitors, particularly with their effects on pigment and biofilm inhibition. Benzothiazolone derivative QS inhibitor compounds may serve as lead compounds for future studies.

Keywords: Antibacterial activity, benzothiazolone, quorum sensing inhibitors.

ÖZ

Bakterilerin popülasyon yoğunluklarını algulayarak iletişim kurmalarını ve özellikle patojenik bakterilerde biyofilm oluşumu ve virülans faktörü üretimini düzenlemelerini sağlayan çoğunluk algılama (Quorum Sensing, QS), bakteriyel enfeksiyonların kontrolü için yeni bir hedef olarak ortaya çıkmıştır. Bu çalışmada, benzotiyazolon ve azot-süstitüe türevlerinin QS inhibitör etkileri araştırılmıştır. Bu amaçla, sentezlenen bileşiklerin *Chromobacterium violaceum*'ün violacein üretimi üzerine etkileri, *Pseudomonas aeruginosa* tarafından oluşturulan biyofilmin inhibisyonu, biyofilm eradikasyonu, proteolitik aktivitenin inhibisyonu, bakterinin yüzme ve sürünme hareketleri üzerindeki etkileri araştırılmıştır. Sentezlenen bileşikler arasında benzotiyazolon (Q1) ve N-asetilbenzotiyazolon (Q8), özellikle pigment ve biyofilm inhibisyonu üzerindeki etkileriyle en aktif QS inhibitörleri olarak belirlenmiştir. Bulunan benzotiyazolon türevi QS inhibitörü bileşikler gelecekteki çalışmalarda öncü bileşik olabilir.

Anahtar Kelimeler: Antibakteriyel aktivite, benzotiyazolon, çoğunluk algılama inhibitörleri.

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^{*} ORCID: 0000-0002-8250-7096, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, 06330, Ankara, Türkiye

^{**} ORCID: 0000-0002-5007-5209, Department of Biology, Faculty of Science, Gazi University, 06500, Ankara, Türkiye

^{***} ORCID: 0000-0002-5147-7015, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, 06330, Ankara, Türkiye

^{****} ORCID: 0000-0002-5292-9554, Department of Medical Services and Techniques, Vocational School of Health Services, Gazi University, 06830, Ankara, Türkiye

INTRODUCTION

Quorum sensing (QS) is a bacterial communication mechanism that regulates biofilm formation, virulence factors, bioluminescence, and swarming. In the QS system, autoinducers (AIs) released by bacteria activate target genes when the bacterial population increases. Gram-negative bacteria mostly use *N*-acylhomoserine lactones (AHLs), while Gram-positive bacteria communicate via autoinducer peptides (AIPs). There are also QS compounds used for inter-species

communication (Maha Swetha et al., 2024; Vashistha et al., 2023; Wu & Luo 2021; Zhang et al., 2024).

Pseudomonas aeruginosa (*P. aeruginosa*), a multidrug-resistant Gram-negative bacterium, has three major QS signal molecules: *N*-3-oxododecanoyl-L-homoserine lactone (**1**, the Las system), *N*-butyryl-L-homoserine lactone (**2**, the Rhl system), and 2-heptyl-3-hydroxy-4-quinolone (**3**, the *Pseudomonas* quinolone signal system) (Curran et al., 2018; Papenfort & Bassler 2016; Yashkin et al., 2021) (Figure 1.).

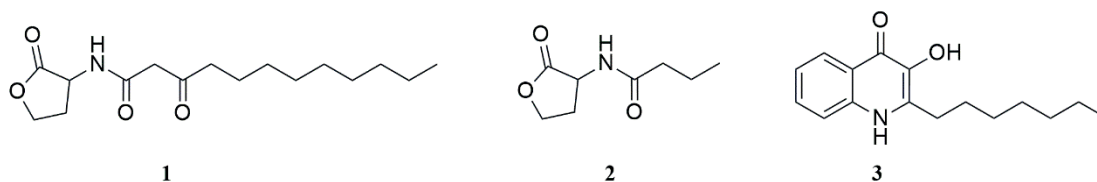


Figure 1. QS signal molecules of *Pseudomonas aeruginosa* (1-3)

Recent studies have shown that inhibition of the QS system can effectively regulate bacterial populations and suppress the expression of virulence factors. This strategy presents a novel and promising avenue for the development of antibacterial agents. Over the past two decades, a wide variety of QS inhibitors have been developed to combat bacterial infections. Notably, most research has focused on Gram-nega-

tive bacteria, especially *P. aeruginosa*, and targeting acyl-homoserine lactone (AHL)-mediated signaling pathways and AHL-related furanones as well as their bioisosteric heterocyclic compounds (**4-13**) (Boursier et al., 2019; Estephane et al., 2008; Goh et al., 2015; Jiang et al., 2020; Li et al., 2018a; Lizarme-Salas et al., 2021; Maiga et al., 2025; Yan et al., 2024; Zhang et al., 2023) (Figure 2.).

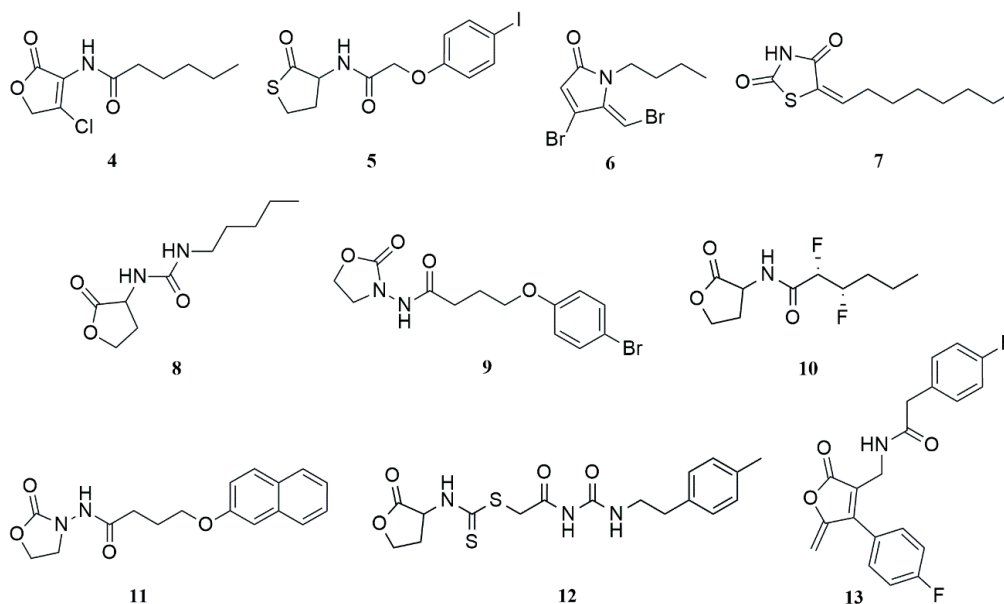


Figure 2. Examples of QS inhibitors

In our previous studies, some *N*-acylbenzoxazolone (14) and *N*-acylbenzimidazolone (15) derivatives were identified as QS inhibitors. In particular, core rings and short-chain acyl derivatives exhibited QS inhibitory activity (Cevizci et al., 2015; Miandji et al., 2012; Önem et al., 2018). As a continuation of this

research, benzothiazolone, which is the bioisostere of benzoxazolone and benzimidazolone heterocyclic compounds, was selected. In the present study, the synthesis of benzothiazolone and *N*-substituted benzothiazolone derivatives (Q1–Q10) and their QS inhibitory activities are presented (Figure 3.).

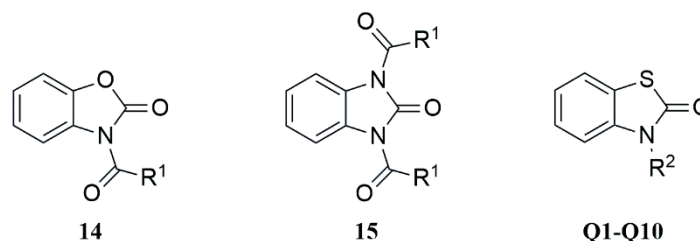


Figure 3. General structures of *N*-acylbenzoxazolones, *N*-acylbenzimidazolones, and *N*-substituted benzothiazolones. R¹: alkyl; R²: H, NH₂, alkyl, acyl

MATERIALS AND METHODS

Chemistry

All reagents and solvents were obtained from commercial suppliers and used without further purification. Melting points were determined using a Schmelzpunkt SMP-II Digital Melting Point Apparatus and are reported uncorrected. ¹H-NMR and ¹³C-NMR spectra were recorded using a Bruker Avance Neo 500 MHz High-Performance Digital FT-NMR spectrometer at the Gazi University Basic and Engineering Sciences Central Laboratory Application and Research Center (GUTMAM). HRMS spectra were obtained using a Waters LCT Premier XE orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer with ESI(+) ionization method. FT-IR spectra were acquired using a Perkin Elmer Spectrum 400 FTIR-ATR spectrometer. Compounds Q1, Q2, Q4, Q5, Q7–Q10 were previously reported (Ashurov et al., 2018; Carato et al., 2004; Dünder et al., 2007; Erdogan et al., 2021; Guenadil et al., 2011; Önkol et al., 2012).

3-Amino-1,3-benzothiazol-2(3*H*)-one (Q3).

6 mmol 1,3-benzothiazol-2(3*H*)-one was dissolved in 5 mL of ethanol, and 0.6 g of NaOH solution in 5 mL of water was added. Then, 6 mmol hydroxylamine-O-sulfonic acid was added portionwise to the

solution. After stirring for 3 h, the resulting precipitate was filtered, washed with water, and dried. The product was obtained in 0.35 g yield (35%). Mp: 133–135 °C. HRMS calc. for C₇H₆N₂OS [M+H]; 167.0279, found: 167.0279. ¹H-NMR (500 MHz, DMSO-*d*₆) δ = 7.61 (1H, d, *J*=7.5 Hz, Ar-H7), 7.32–7.40 (2H, m, Ar-H4,5), 7.18 (1H, t, *J*=7.5 Hz, Ar-H6), 5.62 (2H, s, NH₂) ppm. ¹³C-NMR (125 MHz, DMSO-*d*₆) δ = 111.9 (Ar-C), 118.7 (Ar-C), 123.2 (Ar-C), 123.4 (Ar-C), 127.0 (Ar-C), 138.6 (Ar-C), 168.5 (C=O) ppm. FT-IR: ν̃ = 3318–3191 (NH₂), 1624 (C=O) cm⁻¹.

3-Butanoyl-1,3-benzothiazol-2(3*H*)-one (Q6). 6 mmol 1,3-benzothiazol-2(3*H*)-one was dissolved in 25 mL of acetone, and a solution of 0.4 g of NaOH in 3 mL of water was added. Then, 7.5 mmol butanoyl chloride was added to the solution and stirred at room temperature for 30 min. At the end of the reaction, water was added to the reaction, and the resulting precipitate was filtered, washed with water, dried, and crystallized with ethanol. The product was obtained in 0.70 g yield (52%). Mp: 91 °C. HRMS calc. for C₁₁H₁₁NO₂S [M+H]; 222.0589, found: 222.0595. ¹H-NMR (500 MHz, DMSO-*d*₆) δ = 8.17 (1H, d, *J*=8.4 Hz, Ar-H4), 7.69 (1H, d, *J*=7.7 Hz, Ar-H7), 7.39 (1H, t, *J*=7.8 Hz, Ar-H5), 7.32 (1H, t, *J*=7.5 Hz, Ar-H6), 3.04 (2H, t, *J*=7.3 Hz, -CH₂), 1.64–1.71 (2H, m, -CH₂), 0.96 (3H, t, *J*=7.4 Hz, -CH₃) ppm. ¹³C-NMR (125 MHz, DMSO-*d*₆)

δ = 13.8 (CH₃), 17.7 (CH₂), 40.5 (CH₂), 117.5 (Ar-C), 121.8 (Ar-C), 123.1 (Ar-C), 125.8 (Ar-C), 127.4 (Ar-C), 135.1 (Ar-C), 170.9 (C=O), 174.3 (C=O) ppm. FT-IR: $\tilde{\nu}$ = 2964 (aliphatic CH), 1697 (C=O), 1683 (C=O) cm⁻¹.

Biological activity

Bacterial strains and culture media

The microorganisms used in this study included *Staphylococcus aureus* (*S. aureus*) ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, vancomycin-resistant *Enterococcus faecalis* (*E. coli faecalis*) (VRE) ATCC 51299, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 13883, *P. aeruginosa* PAO1, and *Chromobacterium violaceum* (*C. violaceum*) CV12472.

Determination of antimicrobial activity (minimum inhibitory concentration)

Antimicrobial activity of the chemicals was determined by the broth microdilution method against the tested strains according to the Clinical Laboratory and Standards Institute Guidelines (CLSI, 2020). Serial dilutions of the chemicals, prepared from DMSO stock solutions, were made in Mueller-Hinton broth at concentrations ranging from 0.5 to 64 μ M in 96-well microtiter plates. Azithromycin (Sigma) was used as a positive antibiotic control. Microbial inocula were prepared from a 24 h fresh culture, suspensions in physiological saline solution were adjusted to 0.5 McFarland standard turbidity (1.5×10^8 cfu mL⁻¹), and were diluted 10⁻¹ in physiological saline solution. 5 μ L of the diluted microbial suspension was added to each well. Plates were then incubated for 24 h at 37 °C. After incubation, the minimum inhibitory concentration (MIC) values of the chemicals were determined by the absence of visible turbidity and confirmed by subculturing 10 μ L from non-turbid wells onto an appropriate growth medium. All assays were performed at least three times, and the mean MIC values were selected (Aydın, 2021).

Anti-quorum sensing activity

Screening for violacein production

Agar well diffusion assay was performed with *C. violaceum* ATCC 12472 for the determination of pigment inhibition activity of the synthesized compounds. The bacterial strain was incubated in LB broth at 30 °C for 24 h, and 1% active culture was added to warm LB soft agar (LB Broth containing 0.5% agar) and poured onto LB hard agar. After solidification, 6 mm diameter wells were created, and 50 μ L of each chemical at the appropriate sub-MIC concentration was added. Plates were incubated at 30 °C for 48 h, and the diameter of the zone of violacein pigment inhibition was measured and compared with the control groups. The solvent was used as the negative control, and azithromycin served as the positive control (Patel et al., 2022).

Screening for biofilm inhibition and eradication

The anti-biofilm activity of the synthesized compounds against *P. aeruginosa* PAO1 was evaluated using the crystal violet staining assay. Briefly, 10 μ L of overnight bacterial growth (adjusted to 0.5 McFarland standard) was added to LB broth supplemented with 1% (w/v) glucose, containing sub-MIC concentrations of the test compounds in a dose-dependent manner. The final volume in each well of a 96-well microtiter plate was 200 μ L. Plates were incubated at 37 °C for 24 h to allow biofilm formation. Following incubation, planktonic cells were carefully removed by washing the wells three times with sterile distilled water. The remaining surface-adherent biofilm was fixed with 200 μ L of methanol for 15 min. After discarding the methanol and air-drying the wells, the biofilms were stained with 0.1% (w/v) crystal violet solution for 15 min. Excess stain was removed by washing with sterile distilled water, and the bound dye was then solubilized with 95% ethanol. The absorbance was measured at 570 nm using an ELISA plate reader to quantify biofilm biomass. Each assay was performed in quadruplicate. Wells containing only bacterial culture and medium served as the growth control (Venkatramanan et al., 2020).

The percentage of biofilm inhibition was calculated using the following formula:

$$\text{Biofilm inhibition (\%)} = [(\text{OD growth control} - \text{OD sample}) / \text{OD growth control}] \times 100.$$

The biofilm eradication assay was performed with slight modifications to the biofilm inhibition method described above. Biofilms were first allowed to form in 96-well microtiter plates as described in the biofilm inhibition method. After this initial incubation period, the contents of the wells were carefully discarded. Aliquots of the prepared chemical solutions were added to the microtiter plate wells together with LB broth to give final sub-MIC concentrations per 200 μL well volume. Sterile LB broth was used as a negative control at a 200 μL well volume. The plates were then incubated at 37 °C for 24 h. Following the second incubation period, the plates were prepared for absorbance measurement as described in the biofilm inhibition method (Hamid, 2019). The results were expressed as percentage inhibition using the following equation:

$$\% \text{ Inhibition} = [(\text{Absorbance}(\text{sample}) - \text{Absorbance}(\text{control})) / \text{Absorbance}(\text{control})] \times 100$$

Inhibition of proteolytic activity

The effect of chemicals on the proteolytic activity of the *P. aeruginosa* PAO1 was evaluated using the well-diffusion method on skim milk agar. Agar plates were punched with 6 mm diameter wells and filled with 50 μL of 0.5 McFarland standard turbidity (1.5×10^8 cfu mL^{-1}) of *P. aeruginosa* PAO1 strain and sub-MIC concentrations of the chemicals. Plates were incubated at 37 °C for 24 h and 48 h. Distilled water was used as the negative control. Protease inhibitory activity was estimated by measuring the clear zone around the wells (Kumar et al., 2022; Siriken et al., 2021). The percentage of protease inhibition was calculated using the following formula: $[(\text{Zone diameter}$

of control – Zone diameter of sample) / Zone diameter of control] $\times 100$.

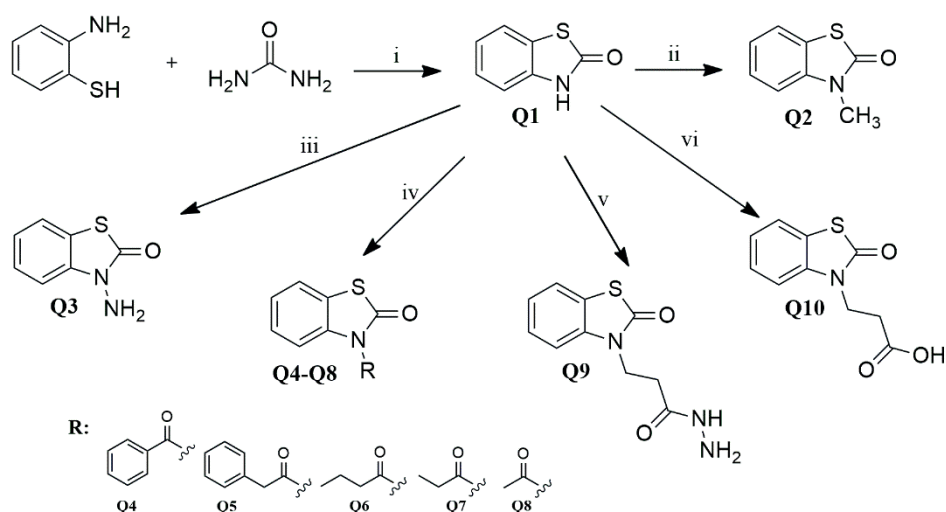
Inhibition of swimming and swarming motility

Swimming and swarming motility inhibition assays were conducted using a slightly modified version of the method described by Li et al. (2018b). Briefly, 5 μL of an overnight culture of *P. aeruginosa* PAO1 (1.5×10^8 cfu mL^{-1}) was inoculated at the center of swimming (1% tryptone, 0.5% NaCl, and 0.3% agar) and swarming (1% peptone, 0.5% NaCl, 0.5% agar, and 0.5% filter-sterilized d-glucose) plates containing sub-MIC concentrations of the chemicals. Plates were incubated at 30 °C for 48 h, and the diameters of the motility zones were measured. The percentage reduction in swimming and swarming motility was calculated using the following formula: $[(\text{Zone diameter of control} - \text{Zone diameter of sample}) / \text{Zone diameter of control}] \times 100$.

RESULTS AND DISCUSSION

Chemistry

The benzothiazolone core (Q1) was synthesized via the condensation of urea and 2-aminothiophenol. The *N*-methyl derivative (Q2) was prepared by reaction with dimethyl sulfate (DMS), and the *N*-NH₂ derivative (Q3) was obtained by reaction with hydroxylamine-O-sulfonic acid (HOSA). *N*-acyl derivatives (Q4-Q8) were synthesized by treating benzothiazolone with the corresponding acyl halides in aqueous sodium hydroxide. For the propanhydrazide (Q9) and propanoic acid (Q10) derivatives, the methyl propanoate intermediate was first prepared, followed by Michael addition with benzothiazolone and methyl acrylate. Subsequently, Q9 was obtained via reaction of the ester with hydrazine hydrate, while Q10 was obtained through ester hydrolysis using concentrated HCl (Scheme 1.).



Scheme 1. Synthesis of compounds Q1-Q10. Reagents and conditions. (i) 180 °C; (ii) DMS, NaOH, water; (iii) HOSA, NaOH, water/ethanol. (iv) Appropriate acyl halide, NaOH, water/acetone; (v) a. methyl acrylate, TEA b. $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, ethanol; (vi) a. methyl acrylate, TEA, b. const. HCl.

Biological activity

Inhibiting intercellular bacterial communication and preventing the production of virulence factors or biofilm formation represents an alternative therapeutic strategy to combat bacterial infections caused by antibiotic-resistant bacterial strains. In this study, we evaluated the anti-virulence potential of a series of synthesized benzothiazolone derivatives against *C. violaceum* ATCC 12472 and *P. aeruginosa* PAO1, two well-characterized Gram-negative pathogens with QS-regulated virulence phenotypes.

First of all, the antibacterial activities of the synthesized compounds were evaluated against a panel of bacterial strains, including *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, vancomycin-resistant *E. faecalis* (VRE) ATCC 51299, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *P. aeruginosa* PAO1, and *C. violaceum* CV12472. MIC assays revealed that the compounds demonstrated weak

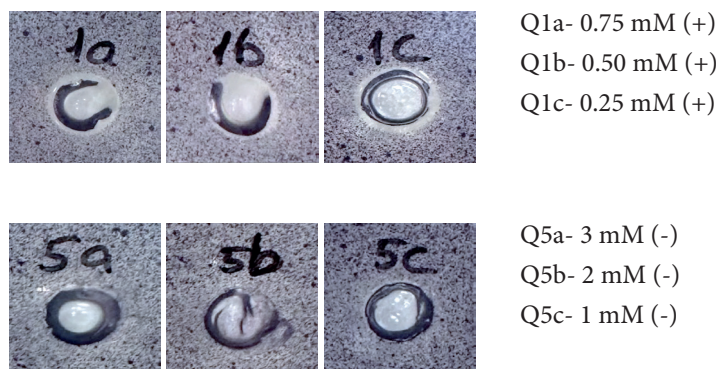
antimicrobial activity against the tested strains. The most active compound against *C. violaceum* ATCC 12472 was benzothiazolone (Q1), with an MIC value of 1 mM, whereas Q1, its *N*-methyl derivative (Q2), and *N*-acetyl derivative (Q8) were effective against *P. aeruginosa* PAO1 at an MIC value of 4 mM (Table 1.).

An agar well diffusion assay was performed using *C. violaceum* ATCC 12472 to evaluate the anti-QS activity of the synthesized compounds through pigment inhibition. The QS inhibition assay revealed that most of the tested compounds reduced violacein pigment production at sub-MIC concentrations, supporting their potential as quorum sensing inhibitors (QSIs). Among them, Q1 was the most potent, showing pronounced QS inhibition at 0.25 mM concentration. Moreover, Q8 and *N*-propanoic acid derivative (Q10) demonstrated effective inhibition at ≥ 0.5 mM. In contrast, the *N*-phenacetyl derivative (Q5) was inactive at sub-MIC concentrations ≤ 3 mM (Table 1. and Figure 4.).

Table 1. Minimum Inhibitory Concentration (MIC) activity and pigment inhibition of the synthesized compounds

Comp.	A	B	C	D	E	F	G	Pigment inhibition
Q1	8 mM	16mM	16mM	4mM	8 mM	4mM	1 mM	≥0.25 mM
Q2	>4 mM	>4mM	>4mM	4mM	>4mM	4mM	2 mM	≥1 mM
Q3	16mM	>16mM	>16mM	>16mM	>16mM	16mM	4 mM	≥1 mM
Q4	16mM	>16mM	>16mM	>16mM	16mM	16mM	8 mM	≥4 mM
Q5	4mM	8mM	8mM	4mM	16mM	8mM	4 mM	-
Q6	>16mM	>16mM	>16mM	>16mM	>16mM	16mM	8 mM	≥4 mM
Q7	4mM	8mM	4mM	8mM	<8mM	8mM	4 mM	≥3 mM
Q8	16mM	16mM	>16mM	8mM	16mM	4mM	2 mM	≥0.5 mM
Q9	>32mM	>32mM	>32mM	>32mM	>32mM	32mM	8 mM	≥7 mM
Q10	2mM	8mM	8mM	4mM	8mM	8mM	2 mM	≥0.5 mM

A: *S. aureus* ATCC 25923, B: Methicillin-resistant *S. aureus* (MRSA) ATCC 43300, C: Vancomycin-resistant *E. faecalis* (VRE) ATCC 51299, D: *E. coli* ATCC 25922, E: *K. pneumoniae* ATCC 13883; F: *P. aeruginosa* PAO1, G: *C. violaceum* CV12472.

**Figure 4.** Representative examples of the pigment inhibition assay.

The crystal violet staining assay was used to determine biofilm inhibition and eradication activities of the synthesized compounds against *P. aeruginosa* PAO1. All compounds were tested at three sub-MIC concentrations (Table 2). Among the tested derivatives, Q1, Q2, Q5, Q7, and particularly Q8 exhibited promising biofilm inhibition and eradication activities. Analysis of intra- and inter-group results revealed that Q8 was the most effective in inhibiting biofilm formation, achieving 85.15% inhibition at 1 mM concentration. In the biofilm eradication assay, Q1 and *N*-methyl derivative Q2 displayed activity, eradicating over 80% of pre-formed biofilms at 2 mM. This is

particularly noteworthy since the removal of mature biofilms is often more difficult than preventing their formation. These results suggest that the compounds not only interfere with initial biofilm development but may also disrupt established biofilm structures.

The effects of the synthesized compounds on the proteolytic activity of *P. aeruginosa* PAO1 were assessed using the well-diffusion method on skim milk agar. Among the tested compounds, Q2 exhibited 12.80% inhibition at 3 mM, and Q3 showed 10.34% inhibition at 15 mM concentration. The remaining compounds displayed less than 10% inhibition at the sub-MIC concentrations tested.

Table 2. % Biofilm inhibition and eradication of the synthesized compounds at sub-MIC values

Comp.	<i>P. aeruginosa</i> PAO1 MIC	Biofilm inhibition/ Biofilm eradication (%)		
		sub-MIC values (mM)		
		3 mM	2 mM	1 mM
Q1	4mM	91.04±0.54 / 86.89±3.48	69.38±4.51 / 80.78±4.11	19.77±1.03 / ni
Q2	4mM	80.57±3.61 / 83.88±3.26	45.08±2.62 / 81.69±5.67	40.44±2.49 / ni
Q8	4mM	90.97±1.05 / 75.09±7.54	90.79±0.65 / 70.48±2.05	85.15±3.57 / ni
		7 mM	6 mM	4 mM
Q5	8mM	90.52±0.49 / 81.49±6.22	90.07±0.61 / 78.07±5.59	89.63±0.27 / 70.17±11.8
Q7	8mM	90.52±0.70 / 80.56±3.22	89.66±1.89 / 72.85±2.89	88.08±2.60 / ni
Q10	8mM	83.52±5.29 / 49.13±1.62	87.03±4.63 / 54.11±7.95	ni / ni
		15 mM	10 mM	5 mM
Q3	16mM	91.50±0.70 / 66.58±5.65	88.98±3.67 / 56.63±7.47	85.36±5.74 / 40.69±5.46
Q4	16mM	90.30±1.53 / ni	89.24±1.93 / ni	ni / ni
Q6	16mM	89.24±0.04 / 52.44±3.35	85.76±3.49 / ni	71.09±0.04 / ni
		30 mM	20 mM	10 mM
Q9	32mM	87.68±4.96 / 77.28±2.32	86.59±4.43 / 88.98±0.05	88.68±3.24 / 68.60±2.52

The motility assays provided further insight into the anti-virulence profiles of the compounds. Swimming and swarming motility inhibition assays were conducted using *P. aeruginosa* PAO1 (Figure 5). All compounds demonstrated inhibitory effects on both types of motility to varying degrees. Notably, swarming motility was more significantly inhibited (36.70%-67.40%) compared to swimming motility (12.53%-26.76%) across all tested compounds. Among these,

Q6 (*N*-butanoyl derivative) exhibited the highest inhibitory activity, with 67.40% swarming and 15.49% swimming inhibition. Similarly, Q5 (*N*-phenylacetyl derivative) showed high swarming inhibition (60.44%). Q1, which was the most active compound in the violacein pigment inhibition assay and also a potent biofilm eradicator, inhibited swarming by over 60%. In addition, Q1 and Q10 inhibited swimming motility by more than 25%.

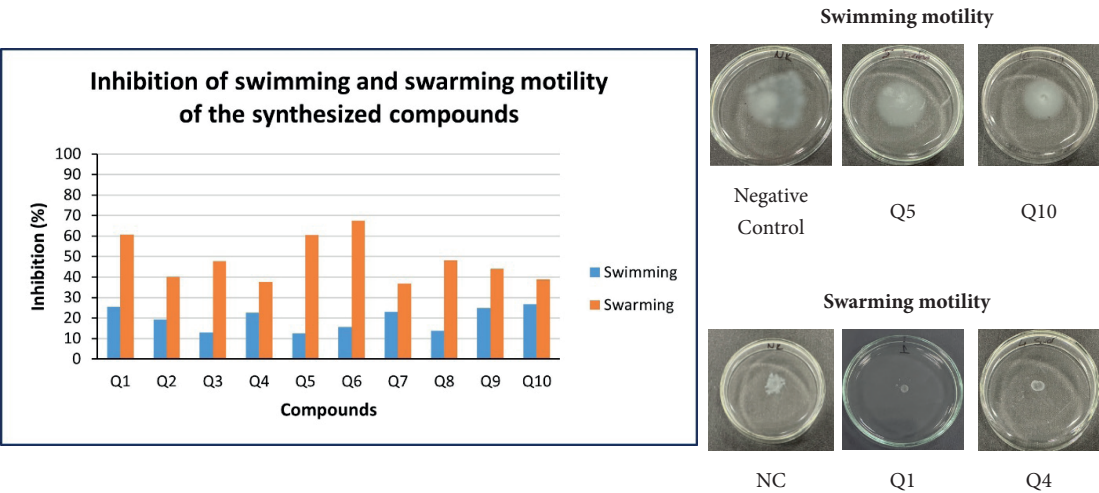


Figure 5. Percentage inhibition of swimming and swarming motility of the synthesized compounds at 1 mM, with representative plate images.

CONCLUSION

In summary, the synthesized benzothiazolone derivatives exhibited promising anti-virulence properties against *C. violaceum* ATCC 12472 and *P. aeruginosa* PAO1, targeting QS, biofilm formation, and motility without exerting strong bactericidal effects. The benzothiazolone core (Q1), *N*-acetyl derivative (Q8), and *N*-propanoic acid derivative (Q10) significantly inhibited violacein production at sub-MIC concentrations, indicating their potential as quorum sensing inhibitors and in agreement with our previous studies (Cevizci et al., 2015; Miandji et al., 2012; Önem et al., 2018). Crystal violet biofilm assays further confirmed the anti-biofilm potential of these compounds: Q8 demonstrated the highest inhibition of biofilm formation (85.15% at 1 mM), while Q1 and *N*-methyl derivative Q2 were particularly effective in eradicating mature biofilms (over 80% at 2 mM). Motility assays showed that all compounds interfered with swimming and swarming behaviors to varying degrees, with swarming motility being more susceptible. Q6 (*N*-butanoyl derivative) exhibited the highest swarming inhibition (67.40%), while Q1 and Q10 also inhibited swimming motility (>25%). These findings demonstrate that several of the synthesized compounds exhibit multi-targeted anti-virulence activity at sub-MIC levels. Thus, benzothiazolones may serve as valuable scaffolds for the development of novel therapeutics that target bacterial communication and virulence, a promising approach to combat antibiotic-resistant infections.

AUTHOR CONTRIBUTION STATEMENT

Design, synthesis and structure elucidation of the title compounds (ÖKÖ and YD), biological activity (MBK and EB). The manuscript preparation and editing (ÖKÖ, YD, MBK, and EB).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Antibiofilm Potential of Ceragenins Against Vancomycin-Resistant *Enterococcus* spp.

Müzeyyen AYDIN^{*}, Fatimanur OLCAY^{**}, Mayram HACIOĞLU^{***},
Gözde HASBAL-ÇELİKOK^{****}, Neşe İNAN^{****}, Paul B. SAVAGE^{*****}

Antibiofilm Potential of Ceragenins Against Vancomycin-Resistant *Enterococcus* spp.

SUMMARY

Enterococcus spp., especially vancomycin-resistant enterococci (VRE), are listed by the World Health Organization as priority pathogens requiring new antimicrobial treatments. Ceragenins (CSAs) are a novel class of broad-spectrum antimicrobials that are relatively easy and cost-effective to produce. This study evaluated the antibiofilm effects, anti quorum-sensing (QS), and antioxidant capacities of ceragenins (CSA-44, CSA-90, CSA-192) and linezolid against VRE isolates from various clinical samples. CSA-44 and CSA-192 most effectively inhibited initial adhesion and biofilm formation. For mature biofilms, CSA-90 showed the greatest inhibition (~66%) while CSA-44 and CSA-192 reached ~50%. Linezolid inhibited biofilms by 39–60%, depending on concentration. No anti-QS or antioxidant activity was observed for ceragenins at the tested levels. These findings suggest ceragenins have strong potential as therapeutic agents against VRE, particularly in biofilm-related infections. Further *in vivo* research is needed to confirm their efficacy and safety for clinical use.

Keywords: Biofilm, cationic antimicrobial peptides, *Enterococcus*, linezolid, vancomycin resistance.

Vankomisine Dirençli Enterokok Türlerine Karşı Cerageninlerin Antibiyofilim Etkinliği

ÖZ

Enterococcus spp., özellikle vankomisine dirençli enterokoklar (VRE), Dünya Sağlık Örgütü tarafından yeni antimikrobiyal tedaviler gerektiren öncelikli patojenler olarak belirlenmiştir. Cerageninler (CSA'lar), üretimi nispeten kolay ve uygun maliyetli yeni bir geniş spektrumlu antimikrobiyal sınıftır. Bu çalışmada, çeşitli klinik örneklerden izole edilen VRE'lere karşı cerageninlerin (CSA-44, CSA-90, CSA-192) ve linezolidin antibiyofilim, anti quorum-sensing (QS) ve antioksidan etkileri değerlendirilmiştir. CSA-44 ve CSA-192, biyofilmi yapışma ve oluşum aşamasında en etkili şekilde inhibe etmişlerdir. Olgun biyofilmler için CSA-90 en yüksek inhibisyon oranını (~%66) gösterirken, CSA-44 ve CSA-192 ~%50'ye varan oranlarda inhibisyon göstermiştir. Linezolid, konsantrasyona bağlı olarak biyofilmleri %39-60 oranında inhibe etmiştir. Çalışılan konsantrasyonlarda cerageninler için herhangi bir anti-QS veya antioksidan aktivite gözlemlenmemiştir. Bu bulgular, cerageninlerin özellikle biyofilim ile ilişkili enfeksiyonlarda VRE'ye karşı terapötik ajanlar olarak güçlü bir potansiyele sahip olduğunu göstermektedir. Klinik kullanım için etkinlik ve güvenilirliklerini doğrulamak amacıyla daha fazla *in vivo* araştırma yapılması gerekmektedir.

Anahtar Kelimeler: Biyofilim, katyonik antimikrobiyal peptitler, *Enterococcus*, linezolid, vankomisin direnci.

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^{*} ORCID: 0000-0002-8082-9470, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, İstanbul University, İstanbul, Türkiye.

^{**} ORCID: 0000-0001-8442-8538, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, İstanbul University, İstanbul, Türkiye.

^{***} ORCID: 0000-0003-0823-631X, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, İstanbul University, İstanbul, Türkiye.

^{****} ORCID: 0000-0002-0216-7635, Department of Biochemistry, Faculty of Pharmacy, İstanbul University, İstanbul, Türkiye.

^{*****} ORCID: 0000-0002-1559-6244, Dr. Abdurrahman Yurtaslan Ankara Oncology Hospital, Medical Microbiology Laboratory, Ankara, Türkiye.

^{*****} ORCID: 0000-0002-4642-6109, Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA.

INTRODUCTION

Enterococcus spp. are Gram-positive bacteria found in soil, surface water, seawater, plants, and fermented foods. *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are the dominant species in this genus. This facultative anaerobic genus is highly tolerant to a wide range of environmental conditions, including extremes of pH, temperature, and salt concentration. This tolerance contributes to their colonisation and persistence in various environments. They are primarily commensals in the gastrointestinal tract but can cause a range of infections. The main infections they cause include bacteremia, endocarditis, and urinary tract infections. In addition to their potential to cause infections, enterococci are also characterised by their resistance to many antibiotics. They show both intrinsic and acquired resistance to many antimicrobials used to treat Gram-positive bacterial infections, particularly vancomycin (Agudelo Higueta & Huycke, 2014; Ch'ng et al., 2019; Garcia-Solache & Rice, 2019).

In 2019 and 2021, 1.27 and 1.14 million deaths, respectively, were known to be directly related to antibiotic-resistant bacteria, and 4.71-4.95 million deaths annually were indirectly related to bacterial antimicrobial resistance. Due to limited treatment options, bacteraemia caused by vancomycin-resistant enterococci (VRE) leads to higher morbidity and mortality than bacteraemia caused by vancomycin-sensitive enterococci. In 2019, *E. faecium* and *E. faecalis* were responsible for 100,000 to 250,000 of these deaths, with vancomycin-resistant strains accounting for a high percentage (Humphreys, 2014; Naghavi et al., 2024). VRE has been recognized as a global public health problem by the World Health Organization (WHO), US Centers for Disease Control and Prevention (CDC), and European Centre for Disease Prevention and Control (ECDC). In recent years, the epidemiology of VRE has changed rapidly and markedly, and an increase in vancomycin resistance, particularly in *E. faecium*, has been observed in many countries.

Linezolid, daptomycin and tigecycline are antibiotics of last resort in the treatment of VRE infections. However, resistance to these antibiotics has also been reported (CDC, 2019; Dadashi et al., 2021; ECDC, 2023; Tacconelli et al., 2018).

It is a global health priority to combat antibiotic resistance in enterococci. Although *E. faecalis* forms thicker biofilms that confer resistance to antibiotics, *E. faecium* is intrinsically more resistant to antibiotics, especially in biofilm form. The underlying mechanism for the emergence of antibiotic resistance to biofilms has not yet been determined, but it may be due to high levels of matrix production in mature biofilms and thus low penetration of antibiotics, or to an increase in antibiotic-tolerant cells, as in other bacterial species (Komiya et al., 2016; Soares et al., 2014). Biofilms are loosely defined as clusters of bacteria in a matrix of their own production. Many species of bacteria can adhere to surfaces and form biofilms. Although these biofilms are found in natural and industrial environments, they can cause resistant infections, particularly in hospitals. *Enterococcus* spp. are one of the common causes of opportunistic infections associated with biofilms. They are responsible for 25% of catheter-associated urinary tract infections and are often found in wounds. They are also increasingly being reported in cases of infectious endocarditis, all of which are associated with biofilms. Enterococcal biofilms are inherently tolerant to antimicrobial agents and therefore represent a serious obstacle to the treatment of infections. In addition to being difficult to eradicate, biofilm-associated enterococcal infections are a source of bacterial dissemination and a reservoir for antibiotic resistance genes (Ch'ng et al., 2019; Stoodley et al., 2002).

Within biofilms, microorganisms modify their behaviours when they detect that their population concentration has reached a certain level, then they begin to communicate through small signalling molecules. This mechanism is known as quorum sensing (QS) and is used to regulate genes associated with vir-

ulence, biofilm formation, antibiotic resistance, etc. Numerous natural and synthetic compounds can behave as anti-QS molecules by targeting QS systems to control biofilms (Shrestha et al., 2022).

As a result of the increase in antibiotic resistance, especially in recent years, the need for new antimicrobial agents is on the rise. Antimicrobial peptides (AMPs) play an important role in the innate immune system and have been investigated for their use in treating infectious diseases. However, their susceptibility to protease degradation, difficulty in large-scale production, and relatively high cost are among the difficulties encountered. Ceragenins (CSAs) are a group of antimicrobial agents that mimic the mechanisms of action of AMPs while eliminating these disadvantages. They are relatively easy to produce on a large scale and are not substrates for proteases as they are not peptide-based. They cause membrane depolarisation by forming transient pores in the membrane and cause cell death. In addition to their broad-spectrum antibacterial effects, they have strong antibiofilm activity. In previous studies, ceragenins were found to be effective against resistant Gram-positive bacteria, and these bacteria did not readily develop resistance to these molecules even after prolonged exposure. These findings suggest that ceragenins may be effective in the treatment of infections caused by drug-resistant *Enterococcus* spp. (Epand et al., 2007; Hacıoğlu et al., 2023). The aim of the present work is to investigate the *in vitro* antibiofilm, anti-QS, and antioxidant activities of ceragenins (CSA-44, CSA-90, and CSA-192) and linezolid against VRE isolates.

MATERIALS AND METHODS

Bacterial strains

A collection of 17 VRE isolated from various clinical specimens, including blood (n=9), urine (n=4), rectal swab (n=3), and vaginal fluid (n=1) was submitted to the Synevo Laboratories Ankara Central Laboratory in Türkiye. *E. faecalis* ATCC 29212 was used as a quality control strain.

Media and solutions

Before all experiments, each isolate was cultured on tryptic soy agar (Difco Laboratories, Detroit, MI, USA). Brain-heart infusion broth (BHIB, Thermo Scientific, Oxoid, USA) was used for the biofilm assays.

Antimicrobial agents

CSA-44, CSA-90, and CSA-192 were synthesized from a cholic acid scaffold method as described previously (Dao et al., 2020; Lai et al., 2008). Linezolid was kindly provided by its manufacturer, Koçak Farma İlaç ve Kimya Sanayi A.Ş. Stock solutions of ceragenins and linezolid were prepared from dry powders in distilled water. Frozen solutions of antimicrobial agents were used within six months.

Biofilm attachment assay

Biofilm attachment assay was performed using a previously described method with some modifications. Overnight cultures of isolates were prepared to a cell density equivalent to 1×10^6 cfu/mL as described above. Clinical isolates of VRE were added to each well of 96-well tissue culture microtiter plates containing 1/10x MIC of CSAs and linezolid, according to the MIC values determined in a previous study conducted by our working group, and the MIC results are presented in the supplementary material (Hacıoğlu et al., 2023). A positive control without antimicrobials and a negative control without cells were also prepared. Plates were incubated at 37°C for 1, 2, and 4 hours. After incubation, the wells were washed twice with physiologically buffered saline (PBS) and measured spectrophotometrically at OD 450 nm on a microplate reader (EON, Biotek, USA) (Hacıoğlu et al., 2021).

Inhibition of biofilm formation

Clinical isolates of VRE (1×10^6 cfu/ml) were added to each well of 96-well tissue culture microtiter plates containing 1x MIC, 1/10x MIC, and 1/100x MIC of CSAs and linezolid, positive control without antimicrobial, and negative control without cells. Plates were incubated at 37°C for 24 hours. At the end

of the incubation period, the wells were washed twice with PBS and measured spectrophotometrically at OD 450 nm using a microplate reader (EON, Biotek, USA) (Hacıoğlu et al., 2021).

Biofilm formation

Suspensions of 1×10^6 cfu/ml were prepared from the overnight cultures of VRE isolates by dilution in BHIB. The prepared bacterial suspensions were transferred to 96-well microtiter plates and incubated at 37°C for 24 hours to achieve biofilm formation.

Effects of ceragenins and linezolid on mature biofilms

Biofilms were formed as described above to evaluate the activities of ceragenins at concentrations between 256-16 µg/mL and linezolid 64-4 µg/mL, on 24-hour-old mature VRE biofilms. Metabolic activities of the biofilms were assessed using the standardized static microtiter plate model and measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay. After biofilm formation, the medium was removed using a multichannel micropipette, and the wells were washed with PBS. CSAs were added to the wells, and the plates were incubated at 37 °C for 24 hours after positive and negative controls were prepared. After incubation, the wells were washed again with PBS. A solution of MTT at a concentration of 5 mg/mL was prepared. MTT solution was added to the wells and the plates were incubated for 3 hours at

35°C in the dark. Formazan crystals formed at the end of the incubation were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and measured spectrophotometrically at OD 450 nm using a microplate reader (EON, Biotek, USA) and evaluated by comparison with the control (Sabaeifard et al., 2014).

Anti-QS assay

The anti-QS activity of ceragenins (50, 25, 12.5 µg/mL) was examined using the biosensor strain *Chromobacterium violaceum* (*C. violaceum*) ATCC 12472. The anti-QS activity was investigated by means of a disk diffusion assay in Luria–Bertani agar (Sigma-Aldrich, St. Louis, MO, USA) plates with the appearance of a colorless ring (Hamidi et al., 2020).

Antioxidant capacities

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH scavenging activities of the ceragenins were determined using a modified Brand-Williams method (Brand-Williams et al., 1995), as described by Hasbal et al. (2015). Specifically, a 10 µL aliquot of each sample solution was combined with 240 µL of a 0.1 mM DPPH solution prepared in methanol. The resulting mixtures were subjected to agitation and subsequently incubated in darkness at ambient temperature for a duration of 30 min. Following incubation, the reduction in absorbance was quantified at a wavelength of 517 nm. Quercetin served as the reference standard. The percentage of DPPH radical scavenging activity was then calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = 1 - \left(\frac{\text{Absorbance of the extract at 517nm}}{\text{Absorbance of the control at 517nm}} \right) \times 100$$

Ferric-reducing antioxidant power (FRAP) assay

The antioxidant capacities of the ceragenins were quantitatively assessed using a modified FRAP assay, as described by Hasbal et al. (2015), based on the methodology outlined by Benzie and Strain (1996). The FRAP reagent was freshly synthesized by combining 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution (in 40 mM hydrochloric acid), and 20 mM ferric chloride solution in

a volumetric ratio of 10:1:1. A 10 µL aliquot of each sample solution was combined with 20 µL of solvent, and the mixture was incubated at 37 °C for 10 min. Subsequently, 270 µL of FRAP reagent was added. Following a 4-minute incubation period, the increase in absorbance was measured at a wavelength of 593 nm. Quercetin was employed as the reference standard. The reducing capacity was expressed as FRAP values, represented as mM Fe²⁺ equivalents, derived from the

standard regression curve ($y=0.5815x-0.0043$, $R^2=1$) generated using ferrous sulfate heptahydrate ($\text{Fe-SO}_4 \cdot 7\text{H}_2\text{O}$) solutions within a concentration range of 0.1 to 1.5 mM.

RESULTS

Inhibition of adhesion

Among the ceragenins, CSA-44 and CSA-192

were the most effective, with adhesion inhibition rates of 18.18% and 20.91% after 1 h incubation, respectively, while CSA-90 was effective with an adhesion inhibition rate of 9.43% after 4 h incubation. It was found that linezolid was not as effective as ceragenins at 1 and 2 h. However, it was more effective than ceragenins after 4 h incubation with an inhibition rate of 11.18% (Figure 1.).

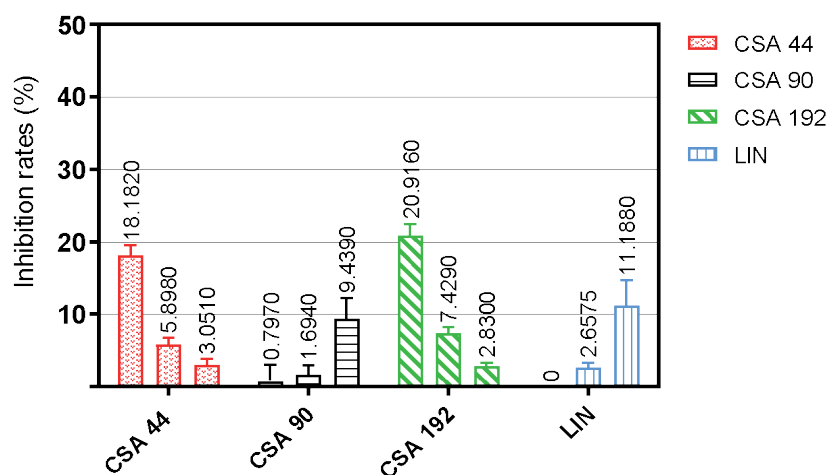


Figure 1. Inhibition of adhesion rates of antimicrobials after 1, 2, and 4 h incubation at MIC/10. The error bars indicate the standard deviations.

Inhibition of biofilm formation

Inhibition of biofilm formation rates depended on concentration; the highest inhibition rates were shown at MICs for all agents, as expected. CSA-44 and CSA-192 were the most efficient agents with inhibi-

tion rates of biofilm formation of 37.21 and 39.31 (%), respectively, at their respective MICs. Linezolid and CSA-90 showed similar inhibition rates at the MICs, while all three ceragenins were more effective than linezolid at the sub-MIC level. Results are shown in Figure 2.

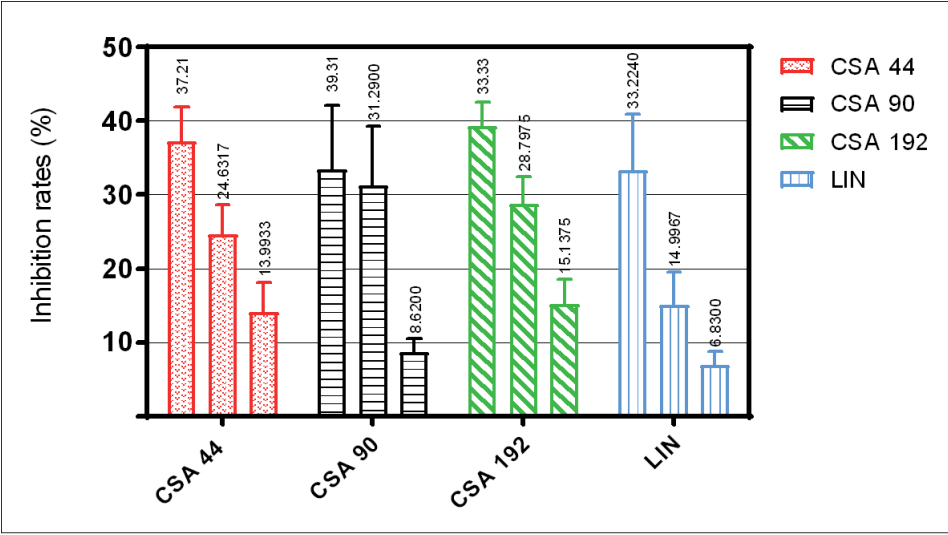


Figure 2. Inhibition of biofilm formation rates of antimicrobials at MIC, MIC/10, and MIC/100 concentrations, respectively. The error bars indicate the standard deviations.

Effects on mature biofilms

The results showed that CSA-44, CSA-90, and CSA-192 inhibited mature biofilms of VRE, at 16 µg/mL, by approximately 30, 39, and 20% respectively. In addition, at 32 µg/mL, CSA-44 and CSA-192 showed

an inhibition of almost 50%, while CSA-90 showed an inhibition of almost 66%. In line with expectations, the inhibition rates increased as the concentration of ceragenins increased. Depending on the concentration, linezolid showed an inhibition rate between 39% and 60% against VRE (Figure 3.).

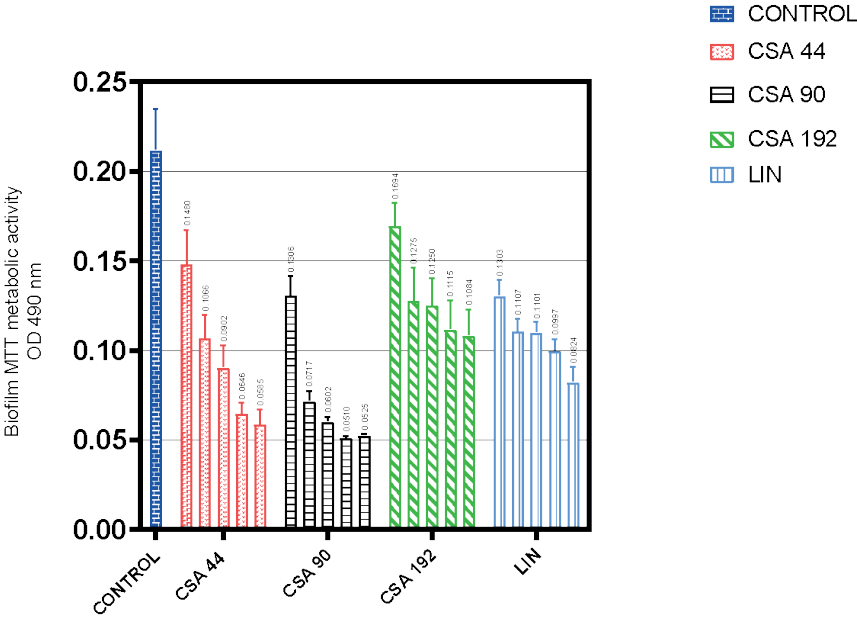


Figure 3. Effects of antimicrobials on mature biofilms, by the MTT method. Bars indicate 16, 32, 64, 128, and 256 µg/mL for ceragenins, 4, 8, 16, 32, and 64 µg/mL for linezolid. 256-16 and 32-2 µg/mL, on 24-hour-old mature VRE biofilms. The error bars indicate the standard deviations.

Anti QS effects of CSAs

In this study, the QS inhibition of *C. violaceum* 12472, a biosensor strain, by ceragenins was investigated using the disk diffusion method, and no QS inhibitory activity of ceragenins was detected at the concentrations studied.

Antioxidant effects of CSAs

The antioxidant capacities of the ceragenins were investigated using both DPPH radical scavenging activity and FRAP assays. For the DPPH radical scavenging activity assay, the samples were evaluated at concentrations of 100, 32, 16, 8, 4, 2, and 1 µg/mL. However, no detectable DPPH radical scavenging activity was observed across these concentrations. Similarly, under the tested concentrations, the ceragenins exhibited no ferric reducing antioxidant power, and FRAP values were undetectable. In contrast, quercetin, at a final concentration of 10 µg/mL, exhibited a DPPH radical scavenging activity of $87.437 \pm 0.175\%$. Furthermore, quercetin at a concentration of 125 µg/mL demonstrated a FRAP value of 2.312 ± 0.143 mM Fe²⁺ equivalents.

DISCUSSION

Enterococci, members of the gastrointestinal microbiota, are known to be opportunistic pathogens, particularly in critically ill and immunocompromised patient groups. Since the emergence of VRE in the late 1980s, clinicians have faced significant challenges in the management of infections caused by these pathogens. Although they have relatively low virulence potential, their inherent tolerance and ability to establish persistent adaptation to common broad-spectrum antimicrobials have led to enterococci becoming a leading cause of healthcare-associated infections. In addition, resistance continues to emerge against new antibiotics such as daptomycin and linezolid, which have good treatment efficacy (Miller et al., 2020; Weiner-Lastinger et al., 2020). Moreover, the increase in biofilm-associated infections, which are known to be highly resistant to antimicrobial agents, and limited treatment options, reveal the need for new antimicrobial agents.

Biofilm, the form in which bacteria can live in complex communities embedded in the polymeric matrix they form by adhering to surfaces, is relatively common. Enterococci have an enhanced ability to adhere and therefore to form biofilms. These characteristics generally make biofilm removal difficult. Due to the increased binding surface, formation characteristics, and the polymicrobial nature of the biofilm itself, removal of mature biofilms is generally more difficult. In addition, previous studies have shown that biofilms play a role in most nosocomial enterococcal infections (Weiner-Lastinger et al., 2020). In line with emerging areas of research focusing on the prevention of adherence and biofilm formation of microorganisms, in the present study, we investigated the inhibition of biofilm attachment and biofilm formation by CSA-44, CSA-90, CSA-192, and linezolid against clinical VRE isolates. We found that CSA-44 and CSA-192 were more efficient in inhibiting adhesion after 1 h incubation, whereas CSA-90 and linezolid were more efficient after 4 h incubation. CSA-44 and CSA-192 were found to be the most efficient agents with inhibition rates of biofilm formation (after 24 h incubation) of 37.21 and 39.31 (%), respectively, at the MIC, and all three ceragenins were more effective than linezolid at sub-MIC levels. CSA-192 was the most effective in inhibiting adhesion and biofilm formation. In the study by Tekintaş et al. (2024), inhibition of biofilm formation of VRE isolates by CSA-44 was assessed at MIC (2-4 µg/ml) and MIC/2 concentrations. Higher inhibition rates were observed at the MIC concentration, with the inhibition of biofilm formation rates in the range of 49-97 (%) (Tekintaş et al., 2024). Another study observed the antibiofilm activity of CSA-44 against *E. faecalis* and *C. albicans* and found that CSA-44 inhibited biofilm formation and decreased the amount of biofilm formed by these microorganisms on teeth and dental composite (Tokajuk et al., 2022).

Since mature enterococcal biofilms are inherently resistant to antimicrobial agents, there is a great need

for new treatment options, as current antimicrobial agents are often inadequate for the treatment of biofilm-associated infections. Therefore, in our study, we also evaluated ceragenins and linezolid at different concentrations against mature VRE biofilms. CSA-44 and CSA-192 showed an inhibition of almost 50%, while CSA-90 showed an inhibition of almost 66% at a concentration of 32 µg/mL. As expected, the inhibition rates increased with increasing concentration of ceragenin. Depending on the concentration, linezolid showed an inhibition rate between 39% and 60%. Several studies have demonstrated the potent antibiofilm activity of CSA-44 in the concentration range of 10–20 µg/ml against mature biofilms formed by *E. faecalis* (Czarnowski et al., 2024; Tokajuk et al., 2022).

Although previous studies have also shown that CSA-44 inhibited the adhesion and biofilm formation of various microorganisms, such as *Achromobacter spp.* (Damar-Çelik et al., 2021), *Pseudomonas aeruginosa* (Bozkurt-Güzel et al., 2019), *Escherichia coli* (Bozkurt-Güzel et al., 2018), *Klebsiella pneumoniae* (Chmielewska et al., 2020) and *Candida spp.* (Bozkurt-Güzel et al., 2018). In this study, we have found that other ceragenins, especially CSA-192, also have significant antibiofilm activity against biofilms of VRE at early stages and CSA-90 against mature VRE biofilms.

In the present study, CSA-44 and CSA-192 exhibited stronger inhibition of early-stage biofilm adhesion, whereas CSA-90 was more effective against mature biofilms. Ceragenins are based on the bile acid cholic acid and mimic the amphiphilic morphology of AMPs. They affect biofilm formation by lysing cells, damaging cell membranes, and leaking cell contents, thereby destroying bacterial and fungal cells. CSA-44 contains an eight-carbon chain, but it also contains esters rather than ether bonds. CSA-192 is closely related to CSA-13, which previous studies have shown to rapidly penetrate biofilms and cause cell death

without significantly altering the extracellular matrix. The ability of CSA-44 and CSA-192 to reduce biofilm adhesion and formation observed here demonstrates that these agents can maintain their activity within the composing biofilm extracellular matrix. In contrast, CSA-90 has two short (5-carbon) alkyl chains and does not display the activity observed with single alkyl chains. This can be by the fact that ceragenins with ester substituents (e.g., CSA-44) tend to act at the initial steps of biofilm formation, whereas CSA-90 can more readily permeate biofilm matrices (Bozkurt-Güzel et al. 2018; Tokajuk et al., 2022).

As QS inhibition plays an important role in biofilm disruption by interfering with microbial communication, we investigated the anti-QS efficacy of CSAs. According to the results, although they showed antibiofilm activity, they did not show anti-QS activity. This can be explained by the fact that antimicrobial compounds can exert antibiofilm activity through a variety of mechanisms, including direct bacterial killing, extracellular matrix degradation, and inhibition of adhesion or metabolic pathways (Roy et al., 2018).

The antioxidant capacities of the ceragenins were also investigated using DPPH radical scavenging activity and FRAP assays. Oxidative stress describes the imbalance between the antioxidant defence mechanisms and the levels of reactive oxygen and nitrogen species. Antioxidants are compounds that are able to inhibit oxidation by neutralising the damaging effects of free radicals, and they protect key cellular components. While it is generally posited that elevated antioxidant levels correlate with enhanced antimicrobial activity (Ispiryan et al., 2024), the absence of antioxidant activity observed in ceragenins at the studied concentrations suggests a mechanism of action distinct from traditional antioxidant pathways. This finding prompts the hypothesis that ceragenins primarily exert their antimicrobial effects through membrane depolarization, and potentially may in-

volve contributions from oxidative stress, rather than solely relying on direct antioxidant activity. Given the complexity of antimicrobial compound mechanisms, where multiple pathways may operate concurrently, and the cationic nature of ceragenins, which might induce oxidative stress, further investigation into the possible interaction of ceragenins with oxidative stress pathways is warranted to fully elucidate their antimicrobial mechanisms.

CONCLUSION

The emergence and spread of VRE presents a growing issue in clinical microbiology and infectious disease management and requires new antimicrobial agents. According to the present study, ceragenins appear to be potential agents with antibiofilm activity against VRE strains. Due to its high resistance to antimicrobial agents, biofilm treatment is extremely challenging. Promisingly, in biofilm assays, ceragenins were found to be more effective than linezolid, which is commonly used to treat infections but to which resistance is increasing. However, more research is needed to fully determine their effectiveness and safety in clinical use. This study highlights the need for continued research in this area and suggests that ceragenins may be a potential treatment option the combat against VRE.

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AUTHOR CONTRIBUTION STATEMENT

Concept – MA, FO, MH; Design – MA, FO, MH; Supervision – MA, FO, MH; Resources – MH; Materials – MH, N., PBS; Data Collection and/or Processing – MA, FO, MH, GHC, NI; Analysis and/or Interpretation – MA, FO, MH, GHC, NI, PBS; Literature Search – MA, FO, MH; Writing – MA, FO, MH, GHC, NI, PBS; Critical Reviews – MA, FO, MH, GHC, NI, PBS.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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D-Optimal Design-Based Formulation and Evaluation of Tamanu Oil (*Calophyllum inophyllum* L.) Emulgel for Open Wound Healing and Erythema Reduction in Male Wistar Rats

Rita RAKHMAWATI[°], Anif Nur ARTANTI^{**}, Rizka AYUNINGTYAS^{***},
Kuni HANIFAH^{****}, Fitrawan Hernuza PRIBADI^{*****}, Nestri HANDAYANI^{*****}

D-Optimal Design-Based Formulation and Evaluation of Tamanu Oil (Calophyllum inophyllum L.) Emulgel for Open Wound Healing and Erythema Reduction in Male Wistar Rats

Erkek Wistar Sıçanlarında Açık Yara İyileşmesi ve Eritem Azaltılması İçin Tamanu Yağı (Calophyllum Inophyllum L.) Emülgelinin D-Optimal Tasarım Temelli Formülasyonu ve Değerlendirilmesi

SUMMARY

Tamanu oil (*Calophyllum inophyllum* L.) is recognised for its wound-healing potential; however, its oily consistency and formulation instability restrict its topical application. To address these limitations, this study aimed to develop and optimise a stable tamanu oil-based emulgel using a D-optimal mixture design in Design Expert[®] software. It then evaluated its wound healing activity in an open wound model. The formulation was optimised by varying the concentrations of hydroxypropyl methylcellulose (HPMC) and propylene glycol, with pH and viscosity as response variables. The selected formulation (24.72% HPMC and 75.28% propylene glycol) was assessed in vivo in male Wistar rats. Wound healing progression was monitored over 21 days and analysed using ImageJ. Percentage wound closure and erythema duration were statistically evaluated using one-way ANOVA, followed by LSD post hoc analysis for wound closure (AUC-based) and Duncan's test for erythema. The optimised emulgel showed favourable physicochemical properties and maintained stability over four weeks. In vivo, it achieved 96.66% wound closure by day 15 and complete closure by day 21, with a significantly lower AUC (6.53 ± 1.08 ; $p < 0.05$) than the control groups. Erythema resolved more rapidly in the tamanu group, supporting its anti-inflammatory potential.

Keywords: *Calophyllum inophyllum*, D-optimal design, emulgel formulation, tamanu oil, wound healing.

ÖZ

Tamanu yağı (*Calophyllum inophyllum* L.), yara iyileştirici potansiyeli ile tanınmaktadır; ancak yağlı yapısı ve formülasyon kararsızlığı, topikal uygulama alanını kısıtlamaktadır. Bu sınırlamaları aşmak amacıyla, bu çalışma Design Expert[®] yazılımında D-optimal karışım tasarımı kullanılarak kararlı bir tamanu yağı bazlı emüljel geliştirmeyi ve optimise etmeyi, ardından açık yara modelinde yara iyileştirici etkinliğini değerlendirmeyi amaçlamıştır. Formülasyon optimizasyonu, hidroksipropil metilselüloz (HPMC) ve propilen glikol konsantrasyonlarının değiştirilmesi ile gerçekleştirilmiştir; pH ve viskozite yanıt değişkenleri olarak belirlenmiştir. Seçilen formülasyon (%24,72 HPMC ve %75,28 propilen glikol) erkek Wistar sıçanlarında in vivo olarak test edilmiştir. Yara iyileşme süreci 21 gün boyunca takip edilmiş ve ImageJ yazılımı kullanılarak analiz edilmiştir. Yara kapanma yüzdesi ve eritem süresi, tek yönlü ANOVA ile istatistiksel olarak değerlendirilmiş; yara kapanması (AUC temelli) için LSD post hoc analizi, eritem için ise Duncan testi uygulanmıştır. Optimise edilen emüljel, uygun fizikokimyasal özellikler sergilemiş ve dört hafta boyunca kararlılığını korumuştur. In vivo sonuçlara göre, 15. günde %96,66 oranında yara kapanması ve 21. günde tam kapanma elde edilmiş; kontrol gruplarına kıyasla anlamlı derecede düşük bir AUC değeri ($6,53 \pm 1,08$; $p < 0,05$) gözlenmiştir. Eritem, tamanu grubu ile daha hızlı iyileşmiş olup bu bulgu, ürünün antiinflamatuar potansiyelini desteklemektedir.

Anahtar Kelimeler: *Calophyllum inophyllum*, D-optimal tasarım, emüljel formülasyonu, tamanu yağı, yara iyileşmesi.

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[°] ORCID: 0000-0001-8905-5531, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

^{**} ORCID: 0009-0001-8015-2850, Department of Pharmacy, Vocational College, Universitas Sebelas Maret, Surakarta, Indonesia

^{***} ORCID: 0009-0008-2472-1247, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

^{****} ORCID: 0009-0008-1866-4557, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

^{*****} ORCID: 0009-0001-3730-4935, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

^{*****} ORCID: 0000-0003-1945-9500, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

[°] Corresponding Author; Rita Rakhmawati

E-mail: ritarakhmawati@staff.uns.ac.id

INTRODUCTION

Wound healing is a multifaceted and tightly regulated biological process involving an orchestrated sequence of overlapping phases, such as hemostasis, inflammation, proliferation, re-epithelialisation, angiogenesis, and tissue remodelling. This physiological response is essential for restoring injured skin's structural and functional integrity (Mansour et al., 2025). However, in excisional open wounds, healing can be delayed or impaired, resulting in chronic wounds, infection susceptibility, and poor tissue regeneration (Maqbool et al., 2025). Therefore, clinical management of wounds demands effective therapeutic agents and delivery systems that enhance healing outcomes while ensuring patient compliance.

One of the critical components in modern wound care is the use of topical formulations or wound dressings designed to protect the wound, provide a moist environment, absorb exudates, and deliver bioactive substances directly to the site of injury (Minsart et al., 2022; Su et al., 2023). To modulate the wound microenvironment, ideal wound care products should offer additional pharmacological benefits, such as anti-microbial, anti-inflammatory, and antioxidant activities (Moradifar et al., 2025). Despite the availability of synthetic wound dressings and pharmaceutical products, the demand for plant-based therapies is increasing due to their affordability, multi-target mechanisms, and traditional validation across various cultures (Elkordy et al., 2021; Nasim et al., 2022).

Calophyllum inophyllum L., widely known as tamanu, is a tropical tree native to Southeast Asia and the Pacific Islands. Its seed-derived oil, tamanu oil, has a long history of use in traditional medicine for treating skin ailments, burns, and wounds (Ferdosh, 2024; Krishnappa et al., 2024). Tamanu oil has been traditionally used by coastal and island communities in Indonesia, Polynesia, and Melanesia as part of their cultural healing practices. In this study, we take an ethnopharmacological approach to examine and improve its therapeutic use through innovative formula-

tion methods. Phytochemical analyses have identified calophyllolide, inophyllums, calanolides, fatty acids (such as oleic acid and linoleic acid), and tamanolides as key bioactive constituents responsible for its therapeutic effects (Ferdosh, 2024). These compounds have demonstrated anti-inflammatory, antibacterial, antioxidant, and tissue-repairing activities in various experimental models. Calophyllolide, for instance, was shown to enhance re-epithelialisation, reduce fibrosis, and stimulate wound closure in rats, outperforming standard treatments such as povidone-iodine (Nguyen et al., 2017). Furthermore, Erdogan et al. (2021) reported that topical application of tamanu oil in full-thickness wound models improved collagen density and accelerated wound contraction. Additionally, cold-pressed tamanu oil is rich in unsaturated fatty acids such as oleic and linoleic acids (Rakhmawati et al., 2024a), which are known to promote keratinocyte migration, modulate inflammation, and accelerate dermal tissue regeneration (Tarigan et al., 2024; Wang et al., 2024).

While these findings highlight the therapeutic promise of tamanu oil, its direct application in clinical or experimental settings poses certain limitations. The oil is inherently greasy, sticky, and difficult to spread uniformly, which hinders patient comfort and dosage accuracy. Moreover, the physical instability of tamanu oil in its native form raises concerns regarding its shelf life and consistency during topical use (Krishnappa et al., 2024). Incorporating tamanu oil into an emulgel-based delivery system represents a rational formulation strategy to address these challenges. Emulgels combine the emollient and solubilising properties of emulsions with the non-greasy, bio-adhesive, and cooling features of gels, thereby offering a favourable vehicle for lipophilic natural products (Ganju et al., 2024; Kumbhar et al., 2025). Emulgels are particularly advantageous for dermal applications, as they enhance drug permeation through the skin, provide sustained release, and improve user acceptability (Suen et al., 2024).

Despite tamanu oil's well-established wound healing potential and the growing interest in emulgel-based topical systems, studies exploring its incorporation into optimised formulations tailored for excisional open wounds remain limited. Previous research has primarily focused on evaluating the wound-healing activity of pure tamanu oil in incision wound models, whereas the present study investigates its potential in open wound healing. For instance, Rakhmawati et al. (2024a) demonstrated that cold-pressed tamanu oil exhibited superior fatty acid characteristics and significantly enhanced wound closure in vivo compared to hot-pressed tamanu oil and controls. In a separate study, the same authors further confirmed the efficacy of tamanu oil in promoting wound healing in diabetic rat models, highlighting its potential as a natural therapeutic agent (Rakhmawati et al., 2024b). However, these investigations evaluated tamanu oil solely in its unformulated state, overlooking the critical role of delivery systems in maximising therapeutic outcomes. Without an optimised formulation, challenges remain in ensuring sufficient skin permeation, maintaining stability, and enhancing user comfort during application. Moreover, while the anti-inflammatory properties of tamanu oil are widely recognised, its impact on erythema, a common clinical indicator of inflammation during the early stages of wound healing (Johnson et al., 2024), has not been sufficiently investigated in controlled animal studies. This represents an important knowledge gap, particularly considering that erythema, if prolonged or excessive, may signal persistent inflammation and delayed wound resolution (Roşca et al., 2025). Therefore, further investigation into the efficacy of tamanu oil in modulating both wound closure and erythema through a stable and practical emulgel delivery system is essential to realise its potential as a natural topical therapeutic agent fully.

Therefore, this study aimed to optimise a stable and practical tamanu oil-based emulgel formulation using a D-optimal design approach within the Design Expert® software. This approach, grounded in re-

sponse surface methodology (RSM), enables efficient exploration of formulation variables through minimal experimental runs while evaluating each factor's individual and interactive effects. Furthermore, the optimised formulation was evaluated for its therapeutic performance in wound closure and erythema reduction using an excisional open wound model in male Wistar rats.

MATERIALS AND METHODS

Materials

Cold-pressed *Calophyllum inophyllum* (tamanu) oil was used as the active ingredient. Hydroxypropyl methylcellulose (HPMC; Brataco) served as the gelling agent, while propylene glycol (Brataco) functioned as the humectant. Additional excipients included liquid paraffin (Brataco) as the emollient, Tween 80 and Span 80 (Brataco) as surfactants, phenoxyethanol as a preservative, and menthol as a fragrance-correcting agent. Distilled water was used as the solvent. Commercial product gel was used as the positive control. Other materials included alcohol swabs, surgical scalpels (B-Braun), and BR® rodent feed.

Animals

Twenty-four healthy male Wistar rats (150–200 g, 2–3 months old) were obtained from the Laboratory of Animal Development, LIPI, Yogyakarta. Animals were acclimatised for seven days in cages containing 2–3 cm thick husk bedding, which was replaced every three days. Rats were fed 15 g of standard chow daily. Ethical approval was obtained from the Ethics Committee of Universitas Gadjah Mada (Approval No. 110/UN27.06.11/KEP/EC/2022).

Emulgel formulation and optimization

Based on preliminary tests, Tamanu oil emulgel was formulated using HPMC and propylene glycol in a 1:5 ratio (w/w). Emulsification was achieved using a combination of Tween 80 and Span 80, forming an oil-in-water (o/w) emulsion system. A D-optimal mixture design was employed using Design Expert® software (version 9, Stat-Ease Inc.) to optimise the

concentrations of HPMC and propylene glycol. Thirteen formulations were generated, and responses were evaluated based on pH and viscosity. The optimal formulation was selected based on the desirability index (value = 1) and verified using a one-sample t-test. A formulation was considered valid if the experimental results did not significantly differ from the predicted values ($p > 0.05$).

Evaluation of the physicochemical properties of the optimised emulgel

The optimised formulation was evaluated for organoleptic characteristics, homogeneity, pH, viscosity, spreadability, and adhesion. Homogeneity was assessed microscopically by spreading the gel between two glass slides and observing the distribution of particles. The pH was measured using a digital pH meter, with the acceptable range for topical application being 4.5 to 6.5 to ensure compatibility with the skin. Viscosity was determined using a Rion viscometer equipped with spindle number 2, where an ideal range between 50 and 1000 dPas was considered acceptable for semi-solid preparations (Tahar et al., 2023). Spreadability was evaluated by placing 0.5 g of the emulgel between two glass plates under a 150 g load for one minute, and the resulting spread diameter was measured; values between 5 and 7 cm were deemed acceptable. Adhesion was assessed using a glass-slide assembly, where the time required for the upper slide to detach under an 80 g load, following an initial loading of 1 kg for five minutes, was recorded as the adhesion time (Rahmani & Zulkarnain, 2023).

Evaluation of open wound healing activity

The wound healing activity of the optimised tamanu oil emulgel was evaluated using an open excision wound model in male Wistar rats. The number of animals per group was determined using Federer's formula to ensure statistical validity, as follows (Tahar et al., 2023):

$$(t - 1)(n - 1) \geq 15$$

With four treatment groups ($t = 4$), the minimum

number of animals per group was calculated to be at least six ($n \geq 6$), resulting in a total of 24 animals randomly assigned to each group.

Before wound induction, the rats were anaesthetised with ketamine, and the dorsal fur was shaved and disinfected using 70% ethanol. A 1 cm × 1 cm excision wound with a depth of 2 mm was created on the upper dorsal region using a sterile scalpel, limited to the dermal layer without damaging the underlying muscle, following the method of Rakhmawati et al. (2024a). The treatment formulations (0.5 g) were applied topically once daily for 21 consecutive days.

The treatment groups included a normal control (untreated), a negative control (gel base without tamanu oil), a positive control (commercial product[®]), and the test group, which received 20% (w/w) tamanu oil emulgel for its wound healing potential (Krishnapa et al., 2024).

Daily visual observation assessed changes in wound colouration (from red to white), scab formation, and re-epithelialisation. Wounds were photographed on days 0, 3, 6, 9, 12, 15, 18, and 21 using a fixed camera distance and scale for quantitative analysis of wound area using ImageJ software.

Data analysis

Digital photographs of wounds were analysed using ImageJ software. The wound healing progress was calculated as percent wound closure using the following formula (Rakhmawati et al., 2024a):

$$\%P = \frac{(do - dx)}{do} \times 100$$

Where:

do = initial wound area (day 0)

dx = wound area on day x

The total area under the curve (AUC) of wound area over time was calculated to reflect overall wound healing dynamics across the study period, using the following rule:

$$AUC_{tn-1}^{tn} = \frac{L_{tn-1} + L_{tn}}{2} \times (t_n - t_{n-1})$$

Where:

L_{t_n} and $L_{t_{n-1}}$ = mean wound area at time t_n and t_{n-1} , respectively.

All data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using IBM SPSS® version 21.0. Wound closure percentage and AUC values were analysed using one-way ANOVA, followed by Least Significant Difference (LSD) post hoc tests if p-values were below 0.05. A significant difference ($p < 0.05$) compared to the control groups indicated wound healing efficacy.

Additionally, time to erythema resolution was recorded through daily macroscopic observation for 21 days. These data were also analysed using one-way ANOVA, followed by Duncan's multiple range test if ANOVA indicated significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Optimisation of tamanu oil (*Calophyllum inophyllum* L.) emulgel formulation

The optimisation of tamanu oil emulgel formulation aimed to develop a physically stable and pharmaceutically acceptable topical dosage form. This study employed a combination of Tween 80 (1.08%) and Span 80 (0.42%) as emulsifying agents, resulting in a hydrophilic-lipophilic balance (HLB) value of 12.004, which supports the formation of oil-in-water (O/W) emulsions. O/W emulsions are preferred for topical applications due to their better patient acceptability, non-greasy feel, and easier washability (Ali et al., 2022). The overall formulation for five trial batches (F1–F5) is presented in Table 1.

Table 1. Formulation of tamanu oil emulgel formulations (75 g per tube) for trial batches F1–F5

Ingredients	Concentration of Ingredients in Gel Formula (g)				
	F1	F2	F3	F4	F5
Tamanu oil	15	15	15	15	15
HPMC	0.75	1.5	2.25	3	3.75
Propylene glycol	3.75	3	2.25	1.5	0.75
Parafin liquidum	3.75	3.75	3.75	3.75	3.75
Tween 80	0.81	0.81	0.81	0.81	0.81
Span 80	0.315	0.315	0.315	0.315	0.315
Phenoxyetanol	0.375	0.375	0.375	0.375	0.375
Menthol	0.75	0.75	0.75	0.75	0.75
Aquadest	ad 75	ad 75	ad 75	ad 75	ad 75

A key component of the formulation optimisation was the selection of appropriate concentrations of HPMC (hydroxypropyl methylcellulose) as a gelling agent and propylene glycol as a cosolvent and humectant. These two components were selected as independent variables in a mixture design optimisation using the D-optimal method in Design Expert 9 software. The design space was defined with HPMC ranging from 16.67% to 33.33%, and propylene glycol from 66.67% to 83.33%, keeping the total proportion of both components at 100%.

The experimental design generated 13 run formulations with varying HPMC and propylene gly-

col ratios, while maintaining the total concentration of both components at 100%. Several formulations shared identical compositions, such as run 2 with runs 8, 11, and 13; run 3 with runs 9 and 12; and run 4 with runs 5, 6, and 10. This repetition reflects the software's strategy in placing replicates at critical design points, particularly at the centre or vertices of the design space, to evaluate model robustness, minimise experimental error, and improve the reliability of the response surface model. Each formulation was evaluated for pH and viscosity, essential parameters for topical emulgels. The detailed composition of each run and the corresponding response values for pH and

viscosity are presented in Table 2. A pH within the range of 5.5 to 6.5 is considered compatible with the skin and avoids irritation (Khan et al., 2024), while viscosity affects the preparation’s spreadability, retention, and overall patient compliance (Humaira et al., 2025).

Table 2. Experimental design matrix and observed viscosity values for tamanu oil emulgel formulations based on D-optimal mixture design involving HPMC and propylene glycol

Run	A: HPMC (%)	B: Propylene glycol (%)	Parameter	
			pH	Viscosity (dPas)
1.	20.835	79.165	6.42	490
2.	33.330	66.670	6.36	600
3.	25.000	75.000	6.40	495
4.	16.670	83.330	6.44	253
5.	16.670	83.330	6.45	420
6	16.670	83.330	6.44	490
7.	29.165	70.835	6.37	595
8.	33.330	66.670	6.35	650
9.	25.000	75.000	6.41	450
10.	16.670	83.330	6.44	460
11.	33.330	66.670	6.36	645
12.	25.000	75.000	6.40	410
13.	33.330	66.670	6.34	660

The response analysis showed that both pH and viscosity followed linear models with statistically significant effects from the variation in HPMC and propylene glycol concentrations ($p < 0.05$) (Figures 1 and 2.). The lack-of-fit test results were non-significant ($p = 0.56$ for pH, $p = 0.47$ for viscosity), indicating that the linear models adequately predicted the observed responses.

The optimal formulation was determined using

a desirability function approach, yielding a value of 0.787. Although not reaching the theoretical maximum of 1.0, this score represents a highly acceptable result within pharmaceutical formulation standards. In D-optimal mixture designs, desirability values above 0.7 are typically considered robust and indicate a satisfactory balance between multiple responses (Araghi et al., 2023). The value of 0.787 thus reflects the best achievable compromise between pH and viscosity within the studied design space.

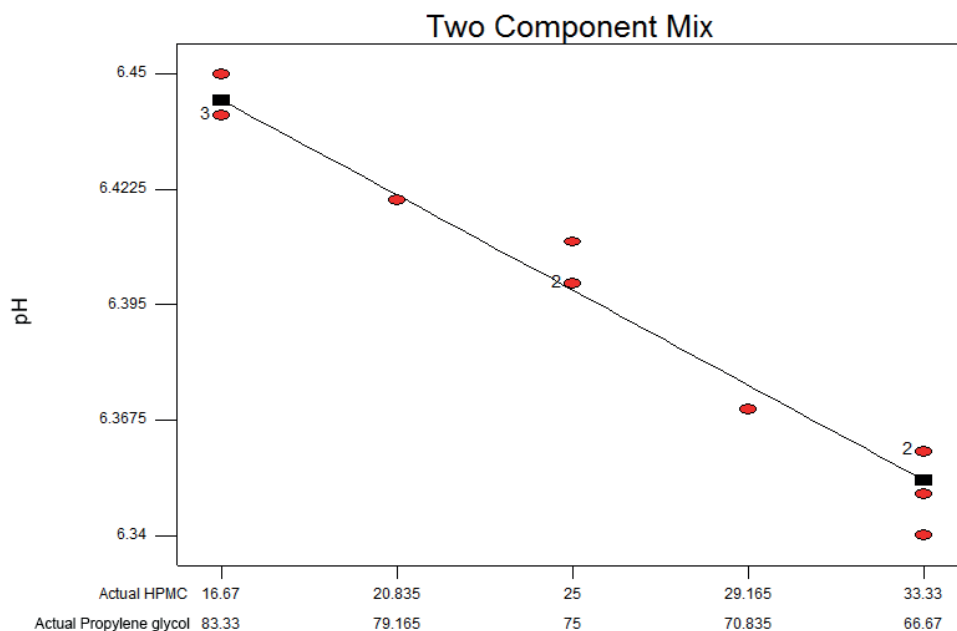


Figure 1. Response surface plot showing the effect of HPMC and propylene glycol concentrations on the pH of tamanu oil emulgel formulations

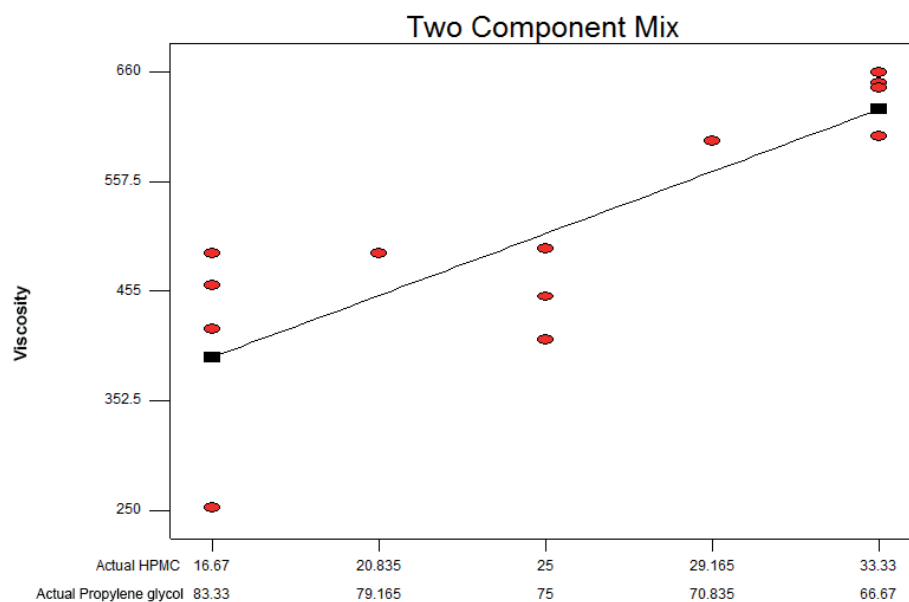


Figure 2. Response surface plot illustrating the influence of HPMC and propylene glycol concentrations on the viscosity of tamanu oil emulgel formulations

This optimal formula comprised 24.72% HPMC and 75.28% propylene glycol, with predicted values of pH 6.39 and viscosity 505.18 dPas (Table 3.). The formulation was subsequently verified in triplicate,

showing observed values (mean $\pm$ SD) of pH 6.39 ± 0.031 and viscosity 498.33 ± 7.64 dPas, with no statistically significant difference from predicted values ($p > 0.05$, Table 4.).

Table 3. Predicted optimal formulation of tamanu oil emulgel and corresponding response values (pH and viscosity) generated by Design Expert® software

HPMC (%)	Propylene glycol (%)	pH	Viscosity	Desirability
24.72	75.28	6.39	505.18 dPas	0.787

Table 4. Predicted optimal formulation of tamanu oil emulgel and corresponding response values (pH and viscosity) generated by Design Expert® software

Parameter	Prediction	Observation				<i>p</i> -value
		R1	R2	R3	Mean ± SD	
pH	6.39	6.38	6.42	6.36	6.39 ± 0.031	0.528
Viscosity (dPas)	505.18	505	490	500	498.33 ± 7.637	0.264

R1= Replication 1; R2= Replication 2; R3= Replication 3

This combination was deemed optimal because it produced a gel with acceptable viscosity and pH, ensuring ease of application, adequate retention on the skin, and safety for prolonged topical use. A balance between HPMC and propylene glycol is critical, as excessive HPMC increases viscosity excessively, impairing spreadability (Narulita et al., 2024), while excess propylene glycol can lower viscosity and compromise emulgel structure (Otake et al., 2025).

In addition to their functional roles, HPMC and propylene glycol also contribute to formulation stability. HPMC serves as a viscosity enhancer and helps stabilise emulsions by forming a three-dimensional gel matrix that entraps oil droplets, preventing phase separation and improving shelf-life (Ferdaus et al., 2025). Propylene glycol, on the other hand, improves sensory feel and acts as a penetration enhancer, which is beneficial for topical delivery systems (Mistry & Notman, 2024).

These findings are supported by Andina et al. (2021), who optimised a green tea gel formulation us-

ing the D-optimal method and found that increasing HPMC concentration significantly raised viscosity, while propylene glycol functioned as a viscosity-modifying and spreadability-enhancing agent. Their study demonstrated that a careful balance between these two components is essential to achieve desirable gel characteristics, aligning with the current formulation approach.

Physicochemical performance of the optimised emulgel

The optimised tamanu oil emulgel exhibited favourable physical and physicochemical characteristics over a four-week storage period (see Table 5). Organoleptically, the formulation retained a stable greenish colour, semi-solid consistency, and characteristic tamanu scent throughout the observation period. The absence of coarse particles under microscopic evaluation further confirmed the homogeneity and colloidal stability of the formulation, suggesting effective emulsification and polymer dispersion (Bruno et al., 2022; Silva et al., 2016).

Table 5. Physicochemical characteristics of the optimised tamanu oil emulgel over 4 weeks of storage (mean±SD)

Week	pH	Viscosity (cPs)	Spreadability (cm)	Adhesiveness (s)
1	6.42±0.33	498.33 ± 7.64	5.87 ± 0.15	3.86 ± 0.41
2	6.32±0.35	473.33 ± 15.28	6.33 ± 0.43	3.47 ± 0.43
3	6.24±0.43	453.33 ± 15.28	6.67 ± 0.55	3.11 ± 0.28
4	6.18±0.41	431.67 ± 10.41	7.28 ± 0.43	2.85 ± 0.46

The pH values remained within the physiologically acceptable range (6.18–6.42), showing no statistically significant difference over time ($p > 0.05$). Maintaining a pH within this range is crucial for dermal compatibility, as formulations between 5.5 and 6.5 preserve the skin barrier's integrity and minimise irritation risk (Khan et al., 2024). Previous studies have shown that pH values exceeding the skin's natural pH may alter enzymatic activity in the stratum corneum, potentially disrupting barrier function and microbiome balance (Kuo et al., 2020; Rajkumar et al., 2023).

A key physicochemical change was observed in the viscosity, which showed a statistically significant decline from 498.33 ± 7.64 cPs in week 1 to 431.67 ± 10.41 cPs in week 4 ($p = 0.001$). The observed decrease in viscosity over the four-week storage period may be attributed to the gradual weakening of the HPMC gel network. Ding et al. (2014) reported reduced molecular interaction in HPMC-based systems can lower viscosity. Noval et al. (2020) found that storage led to viscosity decline in HPMC gels due to changes in hydrogen bonding and flow behaviour. These findings suggest that storage conditions likely contributed to the reduced structural integrity of the emulgel matrix. Despite the reduction, the viscosity remained within the optimal range for semi-solid topical systems, which ensures sufficient retention at the application site and controlled drug release. These viscoelastic properties are crucial for maintaining formulation integrity while allowing user-friendly application (Botan et al., 2024).

The spreadability significantly increased from 5.87 ± 0.15 cm in week 1 to 7.28 ± 0.43 cm in week 4 ($p = 0.018$), correlating inversely with the observed decrease in viscosity. As the internal gel structure relaxes, less force is required to spread the formulation, resulting in improved spreadability. This property is essential to ensure uniform distribution of the active ingredient over the skin surface and improve patient compliance. An increase in spreadability also enhances the surface area for drug absorption, thereby potentially improving therapeutic efficacy (Ding et al., 2014).

The adhesion time decreased slightly over the storage period, from 3.86 ± 0.41 to 2.85 ± 0.46 seconds, although this change was not statistically significant ($p > 0.05$). This trend follows the viscoelastic behaviour of the gel, where reduced viscosity and lower gel strength diminish the formulation's ability to resist detachment from the skin (Rahayu et al., 2024). Nevertheless, the final adhesion value remained acceptable for topical application (greater than one second), indicating that the gel could maintain contact long enough to facilitate drug permeation (Ridwanto et al., 2023).

These findings indicate that the optimised emulgel formulation achieved a suitable balance of physicochemical characteristics essential for dermal delivery. The stable pH and viscosity values suggest good formulation robustness, while the improved spreadability and maintained adhesion highlight its potential for enhanced patient compliance and therapeutic performance.

Wound healing activity in the excisional open wound model

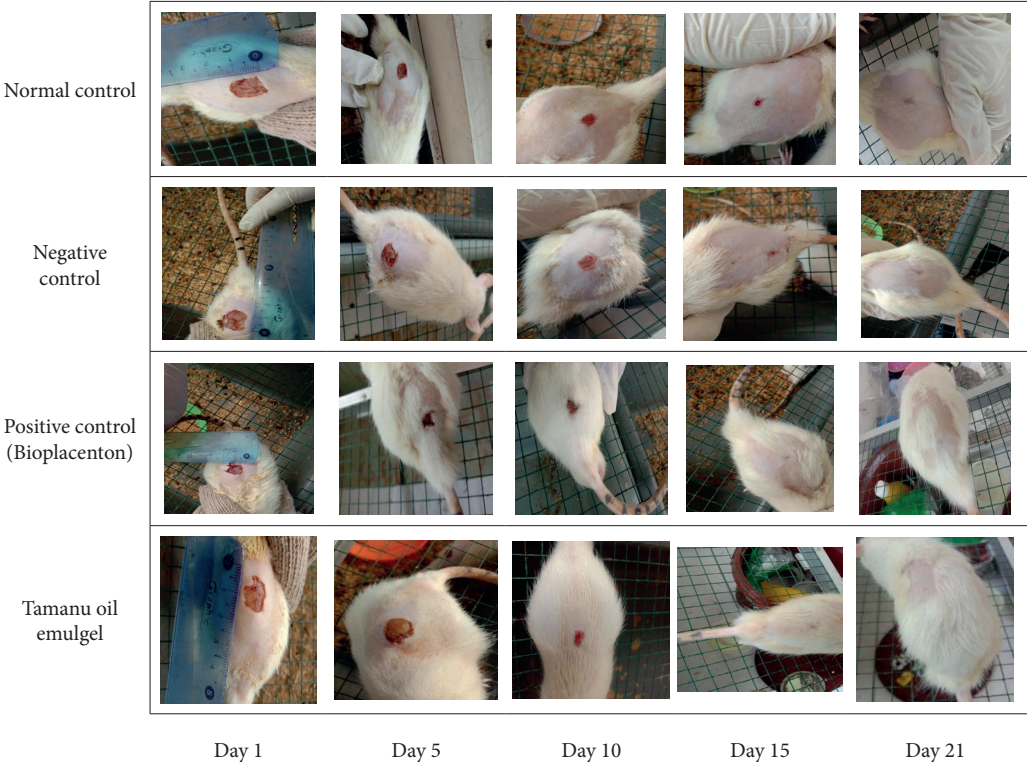
The wound healing activity of the optimised tamanu oil emulgel was evaluated over 21 days using an excisional open wound model in male Wistar rats. Open wounds are injuries involving a break in the skin barrier, exposing underlying tissue to potential infection and delaying the healing process if not properly managed (Sharma et al., 2021). Morphologically (Figure 3A), the wounds in all treatment groups gradually healed from day 1 to day 21. On day 1, all rats exhibited fresh, open wounds with visible erythema and swelling. On day 10, scab formation was evident in all groups. However, the tamanu oil emulgel group displayed smaller scabs with well-defined edges, suggesting active re-epithelialisation (Cioce et al., 2024). By day 15, the emulgel-treated wounds had lost most of their scabs, revealing newly formed epithelial tissue with minimal signs of inflammation. In contrast, wounds in the untreated and negative control groups retained larger scabs and incomplete closure. Com-

plete epithelialisation was observed in the emulgel group by day 21, where wound areas were fully closed and indistinguishable from surrounding skin (Liu et al., 2025).

The percentage of wound closure was calculated based on the reduction in wound size over time. Figure 3B shows the average wound closure percentage

across all groups over the 21 days. Tamanu oil emulgel group exhibited the fastest and most complete wound closure, achieving 96.66% closure by day 15 and 100% closure by day 21. In contrast, none of the control groups reached complete closure by day 21, with the positive control showing intermediate efficacy, outperforming the negative and normal controls, but not surpassing the tamanu oil emulgel.

(A)



(B)

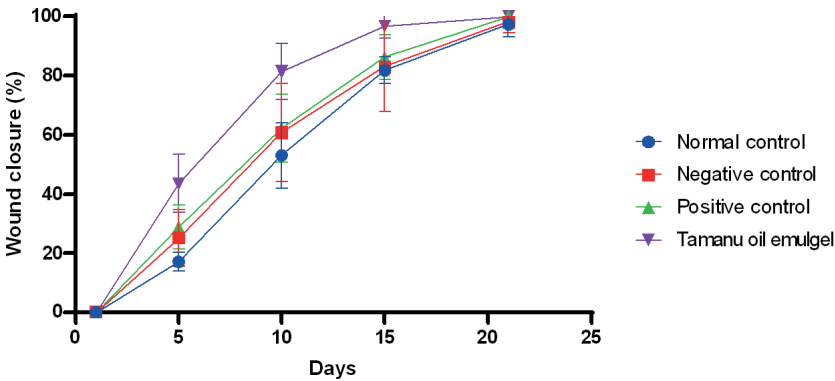


Figure 3. Macroscopic evaluation and quantification of wound closure over 21 days. (A) Representative macroscopic images of wound healing progression in rats (n = 6 per group) from each treatment group on days 1, 5, 10, 15, and 21 following wound excision. (B) Percentage of wound closure (mean ± SD, n = 6) at each observation point (days 1, 5, 10, 15, and 21) across all groups.

The wound healing progression in this study aligns with the typical phases of cutaneous wound repair, which consist of inflammation (days 0–4), proliferation (days 5–21), and remodelling (beyond day 21) (Gupta et al., 2023; Nasiry et al., 2022). On day 1, all groups showed minimal closure, reflecting the early inflammatory response of edema and erythema. By day 5, the tamanu oil emulgel group exhibited a notably higher wound closure (43.56%) than controls, suggesting an accelerated transition into the proliferative phase, likely driven by anti-inflammatory and regenerative phytochemicals in tamanu oil. Continued improvements were observed on days 10 and 15, where the emulgel group consistently outperformed other treatments, reaching 81.29% and 96.66% wound closure, respectively. These findings suggest that the optimised emulgel formulation enhances active compound delivery and supports sustained fibroblast activity, collagen synthesis, and re-epithelialisation throughout the proliferative phase. By day 21, near-complete healing (100%) was observed in the tamanu group, indicating a potential role of the formulation in promoting faster tissue remodelling (Cioce et al., 2024; Stojanovic et al., 2024). The prolonged inflammatory signs and delayed scab shedding in other groups suggest a slower resolution of inflammation and suboptimal transition to the proliferative phase, which may explain their lower wound closure percentages (Binsuwaidan et al., 2024).

To comprehensively assess the wound healing performance of the tested formulations, the area under the curve (AUC) of wound size reduction was calcu-

lated over the 21-day treatment period. AUC serves as an integrated indicator of the healing trajectory, capturing both the rate and extent of wound closure over time, rather than relying solely on endpoint measurements (Lammert et al., 2024). As shown in Table 6, the tamanu oil emulgel group exhibited the lowest total AUC, indicating a consistently rapid wound contraction and superior healing kinetics. Statistical one-way ANOVA analysis demonstrated significant differences among the treatment groups ($p = 0.009$). Post hoc LSD tests confirmed that the tamanu oil emulgel significantly outperformed both the normal and the negative control groups ($p < 0.05$), although no statistically significant difference was observed when compared to the Bioplacenton® group.

This finding demonstrates that the tamanu oil emulgel in this study has a notably lower AUC value than the cold-pressed tamanu oil reported by Rakhmawati et al. (2024a), which was 11.67 ± 0.78 . The marked decrease in AUC reflects a significantly enhanced wound healing rate, underscoring the superior efficacy of the emulgel formulation over the unformulated oil. This improvement can be attributed to the emulgel's ability to enhance skin permeation and retention of active compounds and its improved physicochemical stability and patient acceptability (Suena et al., 2024). Collectively, these factors contribute to more efficient and sustained delivery of tamanu oil's bioactive components, thereby accelerating the wound closure process and optimising therapeutic outcomes.

Table 6. The area under the curve (AUC) of the wound area is over 21 days for each treatment group. Data are presented as individual values per animal and as mean $\pm$ standard deviation (SD).

Treatment Group	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean $\pm$ SD
Normal Control	9.52	8.30	11.41	10.31	10.54	10.36	10.07 ± 1.06^a
Positive Control	7.86	9.59	10.13	7.33	6.13	9.40	8.40 ± 1.55
Negative Control	12.12	10.89	8.27	8.21	6.58	6.62	8.78 ± 2.27^a
Tamanu Oil Emulgel	7.24	6.94	7.48	4.46	6.47	6.65	6.53 ± 1.08^a

Superscript letter ^a indicates that the tamanu oil emulgel group showed a statistically significant difference ($p < 0.05$) compared to the normal control and negative control groups based on LSD post hoc analysis.

In addition to wound size, erythema resolution was monitored as a marker of inflammation attenuation. Erithema represents the visible manifestation of localised vascular response and leukocyte recruitment during the inflammatory phase of wound healing, typically peaking within the first four days post-injury (Pan et al., 2022; Wong et al., 2023). The average duration for erythema disappearance in the

tamanu oil emulgel group was 9.5 days (Table 7.). One-way ANOVA followed by Duncan's post hoc test confirmed statistically significant differences ($p < 0.05$) between the tamanu oil group and the normal and negative control groups. However, there was no significant difference between the tamanu oil group and the positive group, indicating comparable anti-inflammatory performance.

Table 7. Duration (in days) required for erythema disappearance at wound sites in each treatment group. Data are presented as individual values per animal and as mean $\pm$ standard deviation (SD).

Treatment Group	Duration of Wound Erythema Resolution (Days)						Mean $\pm$ SD
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	
Normal control	15	14	18	17	14	16	15.67 $\pm$ 1.63 ^a
Positive control	13	19	18	10	10	16	14.33 $\pm$ 3.87
Negative control	19	16	14	10	12	12	13.83 $\pm$ 3.06 ^a
Tamanu oil emulgel	7	11	10	7	12	10	9.50 $\pm$ 1.87 ^a

Superscript letter ^a indicates that the tamanu oil emulgel group showed a statistically significant difference ($p < 0.05$) compared to the normal control and negative control groups based on Duncan post hoc analysis

Several recent studies have explored the wound healing potential of tamanu oil, highlighting its promising pharmacological activities in skin regeneration, including anti-inflammatory, anti-microbial, and anti-erythema effects. Ansel et al. (2016) demonstrated that tamanu oil extract promoted wound repair in human keratinocytes and dermal fibroblasts by up-regulating genes associated with cell adhesion, proliferation, and O-glycan biosynthesis. Building on this, Nguyen et al. (2017) isolated calophyllolide, one of tamanu oil's major bioactive components, and reported its ability to reduce fibrosis, shift macrophage polarisation toward the M2 phenotype, and enhance complete re-epithelialisation within 14 days in excisional wound models. In a subsequent in vivo study, Erdogan et al. (2021) observed a significant improvement in wound contraction in rats treated with tamanu oil, alongside increased macrophage infiltration and more mature granulation tissue formation by day 7. Rakhmawati et al. (2024a) further confirmed the efficacy of cold-pressed tamanu oil, which exhibited the fastest wound healing rate among all treatment groups, as indicated by the lowest area under the curve (AUC)

value. This result was supported by its favourable physicochemical properties, namely lower acid and peroxide values, as well as its higher content of linoleic and oleic acids. These fatty acids have been implicated in key mechanisms of tissue repair. Oleic acid has been shown to modulate inflammatory responses by increasing IL-10 expression and reducing COX-2 levels, leading to enhanced collagen deposition and balanced cytokine activity (Cardoso et al., 2011). Similarly, linoleic acid accelerated wound closure in porcine burn models, with complete healing observed in 37 days compared to 45 days in untreated controls (Zhao et al., 2022). Both fatty acids are believed to support tissue remodelling through the regulation of matrix metalloproteinase (MMP) activity, further underscoring their contribution to the wound healing process.

Despite this growing body of evidence, the application of *Calophyllum inophyllum* oil in statistically optimised delivery systems for open wound healing remains limited. However, the application of *Calophyllum inophyllum* oil in optimised delivery systems for open wound healing remains scarcely investigated. This study presents novel findings on formulating a ta-

manu oil emulgel using a D-optimal design approach, emphasising its potential as a scientifically designed, plant-based topical therapy. To our knowledge, this is the first investigation to evaluate such a formulation in an excisional wound model, supporting its promise as a natural and effective candidate for open wound management.

An emulgel system likely enhanced the formulation's effectiveness by improving skin adhesion, drug retention, and user acceptability. Its ability to deliver lipophilic compounds while maintaining a moist wound environment may have supported tissue regeneration more efficiently than conventional preparations (Afzal et al., 2023; Milutinov et al., 2023).

Despite the promising outcomes observed, such as accelerated wound closure and shorter erythema duration, this study is limited to macroscopic evaluations without histological or molecular confirmation of healing mechanisms. Further studies are needed to validate the underlying biological pathways and assess long-term safety.

CONCLUSION

This study successfully developed and optimised a tamanu oil-based emulgel using a D-optimal design approach. The selected formulation, 24.72% HPMC and 75.28% propylene glycol, exhibited desirable physicochemical properties, including suitable pH, viscosity, spreadability, and physical stability for up to four weeks. In vivo testing in male Wistar rats with excisional wounds showed that the optimised emulgel achieved 96.66% wound closure by day 15, indicating accelerated healing. Complete closure was observed by day 21, with a significantly lower wound AUC than the control groups. Erythema resolved faster in the treated group, suggesting effective inflammation control. These findings demonstrate the synergistic therapeutic effect of tamanu oil's bioactives and the enhanced dermal delivery afforded by the emulgel system.

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AUTHOR CONTRIBUTION STATEMENT

RR: Concept and design, drafting the manuscript, supervision, final approval; ANA: Concept and design, data analysis, drafting the manuscript, final approval; RA: data acquisition, statistical analysis, drafting manuscript; KH: data acquisition, drafting manuscript; FHP: data analysis, critical revision of the manuscript, final approval; NH: critical revision of the manuscript, final approval.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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An Updated Review on Possible Antidiabetic and Hypolipidemic Molecular Action Mechanisms of Medicinal Plants

İçim GÖKKAYA*, Gülin RENDA**

An Updated Review on Possible Antidiabetic and Hypolipidemic Molecular Action Mechanisms of Medicinal Plants

Tıbbi Bitkilerin Olası Antidiyabetik ve Hipolipidemik Moleküler Etki Mekanizmaları Üzerine Güncellenmiş Bir İnceleme

SUMMARY

The prevalence of diabetes mellitus and its related diseases has been increasing. It is common to use natural resources to support medical treatments for endocrine diseases. The aim of this study was to discuss the possible molecular mechanisms of action of medicinal plants and their secondary metabolites, which have been proven to be effective in diabetes mellitus and dyslipidemia by clinical studies. Literature searches were conducted using international databases (Science Direct, Scopus, and Web of Science), academic search engines (Google Scholar), and the Cochrane Library. Studies published between 2015 and 2023 are included in the manuscript. The most prominent herbs for diabetes mellitus and dyslipidemia were garlic, cinnamon, turmeric, ginseng, walnut, flaxseed, bitter melon, black cumin, pomegranate, fenugreek, and ginger. These plants were effective in diabetes mellitus and dyslipidemia by modulating different signaling pathways [(adenosine monophosphate activated protein kinase (AMPK), nuclear factor kappa B (NF-κB), and c-Jun N-terminal kinases (JNK), etc.)], and by their biological activities, including antioxidant, anti-inflammatory, and antiapoptotic effects. This review elaborates on the mechanisms of action of extracts prepared from medicinal plants and their secondary metabolites regarding glucose and lipid metabolism in the treatment of diabetes mellitus. Medicinal herbs treat diabetes mellitus or ameliorate its progression by acting on multiple targets via different enzymes, receptors, and mediators. However, investigations to elucidate the mechanism of action were conducted in vitro and in vivo. The mechanisms identified in preclinical studies must be corroborated by extensive and multicenter clinical trials. The clarification of the effects and mechanisms of action of medicinal plants by preclinical and clinical studies will contribute to the development of antidiabetic drugs.

Keywords: Diabetes mellitus, dyslipidemia, medicinal plants, molecular mechanism.

ÖZ

Diabetes mellitus ve ilişkili hastalıkların prevalansı giderek artmaktadır. Endokrin hastalıklara yönelik tıbbi tedavileri desteklemek için doğal kaynakların kullanımı oldukça yaygındır. Bu çalışmanın amacı, diabetes mellitus ve dislipidemi etkili olduğu klinik çalışmalarla kanıtlanmış olan tıbbi bitkilerin ve sekonder metabolitlerinin olası moleküler etki mekanizmalarını tartışmaktır. Literatür taramaları uluslararası veri tabanları (Science Direct, Scopus ve Web of Science), akademik arama motorları (Google Scholar) ve Cochrane Kütüphanesi kullanılarak yapılmıştır. 2015-2023 yılları arasında yayımlanan çalışmalar makaleye dahil edilmiştir. Diabetes mellitus ve dislipidemi için en çok öne çıkan tıbbi bitkilerin sarımsak, tarçın, zerdeçal, ginseng, ceviz, keten tohumu, acı kavun, çörek otu, nar, çemen otu ve zencefil olduğu görülmektedir. Bu bitkiler, farklı sinyal yollarını [adenozin monofosfat ile aktive edilen protein kinaz (AMPK), nükleer faktör-κB (NF-κB) ve c-Jun N-terminal kinaz (JNK) vb.] modüle ederek, antioksidan, antiinflamatuvar ve antiapoptotik etkileri içeren biyolojik aktiviteleri ile diyabetes mellitus ve dislipidemi etkili olmuştur. Bu derlemede, tıbbi bitkilerden hazırlanan ekstraktların ve bu ekstraktların bileşimindeki sekonder metabolitlerin diyabetes mellitus tedavisinde glukoz ve lipit metabolizması üzerindeki etki mekanizmaları ayrıntılı olarak ele alınmıştır. Tıbbi bitkiler farklı enzimler, reseptörler ve mediyatörler üzerinden çok sayıda hedefe etki ederek diyabetes mellitusu tedavi etmekte veya progresyonunu hafifletmektedir. Bununla birlikte etki mekanizmasını belirlemeye yönelik çalışmalar in vitro ve in vivo olarak gerçekleştirilmiştir. Preklinik çalışmalarda elde edilen mekanizmaların büyük ölçekli ve çok merkezli klinik çalışmalarla desteklenmesine ihtiyaç vardır. Preklinik ve klinik araştırmalarla tıbbi bitkilerin etki ve etki mekanizmalarının aydınlatılması antidiyabetik ilaç geliştirme çalışmalarına katkı sunacaktır.

Anahtar Kelimeler: Diabetes mellitus, dislipidemi, tıbbi bitkiler, moleküler mekanizma.

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* ORCID: 0000-0003-0803-2886, Department of Pharmaceutical Botany, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Türkiye ; Institute of Health Sciences, Ankara University, Ankara, Türkiye

** ORCID: 0000-0001-6323-0338, Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Türkiye

° Corresponding Author; İçim GÖKKAYA
E-mail: icim1995@hotmail.com ; icimgokkaya@ktu.edu.tr

INTRODUCTION

Diabetes mellitus is defined as a chronic and broad-spectrum metabolic disorder characterized by hyperglycemia resulting from insulin deficiency and/or defects in insulin action, affecting many organs, and causing multisystemic involvement (Petersmann et al., 2019). Diabetes mellitus is classified into four clinical forms, including Type 1 diabetes mellitus, Type 2 diabetes mellitus, gestational diabetes mellitus, and other specific types (TEM, 2020). Type 2 diabetes mellitus, which accounts for 90–95% of all diabetes mellitus cases, is associated with a progressive insulin secretion defect accompanied by a background of insulin resistance (Hoogwerf, 2020). In the 10th edition of the “Diabetes Atlas” published by the International Diabetes Federation (IDF) in 2021, it was reported that 537 million adults aged 20–79 (prevalence: 10.5%) worldwide have diabetes mellitus. It is estimated that the number of people with diabetes mellitus among adults in this age group will reach 643 million in 2030 (prevalence: 11.3%) and 783 million in 2045 (prevalence: 12.2%) (IDF, 2021). Dyslipidemia is a clinical condition characterized by plasma lipoprotein levels outside normal ranges or the dysfunction of lipoproteins. Dyslipidemia is one of the most important etiologies of cardiovascular disease risk in diabetes mellitus. In diabetic dyslipidemia, also known as atherogenic dyslipidemia, low HDL-c, high TG, and LDL-c levels are also accompanied by a predominance of small dense LDL particles (Bahiru, Hsiao, Phillipson, & Watson, 2021). The prevalence of diabetic dyslipidemia is quite high in patients with Type 2 diabetes mellitus, with a prevalence ranging between 72% and 85%. A global survey showed that 65% of patients with diabetes mellitus had LDL-c levels greater than 100 mg/dL at the time of diagnosis and should receive medication for dyslipidemia (Athyros et al., 2018).

Medicinal plants and the secondary metabolites in their composition have important roles in the protection of health and the treatment of diseases. Medic-

inal plants are an important source of raw materials for the development of antidiabetic drugs (Lee et al., 2022). Medicinal plants may help in the treatment of the disease or improve its progression through modifying multiple pathways involved in the pathogenesis of diabetes mellitus. Previous studies have identified the mechanisms of action of hypoglycemic plants, including the enhancement of insulin secretion, an increase in glucose uptake by adipose and muscular tissues, inhibition of intestinal glucose absorption, and suppression of glucose synthesis by hepatocytes (Hui, Tang, & Go, 2009). Some of these mechanisms are mediated by PPAR receptors. PPARs are encoded by different genes that generate nuclear receptor isoforms: PPAR- γ , PPAR- α , and PPAR- δ . PPAR- γ regulates the expression of several factors released from adipose tissue that enhance insulin sensitivity, including adiponectin and leptin, or diminish it, such as resistin and TNF- α . In differentiated adipocytes, PPAR- γ impacts the expression of genes related to glucose homeostasis. PPAR- γ upregulates the expression of GLUT-4 and CAP, which are encoded by the GLUT-4 and c-CAP genes, respectively. PPAR- γ , triggered during the differentiation of preadipocytes into adipocytes, is crucial for lipid metabolism and other pathways, such as inflammation, immunity, and glucose homeostasis. Many medicinal plants affect diabetes mellitus by activating PPAR- γ receptors (Ríos, Francini, & Schinella, 2015).

Incretins are hormones produced by the gut that are secreted in response to the ingestion of food, mainly glucose and lipids. There are two intestinal hormones, GIP and GLP-1, which have the effect of incretin. GLP-1 and GIP are rapidly degraded by the enzyme DPP-4. Extending the half-life of GLP-1, inhibiting DPP-4, or using GLP-1 analogues resistant to DPP-4 degradation are two key goals in antidiabetic drug development studies. Medicinal plants are known as having antidiabetic properties, which are mediated through the modulation of incretin levels or the inhibition of the enzymes α -glucosidase and

α -amylase in the gastrointestinal tract (Ríos et al., 2015).

Recent studies have demonstrated that medicinal plants may effectively treat diabetes mellitus by suppressing inflammation and reducing oxidative stress. The increased production of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α , causes damage to pancreatic β cells, resulting in diminished insulin secretion and insulin resistance by decreasing the efficiency of insulin utilized by peripheral tissues. Inhibition of inflammatory cytokines is an important target for the treatment of Type 2 diabetes mellitus. Inhibiting the NF- κ B-mediated inflammatory signaling pathway reduces the synthesis of pro-inflammatory cytokines. The inactivation of the I κ B α protein, which inhibits NF- κ B, decreases the inflammatory response triggered by the NF- κ B-mediated signaling pathway by effectively inhibiting NF- κ B expression (Pang et al., 2019). JNK is a protein kinase that is a member of the mitogen-activated protein kinase family. It is triggered in response to multiple cellular and environmental challenges, including DNA damage, inflammatory cytokines, radiation, oxidative stress, cell proliferation, and apoptosis. JNK activation has been related to diabetes mellitus, insulin resistance, and obesity. TNF- α can activate several signaling cascades, including the NF- κ B and JNK pathways. Inhibition of the JNK pathway significantly enhances insulin sensitivity and improves glucose tolerance (Yung & Giacca, 2020). Previous studies have demonstrated that medicinal plants inhibit NF- κ B, JNK, IL-1 β , TNF- α , and Toll-like receptor 4 in the management of Type 2 diabetes mellitus (Pang et al., 2019).

Oxidative stress damages human pancreatic cells, resulting in the onset or progression of Type 2 diabetes mellitus. SOD, GSH, and CAT are antioxidant enzymes that efficiently neutralize free radicals in the human body. NO is an important bioactive product that can exacerbate oxidative stress in the human body. iNOS triggers the synthesis of the NO. MDA is a byproduct of lipid peroxidation that elevates oxidative stress in the organism. Medicinal plants are recog-

nized for their efficacy in diabetes mellitus treatment by enhancing antioxidant enzyme levels such as SOD, GSH, and CAT; diminishing MDA and NO levels; and suppressing iNOS (Hui et al., 2009; Pang et al., 2019; Ríos et al., 2015). Recent studies have demonstrated a correlation between gut microbiomes and Type 2 diabetes mellitus. Diseases in the gut microbiome have been revealed to be closely associated with insulin resistance and the development of Type 2 diabetes mellitus. It has been noted that microorganisms such as *Roseburia*, *Blautia*, and *Clostridium* are effective in Type 2 diabetes mellitus by altering the composition of the gut microbiota (Pang et al., 2019).

The absorption, synthesis, transport, and metabolism of lipids are primarily mediated by ABCA1, NPC1, LDLR, HMGCR, SREBP-2, ACLY, PPAR, CETP, CYP7A1, FAS, ACC, AMPK, CPT1A, ChREBP, HSL, ATGL, and LXR- α pathways, targets, and their downstream genes, which are essential for these mechanisms. Among them, the inhibition of ACC reduces free fatty acid concentrations in the bloodstream. ACAT2 is an important enzyme in the production and metabolism of triglycerides, and its inhibition can decrease triglyceride levels in serum. The pathways regulated by AMPK can be classified into four main categories, including protein metabolism, lipid metabolism, glucose metabolism, autophagy, and mitochondrial homeostasis. Activated AMPK decreases lipid synthesis by inhibiting the expression of downstream targets, serving as a crucial mechanism in the control of hyperlipidemia. Medicinal plants regulate these targets and pathways, treating hyperlipidemia related to diabetes mellitus (Liu et al., 2022).

The use of medicinal plants for the management of diabetes mellitus and dyslipidemia is common. Plant-derived extracts and their isolated secondary metabolites have demonstrated efficacy in diabetes mellitus and dyslipidemia in many experimental models (Adel Mehraban et al., 2021; Marmitt, Shahrajabian, Goettert, & Rempel, 2021; Ota & Ulrih, 2017; Ríos et al., 2015; Zhao & Chen, 2018). Considering the increasing prevalence of diabetes mellitus

and dyslipidemia in the community and the importance of medicinal plants as a therapeutic resource for these illnesses, it is key to evaluate the clinical effects of medicinal plants on these diseases and elucidate their potential mechanisms of action. This review aims to clarify the possible mechanisms of action of medicinal plants and their secondary metabolites that have been clinically examined in diabetes mellitus and dyslipidemia, based on current studies.

MATERIALS AND METHODS

Systematic reviews, meta-analyses, and traditional reviews on the use of medicinal plants in diabetes mellitus and dyslipidemia were searched using international databases (Science Direct, Scopus, and Web of Science), academic search engines (Google Scholar), and the Cochrane Library. The medicinal plants and secondary metabolites with the most emphasis and clinically studied efficacy in these publications were determined (Adel Mehraban et al., 2021; Marmitt, Shahrajabian, Goetttert, & Rempel, 2021; Ota & Ulrih, 2017; Rios et al., 2015; Zhao & Chen, 2018). Possible molecular mechanisms of action of the identified medicinal plants and secondary metabolites in the disease group were evaluated. Preclinical studies published between 2015 and 2023 were identified by using the name of the medicinal plant/secondary metabolite, the endocrine disease in which its activity was studied, and the keyword “molecular mechanism of action”.

RESULTS AND DISCUSSION

Allium sativum L. (Garlic)

Allium sativum (Amaryllidaceae) is an aromatic herbaceous annual plant used as a spice, food, and traditional medicine in different regions of the world. Garlic bulbs mainly contain sulfurous compounds such as alliin, allicin, ajoene, diallyl sulfide and its derivatives, allyl methyl sulfide, S-propyl-cysteine-sulfoxide, allicin, and S-methyl cysteine-sulfoxide. Previous studies have reported that *A. sativum* and its components are effective in diseases such as Alzheimer's, diabetes mellitus, obesity, hypertension, and dyslipid-

emia (El-Saber Batiha et al., 2020).

High-fat/sucrose diet-fed male Sprague-Dawley rats were treated with aged black garlic extract (ABG10⁺; 250 mg/kg, orally) rich in S-allyl cysteine and melanoidins for 8 weeks. ABG10⁺-treated rats were found to have elevated POMC gene expression and diminished ObR mRNA levels in the hypothalamus. ABG10⁺ overexpressed the β 3-ADR in visceral adipose tissue. The treatment with ABG10⁺ downregulated the gene expression of PPAR- γ , LPL, ObR, and HSL in subcutaneous adipose tissue. It significantly reduced the levels of PPAR- γ mRNA while overexpressing InsR, GLUT-4, UCP-1, and β 3-ADR in brown adipose tissue. Moreover, the obesity-induced rise in IL-1 β , TNF- α , and iNOS gene expression was suppressed in ABG10⁺-treated rats (Amor et al., 2019). It has been demonstrated that water extract prepared from garlic bulbs (150 mg/kg, 6 weeks, orally) boosted protein levels of ABCA1, HMGCR, and CYP46A1, which play a role in cholesterol homeostasis in the brain and isolated astrocytes of C57BL/6J mice. Similar outcomes were observed in cultured astrocytes that had been treated with allicin (Nazeri, Azizidoost, Cheraghzadeh, Mohammadi, & Kheirollah, 2021). The administration of garlic and fermented garlic (for both products: 300 mg/kg, 1 month, orally) to male Sprague-Dawley rats fed a cholesterol-rich diet significantly inhibited collagen and ADP-induced platelet aggregation and ATP secretion. These effects were seen to be more pronounced in rats treated with fermented garlic. Furthermore, SREBP-2, ACAT-2, and HMG-CoA reductase expressions were downregulated in garlic-treated rats (Irfan et al., 2019). Taken together, garlic extracts and sulfur compounds in its composition are effective in metabolic diseases by modulating pathways regulating appetite, lipid metabolism, and insulin sensitivity, suppressing inflammation, and inhibiting platelet aggregation.

Cinnamomum sp. (Cinnamon)

The dried stem bark of *Cinnamomum verum* J.S. Presl. (syn: *Cinnamomum zeylanicum* Nees, Ceylon

cinnamon), *Cinnamomum cassia* Blume (syn: *Cinnamomum aromaticum* Nees, Chinese cinnamon), *Cinnamomum burmanni* (Nees & T.Nees) Blume (Indonesian cinnamon), and *Cinnamomum loureirii* Nees (Saigon cinnamon, Vietnamese cinnamon) (Lauraceae) are used medicinally (İşcan, Bektaş Sarialtin, Soyseven, & Arli, 2022). The essential oils responsible for biological activity consist of cinnamic acid and its derivatives, phenylpropanoid compounds, and terpenes. The major component of *C. verum* and *C. cassia* essential oils is cinnamaldehyde, which has a phenylpropanoid structure. Moreover, compounds with oligomeric proanthocyanidin, glycoside, lignan, lactone, and coumarin structures were isolated from cinnamon species. Previous studies have demonstrated that different cinnamon species and their secondary metabolites have a wide range of pharmacological effects, including antitumoral, anti-inflammatory, analgesic, antidiabetic, antiobesity, antimicrobial, anti-tyrosinase, cardioprotective, immunomodulatory, cytoprotective, and neuroprotective activities (Hariri & Ghiasvand, 2016; Yanakiev, 2020; Zhang et al., 2019).

Vijayakumar et al. tested the antidiabetic and hypolipidemic mechanisms of action of different extracts prepared from the bark of *C. cassia* in different experimental models, including *in vitro*, *in vivo*, and *in silico*. In the *in vitro* study, water, ethanol, and methanol extracts inhibited α -amylase with IC_{50} values of 0.79, 0.62, and 0.77 mg/mL, respectively (acarbose: 0.56 mg/mL). Water, ethanol, and methanol extracts inhibited the α -glucosidase enzyme with IC_{50} values of 0.91, 0.57, and 0.78 mg/mL, respectively (acarbose: 0.50 mg/mL). In the STZ-induced diabetic rat model, ethanol extract (300, 400, and 500 mg/kg b.wt doses, 28 days, oral) decreased glucose-6-phosphatase and fructose 1,6-bisphosphatase levels while increasing glucokinase levels. The results of molecular docking studies revealed that 9-octadecenoic acid was the compound with the highest affinity for PPAR- α/γ among the active constituents of cinnamon (Vijayakumar et al., 2023). In an HFD-fed obese mouse model,

male C57BL/6 mice were administered 100 or 300 mg/kg of water extract prepared from *C. cassia* bark for 16 weeks. The extract dose-dependently enhanced MyHC, PGC-1 α protein expressions, and AMPK phosphorylation in HFD mice. In the continuation of the study, C2C12 myoblasts were incubated with 0.1 or 0.2 mg/ml extract for 24 hours. The extract significantly raised ATP levels in C2C12 myoblast cells by stimulating mRNA expression of mitochondrial biogenesis-related factors such as PGC-1 α , Nrf-1, and Tfam, inducing phosphorylation of AMPK, and up-regulating expression of ACC. These results suggested that *C. cassia* extract controls weight gain by up-regulating mitochondrial biogenesis in skeletal muscle cells (Song et al., 2017). The results of another *in vivo* study have shown that administration of *C. zeylanicum* powder (5%, 28 days, orally) boosted pancreatic SOD, CAT, and GPx activities in an alloxan-induced mouse model (Beji, Khemir, Wannes, Ayari, & Ksouri, 2018). Kaur et al. reported that treatment with *C. zeylanicum* (2 mg powder/fish/day, 4 weeks, orally) exhibited positive effects on lipid metabolism by downregulating SREBF1, SREBF2, LDLR, and NR2F2 gene expression and upregulating FOXO1 expression in a diet-induced zebrafish model (Kaur et al., 2019). Polyphenol-rich *C. zeylanicum* hydroalcoholic extract was administered to HFD-fed Wistar rats at a dose of 100 mg/kg b.wt diet for 12 weeks. The extract decreased serum and liver MDA concentrations and elevated serum TAC, liver SOD, CAT, and GPx activities. In extract-treated rats, hepatic SREBP-1c, LXR- α , ACLY, FAS, and NF- κ B p65 expression were suppressed, while PPAR- α , IRS-1, Nrf2, and HO-1 expression were induced. Cinnamon polyphenol may ameliorate hyperlipidemia by activating transcription factors and the antioxidant defense signaling pathway in the livers of rats on an HFD (Tuzcu, Orhan, Sahin, Juturu, & Sahin, 2017). Khare et al. studied the effects of cinnamaldehyde, which is the active component of cinnamon, on fasting-induced hyperphagia and related hormone levels, adipose tissue lipolysis, and inflammation in mice fed a high-fat diet. Cinnamal-

dehyde was administered orally at doses of 5 and 10 mg/kg/b.wt for 7 days. Treatment with cinnamaldehyde at both doses reduced serum leptin levels. Cinnamaldehyde administration at 10 mg/kg increased total ghrelin serum levels, although not statistically significant compared to HFD-fed mice. It enhanced the expression of POMC and urocortin in the hypothalamus, while BDNF, CARTPT, and CCK were not affected at a 5 mg/kg dose. At high doses, it significantly raised the expression level of these genes in the hypothalamus. Cinnamaldehyde downregulated the expression of the adipogenic marker gene (CCAAT/enhancer-binding protein; C/EBP- α) and upregulated the expression of the DLK-1 and lipolysis-inducing genes (PNPLA2 and MGLL) at a dose of 10 mg/kg. Cinnamaldehyde treatment at this dose was detected to induce the expression of BMP4, PRDM16, and FOXC2. Cinnamaldehyde is concluded to diminish hunger-induced hyperphagia by enhancing lipolysis in adipose tissue, restoring the circulating leptin/ghrelin ratio to normal levels, and mitigating inflammation. Moreover, cinnamaldehyde at a dose of 10 mg/kg suppressed the inflammatory process by lowering the level of IL-1 β and the expression of COX, MCP1, TNF- α , and IL-6. In the continuation of the study, the effect of cinnamaldehyde on cecal microbiota (*Lactobacillus*, *Bifidobacteria*, and *Roseburia* species) was assessed. It was determined that cinnamaldehyde did not affect the abundance of these bacterial genera (Khare et al., 2016). In an *in vitro* study, trans-cinnamic acid (tCA, 0–200 μ M, 6–8 days) was demonstrated to upregulate the expression of brown adipose tissue-specific markers (PGC-1 α , PRDM16, UCP1) and beige adipose tissue-specific genes (Cd137, Cidea, Cited1, Tbx1, Tmem26) in 3T3-L1 white adipocytes. Similarly, tCA boosted the expression of PGC-1 α , PRDM16, and UCP1 in HIB1B brown adipocytes. Expression of tissue-specific genes (Lhx8, Ppargc1, Prdm16, Ucp1, and Zic1) was induced in HIB1B brown adipocytes incubated with tCA. The expression levels of adipogenic transcription factors (e.g., C/EBP α and PPAR- γ) were measured in both tissues. C/EBP α and PPAR- γ

expression was diminished in white adipocytes while elevated in brown adipocytes. tCA upregulated the expression of mitochondrial biogenic genes such as Cox4, Nrf1, MtDNA, and Tfam in both tissues. In addition to these findings, treatment of 3T3-L1 cells with tCA decreased the expression levels of lipogenic markers (ACC and FAS) through the activation of AMPK and β 3-AR signaling pathways. Further, tCA increased lipolysis by increasing the expression levels of pHSL and ATGL. tCA treatment also significantly enhanced the mitochondrial protein levels of ACOX1 and CPT1. In conclusion, the results revealed that tCA may be effective in the treatment of obesity by reducing adipogenesis and lipogenesis and increasing fat oxidation (Kang et al., 2019). Mechanisms such as increasing glucose uptake in muscle and adipose tissue by inducing GLUT-4 production and translocation, stimulating glycogen synthesis in the liver by inhibiting GSK 3 β , and downregulating PEPCK gene expression are thought to be responsible for the positive effects on diabetes mellitus (Silva et al., 2022). Research suggests that cinnamon and cinnamic acid-derived compounds affect diabetes, obesity, and related metabolic disorders by decreasing carbohydrate digestion, reducing hepatic glucose production, enhancing cellular glucose uptake, inhibiting lipogenesis, facilitating lipolysis, augmenting energy expenditure, and through mechanisms including potent antioxidant and anti-inflammatory properties.

***Curcuma longa* L. (Turmeric)**

The rhizomes of *Curcuma longa* (Zingiberaceae), known as turmeric, are used for therapeutic purposes. Curcuminoids, including curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are responsible for the pharmacological effects. The essential oil extracted from the rhizomes of *C. longa* contains various monoterpene and sesquiterpene compounds such as zingiberene, curcumin, and α - and β -turmerone. Previous studies have reported that *C. longa* and its active component, curcumin, exhibit a wide range of pharmacological effects involving anti-inflammatory, antioxidant, antidiabetic, antiangiogenic, anti-

mutagenic, antimicrobial, and anticancer activities. In recent years, clinical studies have been conducted to evaluate the efficacy of *C. longa* and curcumin in cancer, autoimmune diseases, metabolic diseases, neurological diseases, cardiovascular diseases, lung diseases, liver diseases, and other inflammatory diseases (Karłowicz-Bodalska, Han, Freier, Smolenski, & Bodalska, 2017; Kocaadam & Şanlıer, 2017).

STZ-induced diabetic rats were administered curcumin-loaded PLA-PEG copolymer nanoparticles (20 mg/kg b.wt/day, orally). Curcumin decreased oxidative stress markers such as MDA and NO while elevating glutathione levels. It reduced serum NF- κ B, TGF- β , and COX-2 levels. It also upregulated PPAR- γ mRNA expression levels in rats (El-Naggar, Al-Joufi, Anwar, Attia, & El-Bana, 2019). Zhong et al. administered curcumin at a dose of 100 mg/kg/day orally for 4 weeks to C57BL/6J mice fed a high-fat diet. Curcumin upregulated insulin-stimulated Akt phosphorylation levels in peripheral tissues. It suppressed the expression of genes involved in hepatic gluconeogenesis (Pck1 and G6pc) and de novo lipogenesis (SREBF1) in HFD mice. It upregulated the expression of FGF15, which is a gut-derived hormone. The observed effects were attributed to the modulation of the gut microbiota (Zhong et al., 2022). Curcumin (50 mg/kg/day, 8 weeks, orally) induced the expression of adipose triglyceride lipase, hormone-sensitive lipase, PPAR- γ/α , and C/EBP- α in the adipose tissue of male C57BL/6J obese mice fed a high-fat diet. Besides, it stimulated lipolysis and improved glycolipid metabolism. In a second study, differentiated 3T3-L1 adipocytes were incubated with curcumin (10, 20, and 35 μ M) for 48 hours. Curcumin lowered glycerol release and increased glucose uptake by upregulating PPAR- γ and C/EBP- α (Pan et al., 2017). Maithilikarpagaselvi et al. determined that curcumin treatment (200 mg/kg/b. wt, orally, for 10 weeks) improved insulin resistance in male Wistar rats fed a high fructose diet by decreasing IRS-1 serine phosphorylation and enhancing IRS-1 tyrosine phosphorylation in skeletal muscles. It decreased the expression of the proteins COX-2 and

PKC- θ , as well as TNF- α and CRP levels. It promoted GPx activity while inhibiting MDA and total oxidant status. Further, curcumin suppressed ERK1/2 and p38 protein expression in the skeletal muscle of rats. The study indicated that curcumin ameliorates glucose intolerance and insulin resistance by exerting its antioxidant and anti-inflammatory properties (Maithilikarpagaselvi, Sridhar, Swaminathan, & Zachariah, 2016). In a diet-induced obesity model, male C57BL/6 mice were given curcumin at doses of 40 and 80 mg/kg/day by oral gavage for 12 weeks. It significantly diminished the hepatic mRNA expression of SREBP-1 and SREBP-2. Hepatic mRNA expression of genes involved in cholesterol biosynthesis, such as HMG-CR, FDPS, DHCR24, DHCR7, LSS, MVK, Sc4mol, SS, proprotein convertase subtilisin/kexin type 9, and LDLR, was downregulated by curcumin administration. Expression of genes playing a role in fatty acid metabolism, such as ACC1, ACL, ACS, FAS, FADS1, FADS2, SCD-1, and GPAT, was also suppressed with curcumin. It did not affect hepatic ATP-binding cassette, ABCA1, ABCG5, or ABCG8 mRNA levels. Taken together, this study concluded that curcumin improves lipid and glucose homeostasis through the regulation of SREBP and important downstream target genes (Ding et al., 2016). The authors suggest that *C. longa* and curcuminoids affect diabetes mellitus by enhancing insulin sensitivity, diminishing hepatic glucose production, suppressing oxidative stress and inflammation, and regulating lipid metabolism.

Ginseng

Panax ginseng C.A.Mey. (Asian ginseng, Korean red ginseng) and *Panax quinquefolius* L. (American ginseng) (Araliaceae) are two important ginseng species that are prominent for their effects on diabetes mellitus and other endocrine system diseases. Previous phytochemical studies have shown that roots and rhizomes contain saponins, polysaccharides, polyacetylene, phenol, and alkaloid compounds. "Ginsenosides", classified in three different forms as dammaran (protopanaxadiol and protopanaxatriol), ocotillol, and oleanane type, are compounds with a saponin

structure that are responsible for the pharmacological effects of ginseng plants. Ginseng has been the subject of many preclinical and clinical studies with its cardioprotective, cerebrovascular protective, neuroprotective, antitumoral, anti-inflammatory, hemostatic, anticoagulant, antidiabetic, anti-aging, and immunomodulatory effects (Chen, Balan, & Popovich, 2019; Liu, Lu, Hu, & Fan, 2020).

Shin *et al.* documented that water extract prepared from *P. ginseng* roots (GE, 5%, eight-week diet) decreased body weight, adipose tissue, and adipocyte size without affecting food intake in a testosterone-deficient castrated C57BL/6J mouse model fed a high-fat diet. GE suppressed the mRNA expression of adipogenesis-related genes (SREBP1C, PPAR- γ , FAS, SCD1, and ACC1) in visceral adipose tissues. In a second study, 3T3-L1 adipocytes were incubated with 10 μ g/ml GE and 10 μ M ginsenosides (Rb1 and Rg1) for 24 hours. The extract and ginsenosides inhibited lipid accumulation and mRNA expression of PPAR- γ , C/EBP- α , and SCD1. These effects were potentiated by co-administration of the extract or ginsenosides with testosterone. These results suggested that ginseng exhibits positive effects on obesity and dyslipidemia by inhibiting adipogenic gene expression and acting like testosterone (Shin & Yoon, 2018). Nagar *et al.* injected an ethanol extract of *P. ginseng* roots (KRG) and KRG enriched with ginsenoside Rg3 into the jugular vein of SHR at a dose of 3 mL/kg. The results showed that endothelial nitric oxide synthase phosphorylation levels in the aorta and NO production in plasma were induced (Nagar, Choi, Jung, Jeon, & Kim, 2016). The mechanism of the antidiabetic effect of hydromethanolic extract rich in malonyl ginsenosides (PQ-MGR) prepared from *P. quinquefolius* roots was studied in an HFD/STZ-induced Type 2 diabetes mellitus model. C57BL/6J male mice were administered dietary PQ-MGR at doses of 100 and 300 mg/kg/day for 5 weeks. PQ-MGR administration upregulated protein expression of *p*-PI3K, *p*-AKT, *p*-AMPK, *p*-ACC, PPAR- γ , and GLUT-4 in liver and skeletal muscle while down-regulating protein expression of *p*-IRS1 and *p*-JNK.

This study demonstrated that PQ-MGR improved insulin resistance through inhibition of the JNK signaling pathway and activation of the IRS1/PI3K/AKT signaling pathway (Liu *et al.*, 2021). The activity of the ginsenoside Rb2 on glucose metabolism and fat accumulation in the insulin-sensitive cell line 3T3-L1 adipocytes and high-fat diet-induced obesity mice was investigated. Ginsenoside Rb2 induced glucose uptake in 3T3-L1 adipocytes by activating the PI3K/AKT signaling pathway at a concentration of 25 μ M. Ginsenoside Rb2 suppressed TNF- α -induced activation of JNK, ERK, and P38 and phosphorylation of IRS-1 at Ser307. It also inhibited TNF- α -induced IKK α/β phosphorylation and IKK α/β activation. It reduced the expression of IL-6 and suppressor of SOCS-3. Administration of ginsenoside Rb2 (40 mg/kg/d, 10 days, *i.p.*) to C57BL/6J mice upregulated *p*-AKT (Ser473) expression, which supported the *in vitro* finding. The results indicate that ginsenoside Rb2 enhances the uptake of glucose and improves insulin sensitivity via AKT-dependent pathways. (Dai *et al.*, 2018). Another study treated male Wistar rats with ginsenoside Re at a dose of 25 mg/kg/b.wt once daily by gastric irrigation for 30 days in an HFD/STZ-induced Type 2 diabetes mellitus model. Ginsenoside Re diminished oxidative stress by increasing SOD and CAT activities and lowering MDA levels. Ginsenoside Re was found to activate the AMPK signaling pathway by stimulating the cannabinoid type 1 receptor and CaMK II (Wang *et al.*, 2021). The hypoglycemic, anti-inflammatory, and lipid-lowering effects of 25-OH-PPT (20 and 40 mg/kg, 5 weeks, orally), which is a triterpenoid saponin isolated from the stem and leaves of *P. ginseng*, were examined in STZ-induced hyperglycemic mice and DB/DB mouse models. In the STZ mouse model, 25-OH-PPT at both doses diminished the transcription levels of IL-1, IL-6, and COX-2. It did not affect the transcription of TNF- α . In the DB/DB mouse model, 25-OH-PPT decreased the transcription levels of IL-1, IL-6, and TNF- α . It significantly upregulated skeletal muscle GLUT4 and AMPK transcription levels in the STZ-in-

duced mouse model, while it did not affect transcription in DB/DB mice. In both mouse models, 25-OH-PPT treatment upregulated phosphorylated IR, IRS, and AKT protein expression but did not alter GSK 3 β expression. The findings suggested that 25-OH-PPT increases insulin sensitivity by activating the insulin signaling pathway (Xu et al., 2021). Ginseng and its constituent ginsenosides have demonstrated potential in diabetes management through direct modulation of insulin sensitivity and glucose metabolism at the cellular level, as well as by regulating factors such as inflammation and oxidative stress that increase the severity of diabetes.

***Juglans regia* L. (Walnut)**

The different parts of *Juglans regia* (Juglandaceae), including leaf, fruit (husk, nut), bark, inflorescence, and root, have been used for medicinal purposes for many years. Different parts of the plant contain phenolic compounds (3- and 5-caffeoylquinic acid, 3- and 5-p-coumaroylquinic acid, quercetin and derivatives, taxifolin-pantocid, syringetin-*O*-hexoside, myricetin-3-*O*-glucoside, myricetin-3-*O*-pantocid, and epicatechin), naphthalene derivatives (juglone), and organic compounds (citric acid, malic acid, phosphate, and calcium oxalate). The most important secondary metabolites detected in the leaves and green husk of the walnut are juglone and phenolic compounds. Walnut oil is rich in polyunsaturated fatty acids, tocopherols, and phytosterols. Previous studies have stated that walnuts and their secondary metabolites have broad pharmacological effects, including antioxidant, antimicrobial, anticancer, antidiabetic, antihypertensive, and lipid-lowering activities (Bhat et al., 2023; Delaviz, Mohammadi, Ghalamfarsa, Mohammadi, & Farhadi, 2017).

The effect of an ethanol extract prepared from *J. regia* leaves (200 mg/kg, 21 days, orally) on STZ⁺ nicotinamide-induced Type 2 diabetes mellitus was evaluated using Wistar albino rats. The extract alleviated insulin resistance by increasing ADP and FNDC5 levels. It suppressed inflammation by reducing serum

TNF- α levels (Atila Uslu & Uslu, 2022). *J. regia* oil-derived polyunsaturated fatty acid was administered orally to male Wistar rats at doses of 225, 450, and 900 mg/kg b.wt for 18 days in a model of STZ-induced gestational diabetes mellitus. PUFA administration ameliorated oxidative stress by elevating hepatic SOD, GSH, and CAT activities and lowering MDA content. PUFA suppressed the enhanced expression of SREBP-1, SCD-1, ACC, and FAS mRNA in the liver tissue of gestational diabetes mellitus rats. The observed effects were found to be dose-dependent (Sun et al., 2020). Treatment with walnut-derived peptides (fractions 3–10 kDa and LP5) boosted SOD and GSH activities and inhibited TNF- α , IL-6, and IL-1 β secretion in mice with Type 2 diabetes mellitus induced by STZ and HFD. Walnut-derived peptides augmented liver mitochondrial ATP, COX, SDH, and MMP levels. It upregulated LC3-II/LC3-I and Beclin-1 expression while downregulating p62 expression, which may be associated with activation of the AMPK/mTOR/ULK1 pathway. In conclusion, walnut-derived peptides promoted autophagy through activation of the AMPK/mTOR/ULK1 pathway to ameliorate hyperglycemia in mice with Type 2 diabetes mellitus (Hou et al., 2023). In another study, male Sprague-Dawley rats fed an HFD were given orally walnut meal peptides at doses of 200, 400, and 800 mg/kg for 4 weeks. Walnut meal peptides increased Apo-1 levels while decreasing Apo-B levels. Expression of FAS and HMGR was significantly inhibited in the livers of rats treated with walnut meal peptides. Moreover, walnut meal peptides induced the expression of LCAT and CYP7A1 (Yang, Zhong, Wang, Zhang, & Zhang, 2021). Walnuts and their components are effective in diabetes by improving insulin sensitivity, reducing inflammation and oxidative stress, regulating fat metabolism, and promoting autophagy and mitochondrial function.

***Linum usitatissimum* L. (Flaxseed)**

Linum usitatissimum is used for food and medicinal purposes in four common types: whole flaxseed, ground flaxseed, flaxseed oil, and partially defatted flaxseed meal. Flaxseed is particularly enriched in

α -linolenic acid (ALA), fiber, and lignans (mainly secoisolariciresinoldiglucoside). Flaxseed and its isolated secondary metabolites prevent, regress, and slow the progression of cardiovascular diseases such as lipid-induced coronary artery disease, peripheral artery disease, and stroke by lowering serum lipid levels. Secoisolariciresinoldiglucosides, which are responsible for the biological effects, act by being converted into enterolignans such as enterodiol and enterolactone by intestinal bacteria through biotransformation. Many studies have shown that flaxseed increases the diversity of the gut microbiota and the relative abundance of beneficial bacteria (Parikh et al., 2019; Prasad, Khan, & Shoker, 2020).

Bouzghaya et al. reported that treatment with flaxseed extract (1 mL of aqueous extract per mouse, five weeks, oral) ameliorated oxidative stress in alloxan-induced diabetic mice by decreasing hepatic and renal TBARS content and increasing GP_x, SOD, and CAT activities. Phytochemical analysis has revealed that the water extract contained four phenolic compounds (gallic acid (GA), vanillic acid, resveratrol, and coumaric acid) and three flavonoids (kaempferol, apigenin, and luteolin). The secondary metabolite with the highest amount was detected as GA. In the continuation of the study, GA was treated in mice by gavage at a dose of 50 mg/day for 5 weeks. GA significantly improved the activity of antioxidant markers in the liver and kidney, similar to the findings obtained in the water extract. These results suggested that flaxseed water extract has antihyperglycemic and antioxidative activities associated with its high GA content (Bouzghaya, Amri, & Homblé, 2020). Whole-grain flaxseeds (7.5 g/kg rat/day, 90 days, orally) significantly enhanced the levels of catalase, SOD, GPx, GR, GST, and cellular antioxidant enzymes in heart and liver tissues in male Wistar albino mice fed a high cholesterol diet. Although it did not reach statistical significance, reductions in TBARS levels have been observed in heart and liver tissues (Naik et al., 2018). Ground flaxseed (16.7%) was coadministered with a diet for 6 weeks to C57BL/6J male mice in a model of

STZ-induced diabetes mellitus. Treatment with flaxseed decreased glucose tolerance in the OGTT and raised glycogen levels in the liver. It reduced TNF- α levels in the liver but did not affect IL-1 β or IL-6 levels. It also downregulated proinflammatory gene expression, including TLR4, MyD88, and NF- κ B. Moreover, there was a significant increase in the number of some beneficial bacteria in the gut microbiota of the mice in the treatment group. Taken together, these results demonstrate that it is effective in treating diabetes mellitus by suppressing the TLR4/NF- κ B signaling pathway and improving the gut microbiota (Xia et al., 2022). Zhu et al. showed that flaxseed oil (10% w/w, 5 weeks, oral) lowered plasma lipopolysaccharide, IL-1 β , TNF- α , IL-6, IL-17A, and MDA levels and boosted SOD levels in male Sprague-Dawley rats in a STZ-nicotinamide-induced Type 2 diabetes mellitus model. As a result of the intestinal microbiota analysis, it was found that there was a decline in the relative abundance of *Firmicutes* and *Blautia* and the ratio of *Bacteroidetes* to *Firmicutes* in the intervention group. There was an increase in the relative abundance of *Bacteroidetes* and *Alistipes*. Treatment with flaxseed oil has enhanced the levels of intestinal microbiota metabolites of short-chain fatty acids such as acetic acid, propionic acid, and butyric acid (Zhu et al., 2020). Another study assessed the potential mechanism of action of flaxseed oil on obesity and metabolic disorders in mice fed an HFD. Flaxseed oil was fed to rats for 5 days at three different concentrations: 30% (m/m, LFO), 60% (m/m, MFO), and 90% (m/m, HFO). Flaxseed oil lowered the concentrations of conjugated bile acids such as cholic acid, tauro- α -murocholic acid, and tauro-ursodesoxycholic acid in feces. It improved intestinal microbial dysbiosis by increasing the abundance of Clostridiales and Ruminococcaceae and community diversity. It decreased the expression of the FXR and FGF-15 proteins in the colon. These effects were observed to be more pronounced in the HFO group. In the continuation of the study, the effect of flaxseed oil administration on FXR-related lipid metabolism genes in the liver was tested. It up-

regulated the mRNA expression of bile acid receptors (FXR and SHP) and the expression of lipolysis-related genes (CPT-1 α and ATG5). The mRNA expression of the lipogenesis-related SREBP-1c and PPAR- α genes diminished, while the mRNA expression of FAS was not altered. It induced the mRNA expression of CYP7A1 in the liver but did not change the expression of another bile acid synthesis gene, CYP8B1. Taken together, these findings suggested that flaxseed oil improved serum lipid concentrations in metabolic diseases by regulating the gut microbiota, modulating the FXR-FGF15 signaling pathway, and affecting bile acid metabolism (Yang et al., 2023). The effect of herbacetin (10, 20, and 40 mg/day, intragastric, 5 weeks), a flavonoid isolated from flax seeds, on obesity induced by a fatty diet in male C57BL/6J mice was investigated. Herbacetin significantly reduced body weight, plasma glucose, plasma insulin, and HOMA-IR activity in obesity-associated insulin-resistant mice. It significantly suppressed the mRNA expression of SREBP-1c and 2. It also decreased FAS, hepatic fatty acid β -oxidation, and G6PD activity, whereas it enhanced malic enzyme and CPT activity (Veeramani et al., 2018). In summary, flaxseed presents potential therapeutic benefits for diabetes mellitus and obesity through multiple pathways, including antioxidant and anti-inflammatory properties, modulation of gut microbiota and metabolites, enhancement of lipid metabolism and weight management, and improvement of glucose tolerance.

***Momordica charantia* L. (Bitter Melon or Bitter Gourd)**

The fruits of *Momordica charantia* (Cucurbitaceae), known as bitter melon or bitter gourd, are used for therapeutic purposes. Secondary metabolites in the structure of sterol (daucosterol, β -sitosterol, campesterol, and stigmasterol), terpenoid (charantin A and B, charantagenins D and E, kuguasides A, B, C, and D, charantoside A, momordicosides I, F1, F2, K, L, and U, momordicine I, II, VI, VII, and VIII), phenolic acid (caffeic, coumaric, ferulic, and gallic acids), flavonoid, amino acid, vitamin, peptide, and protein,

ribosome-inactivating proteins, polypeptide-P, inhibitory proteins, and P-insulin were isolated from the fruit and other parts of *M. charantia*. The hypoglycemic effect of *M. charantia* fruits and their isolated secondary metabolites comes to prominence. The hypoglycemic effect has been particularly related to charantin, polypeptide-P, and visin. It also has anti-inflammatory, antioxidant, antimicrobial, antitumoral, immunomodulatory, and hepatoprotective effects (Oyelere et al., 2022; Pahlavani et al., 2019). Ethanol extract prepared from *M. charantia* fruits (MCE) was administered orally for eight weeks at three different doses (100, 200, and 400 mg/kg/day) in a rat model of Type 2 diabetes mellitus induced by an HFD and STZ injection. In a dose-dependent manner, serum levels of TNF- α and IL-6 were significantly decreased in all groups treated with MCE. MCE treatment dose-dependently upregulated Akt-2 gene expression levels while downregulating mRNA expression of PTP-1B, SOCS-3, and JNK. MCE elevated GLUT-4 protein levels in skeletal muscle and liver tissues (Ma, Yu, Xiao, & Wang, 2017). In rats with Type 2 diabetes mellitus induced by an HFD and STZ, treatment with ethanol extract prepared from wild bitter gourd fruits (150 and 300 mg/kg, 4 weeks, orally) enhanced the content of antioxidant enzymes such as SOD, CAT, and GSH in serum and diminished the levels of inflammatory parameters including CRP, TNF- α , and IL-6. In molecular mechanism studies, it upregulated hepatic protein expression levels of p-AKT and p-IRS but downregulated p-GSK3 β (Ser9) protein expression. It exerted its effect by activating the AMPK signaling pathway and inducing protein expression of GLUT-2. All effects were dose-dependent (Sun et al., 2023). Yang *et al.* treated male OLETF rats fed an HFD with freeze-dried *M. charantia* fruit powder at concentrations of 1% and 3% for 6 weeks. It reduced the expression of the proinflammatory cytokine TNF- α and the levels of IL-6 and CCL2 in the liver, muscle, and epididymal adipose tissue. It suppressed the activation of NF- κ B in the liver and muscle. Administration of *M. charantia* at 3% concentration upregulated phospho-insulin receptor substrate-1 (Tyr612) and phospho-Akt

(Ser473) levels while downregulating phospho-NF- κ B (p65) (Ser536) and JNK (Thr183/Tyr185) levels. Taken together, the molecular mechanism mediating the antidiabetic effect of *M. charantia* fruits appeared to be the modulation of NF- κ B and JNK signaling pathways (Yang et al., 2015). Wang et al. administered saponin (L-SMC: 20 mg/kg, M-SMC: 40 mg/kg, H-SMC: 80 mg/kg) and polysaccharides (PMC, 500 mg/kg) isolated from *M. charantia* fruits by oral gavage to male Kunming mice for five weeks in an STZ-induced Type 2 diabetes mellitus model. SMC and PMC improved antioxidant capacity by increasing SOD levels and decreasing MDA levels in the liver. M- and H-SMC treatment suppressed NF- κ B levels. M-SMC induced hepatic AMPK phosphorylation. PMC did not affect AMPK phosphorylation or NF- κ B levels. These results suggested that the *M. charantia* saponin fraction was more effective than polysaccharides, and the possible antidiabetic mechanism of action was related to the regulation of the AMPK/NF- κ B signaling pathway (Wang, Wu, Shi, & Liu, 2019). The effect and mechanism of action of four different cucurbitane types (C1–C4) triterpenoid compounds isolated from ethanol extract prepared from *M. charantia* fruits on diabetes mellitus were evaluated. Among these secondary metabolites, C2 (1.68 mg/kg, *i.v.*) significantly boosted glycogen storage in skeletal muscles in an STZ-induced diabetes mellitus model. The hypoglycemic effect of C2 in diabetic mice was found to be associated with the activation of IRS-1 and its downstream signaling pathways, including Akt, GSK-3 β , Erk1/2, and PKC ζ / λ . It did not have any effect on AMPK or ACC (Han et al., 2018). In another study, 3 β ,7 β ,25-trihydroxycucurbita-5,23(E)-dien-19-al, a cucurbitane triterpenoid isolated from *M. charantia*, was demonstrated to activate PPAR- γ (Noruddin et al., 2021). Bitter melon displays significant antidiabetic properties by enhancing insulin sensitivity and glucose absorption through the activation of insulin signaling pathways (Akt, IRS-1, PPAR- γ) and AMPK. It also mitigates inflammation (via NF- κ B, JNK) and oxidative stress (through the modulation of antioxidant enzymes) that facilitate diabetes progression.

***Nigella sativa* L. (Black cumin or Black seed)**

The dried seeds of *Nigella sativa* (Ranunculaceae), known as black cumin, are used for therapeutic purposes. Thymoquinone, the major component of the essential oil, is responsible for its pharmacological effects. The essential oil also contains compounds such as thymol, carvacrol, phellandrene, α -pinene, and β -pinene. Phenolic acids, flavonoids, alkaloids, saponins, and fatty acids were isolated from different parts of the plant. Previous studies have demonstrated that various extracts from seeds and thymoquinone exhibit antioxidant, anti-inflammatory, antihepatotoxic, analgesic, antineoplastic, antimutagenic, antinephrotoxic, immunomodulatory, hypoglycemic, antiulcer, and antimicrobial activities (Dalli et al., 2021; Gürel & Çiçek Polat, 2025).

Balbaa *et al.* administered black cumin oil at a dose of 100 mg/day for 21 days intragastrically to male albino Sprague-Dawley rats in an experimental diabetes mellitus model induced by HFD and STZ. Black cumin oil reduced serum TBARS, hepatic TBARS, serum NO, hepatic NO, and serum TNF- α levels in diabetic rats. It upregulated the gene expression of IGF-1, PI3K, and IR while suppressing the expression of ADAM-17 (Balbaa, El-Zeftawy, Ghareeb, Taha, & Mandour, 2016). *N. sativa* seed polysaccharides were administered at three different doses of 35, 70, and 140 mg/kg for 4 weeks intragastrically to Kunming mice in an STZ- and HFD-induced Type 2 diabetes mellitus model. The high dose lowered MDA, TNF- α , IL-6, and IL-1 β levels while elevating total antioxidant capacity, SOD, and CAT levels in the skeletal muscle tissues of mice. It also stimulated *p*-AKT and GLUT-4 protein levels and expression. Gut microbiota analysis showed that the abundance of *Muribaculaceae* unclassified and *Bacteroides* suppressed by diabetes mellitus was significantly boosted by black cumin treatment. This study revealed that *N. sativa* seed polysaccharides are effective in treating diabetes mellitus by modulating the gut microbiota and activating the PI3K/AKT signaling pathway (Dong et al., 2020). The hydroalcoholic extract prepared from *N.*

sativa seeds (100, 200, and 400 mg/kg, 6 weeks, orally) significantly improved vasorelaxant responses to acetylcholine at all doses in male Wistar rats in the STZ-induced diabetes mellitus model. It upregulated eNOS mRNA expression while downregulating VCAM-1 and LOX-1 mRNA expression in vascular cells of aortic tissue. These results suggested that *N. sativa* ameliorated vasoreactivity, endothelial dysfunction, and vascular inflammation in the aorta of diabetic rats (Abbasnezhad et al., 2019). Adult female Wistar rats were treated intraperitoneally with black seed oil at a dose of 0.2 mg/kg/day for 6 days a week for 30 days in an STZ-induced diabetes mellitus model. It significantly inhibited the expression of Bax and caspase-3 in abdominal and thoracic aortic sections (Cüce et al., 2016). In another study, black cumin seed oil treatment (400 mg/kg, orally, for 21 days) overexpressed Bcl2 in female Wistar albino rats in a model of STZ-induced diabetes mellitus (Altun, Avci, Yildirim, & Yildirim, 2019). These studies concluded that the cardiovascular effects of black seed oil are associated with suppression of apoptosis (Cüce et al., 2016; Altun et al., 2019). Black cumin oil (2.5 mg/kg/day, 8 weeks, orally) reversed L-NAME-induced high blood pressure in adult male Sprague-Dawley rats. The effects of black cumin oil on blood pressure have been associated with a lowering of the content of the cardiac lipid peroxidation product MDA, inhibition of cardiac NADPH oxidase and angiotensin-converting enzyme activities, and a reduction in plasma nitric oxide, as well as an elevation of HO-1 activity in the heart (Jaarin et al., 2015). Collectively, black cumin and thymoquinone have been proposed to exert antidiabetic effects through various mechanisms of action, such as reducing insulin resistance, accelerating β -cell proliferation, augmenting pancreatic insulin secretion and glucose uptake, and attenuating oxidative stress. It inhibited hepatic gluconeogenesis through the reduction of gluconeogenic precursors such as glycerol, alanine, and lactate. The other possible mechanism is the activation of the insulin signaling pathway, the AMPK signaling pathway, and the PPAR- γ pathways

in skeletal muscle cells, hepatocytes, and adipocytes (Maideen, 2021). Previous studies stated that it exhibits antihyperlipidemic activity by strongly inhibiting the hepatic HMG-CoA reductase enzyme, inducing arylesterase activity, and playing a regulatory role in genes affecting cholesterol metabolism. Furthermore, the positive effect on the lipid profile is attributed to the high antioxidant activity of the plant (Mollazadeh, Mahdian, & Hosseinzadeh, 2019). Collectively, black cumin seed and its phytochemicals seem to improve the management of diabetes and its related complications by augmenting insulin sensitivity, facilitating glucose uptake and secretion, inhibiting hepatic glucose production, reducing oxidative stress and inflammation, positively modulating gut microbiota, and balancing lipid metabolism.

***Punica granatum* L. (Pomegranate)**

The seeds, seed oil, roots, stem bark, leaves, flowers, fruit, and fruit peels of the *Punica granatum* (Punicaceae), also known as pomegranate, are medicinally valuable. Different parts of the plant contain alkaloids (pelletierin, pseudopelletierin, *N*-methylpelletierin, isopelletierin, 1-pelletierin), anthocyanins (delphinidin, cyanidin, pelargonidin), tannins (punicalagin, punicafolin, punicalin α , and β -catechin, ellagitannins), flavonoids, phenolic acids (punicalin, gallic acid, ellagic acid, pyrogallol), sterols, terpenes, lignans, fatty acids, and organic acids. Previous pre-clinical studies have reported that it has pharmacological effects, including antimicrobial, anticancer, anti-inflammatory, antidiabetic, and antiobesity activities (Maphetu, Unuofin, Masuku, Olisah, & Lebello, 2022).

In an STZ-NAD-induced Type 2 diabetes mellitus model, rats were orally administered 100 and 300 mg/kg b.wt of pomegranate aril juice equivalent to punicalagin at a dose of 2.6 and 7.8 mg/kg b.wt, respectively, for 6 weeks. High-dose pomegranate juice ameliorated oxidative stress by significantly reducing serum TNF- α concentration, liver MDA concentration, and NOx production and enhancing GST activity in the liver. It upregulated the mRNA expression of hepat-

ic IRS1 while downregulating the mRNA expression of hepatic JNK in both doses administered (El-Beih, Ramadan, El-Husseiny, & Hussein, 2019). In another study, Sprague-Dawley rats were treated with fresh pomegranate juice (1 mL, 21 days, oral) or powdered pomegranate seeds (100 mg, 21 days, oral) in a STZ-NAD-induced diabetes mellitus model. Pomegranate juice reduced plasma IL-6, TNF- α , and NF- κ B levels (Taheri Rouhi, Sarker, Rahmat, Alkahtani, & Othman, 2017). *P. granatum* flowers were administered to male Sprague-Dawley rats by oral gavage at doses of 50 and 100 mg/kg for 4 weeks in an STZ-induced diabetes mellitus model. It boosted the level of glycogen in the liver and muscles. It increased CAT, SOD, and GSH levels while decreasing MDA levels. It suppressed ER stress by downregulating the expression levels of IRE1 α , eIF2 α , XBP1-s, and CHOP, which are ER stress markers. In conclusion, it has been demonstrated that polyphenol extract prepared from pomegranate flowers reduces insulin resistance through the activation of the Akt-GSK3 β signaling pathway and inhibition of ER stress (Tang et al., 2018). Punicalagin administration (20 mg/b.wt/day, 4 weeks, orally) upregulated the expression of autophagy-related proteins, including PU, LC3B, and p62, in male C57BL/6J mice in an STZ-induced diabetes mellitus model. It significantly reversed the diabetes mellitus-induced increase in phosphorylated Akt/total Akt and phosphorylated FoxO3a/total FoxO3a protein ratios (Zhang et al., 2019). Punicalagin (100, 150, and 200 mg/kg, 4 weeks, orally) decreased gluconeogenesis and augmented glycogenesis by stimulating the PI3K/AKT signaling pathway and regulating the HMGB-1/TLR4/NF- κ B signaling pathway in C57BL/6J mice in the STZ-induced Type 2 diabetes mellitus model (Jin, Zhang, Li, & Zhou, 2020). Treatment with pomegranate ellagic polyphenols (PEP; 50, 150, and 300 mg/kg/day, 14 days, orally) was found to enhance 11 β -HSD2 protein and mRNA levels in the placental tissue of rats with gestational diabetes mellitus in an STZ-induced model. It inhibited cell apoptosis in pancreatic and placental tissues by suppressing *p*-PI3K and *p*-AKT

expressions. It downregulated ANP and chemerin expressions in pancreatic tissue, which are associated with insulin resistance and inflammation. Moreover, it downregulated the mRNA and protein levels of CRP, TNF- α , and IL-6 in pancreatic tissue. It also markedly upregulated the protein and mRNA levels of PPAR- α , TRB3, AKT2, *p*-FOXO1, and GLUT-2. All these effects were stated to be dose-dependent (Sun, Zhou, & Wang, 2019). Ellagic acid-rich pomegranate peel extract (44, 88, and 177 mg/kg/b.wt, week, oral) improved the lipid profile in hamsters by upregulating LXR- α , PPAR- α , PPAR- γ , and their downstream gene ABCA1 (Liu et al., 2015). In addition to these mechanisms, it is stated that the possible mechanisms of action of pomegranate juice may be increased β -cell function, induction of insulin secretion, inhibition of α -amylase, α -glucosidase, and dipeptidyl peptidase-4 enzymes, and protective effects against DNA damage (Virgen-Carrillo, Martínez Moreno, & Valdés Miramontes, 2020). In summary, pomegranate and its components influence diabetes mellitus by enhancing insulin sensitivity and glucose utilization, reducing oxidative stress and inflammation, suppressing endoplasmic reticulum stress, promoting cellular health and survival (through autophagy and anti-apoptotic effects), and improving lipid metabolism.

***Trigonella foenum-graecum* L. (Fenugreek)**

The seeds of the *T. foenum graecum* (Fabaceae) are used for therapeutic purposes. The seeds contain pyridine alkaloids (trigonellin, choline, gentianine, and carparine), steroidal saponins (diosgenin, yamogenin, tigogenin, neotigogenin), flavonoids (apigenin, orientin, luteolin, quercetin, vitexin, and isovitexin), free amino acids (4-hydroxyisoleucine, arginine, lysine), essential oil (n-alkane and sesquiterpene compounds), galactomannan, protein, and fixed oil. It has been reported that fenugreek and its phytochemicals exhibit antimicrobial, anticancer, antioxidant, hypoglycemic, hypolipidemic, hypocholesterolemic, immunomodulatory, and neuroprotective activities (Visuvanathan, Than, Stanslas, Chew, & Vellasamy, 2022).

Fenugreek powder (2 or 8 g per 100 g of chow, orally, for 4 weeks) was administered to male hamsters fed a high-cholesterol diet. High-dose fenugreek seed significantly upregulated LDLR gene expression, but there was no significant change in the low-dose group. These results suggested that fenugreek may be effective in treating dyslipidemia through upregulation of LDLR gene expression (Kassaei, Goodarzi, & Kassaei, 2021). In a second study, the saponin fraction isolated from fenugreek seeds (6, 12, and 24 mg/kg doses, 9 weeks, orally) boosted CYP 7A1, SCARB1, and Niemann-Pick C1-like 1 levels in HFD-fed rats. It also decreased the level of HMG-CoA reductase in the liver and serum. In conclusion, the possible positive effects of the saponin fraction on dyslipidemia were related to accelerated cholesterol metabolism, inhibited cholesterol synthesis, and facilitated reverse cholesterol transport (Chen et al., 2017). It was determined that diosgenin (20 mg/kg b.wt, 20 days, intragastric), which is one of the active components of fenugreek seed, ameliorated oxidative stress in C57BL/KsJ-Lepdb/db/+(db/+) pregnant mice by reducing TBARS content, increasing GSH level, and inducing the activities of antioxidant enzymes such as SOD and CAT. It also downregulated the expression of SREBP-1 and its target genes, including FAS, SCD-1, and acetyl coenzyme A carboxylase (Hua, Li, Su, & Liu, 2016). In a model of STZ-induced diabetes mellitus, male Sprague-Dawley rats were treated orally with trigonelline at a dose of 40 mg/kg/day for 8 weeks. Trigonellin lowered the levels of IL-1 β , IL-6, and IL-18 while elevating the level of IL-10. The protein expressions of p53 and Bax, as well as the activities of caspase-3 and caspase-9, were inhibited following trigonellin treatment. It decreased MDA levels and increased SOD, GSH, and GSH-Px levels. Moreover, it induced protein expression of PPAR- γ and GLUT-4 while suppressing protein expression of TNF- α and leptin. Taken together, it was concluded that trigonellin may act on Type 2 diabetes mellitus through antioxidative, anti-inflammatory, and antiapoptotic effects and by modulating the PPAR- γ /

GLUT-4-leptin/TNF- α signaling pathway (Li, Li, Wang, Lou, & Li, 2019). The effect of a water extract prepared from fenugreek seeds and its active component, 4-hydroxyisoleucine, on insulin resistance was tested using human liver cells (HepG2). The extract and 4-hydroxyisoleucine (for both: 100 ng/mL) markedly stimulated the phosphorylation of IR- β , Akt, GSK-3 α/β , and GLUT-2 under normoglycemic and hyperglycemic conditions. Fenugreek seeds and the active ingredient were observed to exhibit insulin-like antihyperglycemic effects by inducing insulin signaling, modulating the expression of insulin signaling pathway-related genes, and increasing glucose uptake (Naicker, Nagiah, Phulukdaree, & Chuturgoon, 2016). In another study, 4-hydroxyisoleucine (10 μ M for 24 h) upregulated the tyrosine phosphorylation of both IR- β and IRS-1 in TNF- α -induced C2C12 myotubes. It reduced the protein concentration of SOCS-3. It increased immunoprecipitation of the IR- β subunit with IRS-1 while decreasing coimmunoprecipitation of SOCS-3 with IR- β and IRS-1. It also activated AMPK phosphorylation. These results suggested that 4-hydroxyisoleucine ameliorates inflammation-mediated insulin resistance by activating the AMPK pathway and suppressing the co-immunoprecipitation of SOCS-3 with IR- β and IRS-1 (Gautam et al., 2016). INDUS810 isolated from fenugreek seed (200 mg/kg, 15 weeks, *i.p.*) inhibited lipid accumulation while inducing lipolysis activity in 3T3-L1 adipocytes. It upregulated PPAR- α , PPAR- γ co-activator 1 β , sirtuin 1, and sirtuin three protein levels. It did not affect FAS, PPAR- γ , and CCAAT/enhancer-binding protein- α expression. Moreover, it diminished the lipid content in adipocytes by activating AMP-activated protein kinase (Cheng, Yang, Ekambaranellor, Huang, & Lin, 2018). The authors suggested that fenugreek could be effective in diabetes mellitus by improving insulin signaling and glucose uptake, exhibiting significant anti-inflammatory and antioxidant properties, protecting cells from apoptosis, and regulating lipid and cholesterol metabolism.

***Zingiber officinale* Roscoe (Ginger)**

The dried rhizomes of *Zingiber officinale* Roscoe (Zingiberaceae) are used for medicinal purposes and as a spice. Essential oils (α -zingiberene, β -sesquifellandrene, β -bisabolene, (-)- β -phellandrene, and geraniol) and gingerol analogs (gingerol, shogaol, paradol, and zingerone) in the composition of the rhizomes are responsible for the biological activity. The compounds, such as diarylheptanoids, sulfonates, steroids, and monoterpenoid glycosides, were isolated from different parts of the plant. Ginger and its isolated compounds have a wide range of pharmacological activities, including gastroprotective, anticancer, lipid-lowering, antiobesity, antihelmintic, antiallergic, neuroprotective, hepatoprotective, and neuroprotective effects (Zhang et al., 2021).

The hydroethanolic extract prepared from ginger rhizomes was administered orally to male Wistar rats at a dose of 250 mg/kg/day for 5 weeks. The extract suppressed the expression of GPAT, connective tissue growth factor, SREBP-1c, and collagen 1 in the liver. It upregulated both PPAR- α and PPAR- γ expression. Ginger treatment significantly enhanced plasma adiponectin levels while decreasing plasma leptin levels. It did not affect the protein expression of the CYP7A1 enzyme or LXR (de Las Heras et al., 2017). Ahmad et al. administered zingerone (at 50 and 100 mg/kg b.wt/day, 21 days, orally) to Wistar male albino mice in a model of alloxan-induced diabetes mellitus. Zingerone diminished inflammation by inhibiting the NF- κ B signaling pathway and down-regulating the expression of inflammatory cytokines such as IL-1 β , IL-2, IL-6, and TNF- α . It improved oxidative stress by boosting antioxidant enzyme activities such as SOD, CAT, and GPx and lowering MDA levels (Ahmad et al., 2018). 6-gingerol (200 mg/kg/day, 28 days, orally) augmented GLP-1 levels and reduced DPP4 levels and activity by stimulating the GLP1/cAMP/PKA/CREB pathway in Lepr db/db type 2 diabetic mice. It upregulated the gene and protein expression of Rab27a and Slp4-a. It also induced membrane translocation of GLUT-4 by upregulating Rab8 and Rab10.

These results suggested that 6-gingerol increased glucose uptake in the skeletal muscles of mice (Samad et al., 2017). In another study, it was reported that administration of 6-gingerol (0.05%, 4 weeks) with a 20% casein diet suppressed hepatic gene expression of PEPCK and G6Pase in db/db mice. It upregulated the gene expression of glycogen synthase while down-regulating glycogen phosphorylase. Moreover, it significantly reduced TBARS and TNF- α levels (Son et al., 2015). In addition to these mechanisms, there is *in vitro* evidence that ginger and its constituents exert antidiabetic effects by enhancing the viscosity of gastrointestinal contents, delaying gastric emptying, forming a barrier against diffusion, and inhibiting the activities of α -amylase, α -glucosidase, and angiotensin-converting enzymes (Tran, Pham, & Le, 2020). Ginger and its phytochemicals have been shown to be effective in the management of diabetes mellitus by increasing glucose uptake and storage, reducing glucose production, suppressing inflammation and oxidative stress, regulating lipid metabolism, and balancing appetite-related hormones.

CONCLUSION

The increasing prevalence of diabetes mellitus has led to a rise in the use of medicinal plants to support medical treatment. Several studies have investigated the efficacy and mechanisms of action of medicinal plants such as cinnamon, garlic, ginseng, flaxseed, black cumin, pomegranate, and walnut in diabetes mellitus and dyslipidemia. Garlic modulates appetite through increasing POMC expression in the hypothalamus and reducing ObR mRNA levels, while also promoting energy expenditure by β 3-ADR and UCP-1 expression. It also inhibits inflammatory mediators such as IL-6, IL-1 β , and TNF- α . Cinnamon contributes to weight management by modulating glucose metabolism by the inhibition of α -amylase and α -glucosidase and by increasing mitochondrial biogenesis through AMPK activation. Turmeric and curcumin mitigate oxidative stress by lowering MDA and NO levels and reduce inflammation by diminish-

ing NF- κ B and COX-2 levels. It augments insulin signaling by elevating Akt phosphorylation in peripheral organs. It improves glycolipid metabolism by inhibiting hepatic gluconeogenesis and lipogenesis. Ginseng is effective in weight management by downregulating adipogenic genes, including SREBP1C, PPAR- γ , FAS, SCD1, and ACC1. The ginsenosides increase glucose uptake and insulin sensitivity by activating the PI3K/AKT and AMPK signaling pathways, as well as reducing inflammation. Walnuts reduce insulin resistance by increasing ADP and FNDC5 levels. Polyunsaturated fatty acids and peptides in their composition alleviate oxidative stress and enhance hyperglycemia by promoting autophagy via the AMPK/mTOR/ULK1 pathway. Flaxseed has been demonstrated to contribute to managing diabetes by regulating gut flora and enhancing short-chain fatty acid synthesis. It improves lipid profiles by modulating bile acid metabolism by regulating the FXR-FGF15 signaling pathway. Black cumin increases insulin sensitivity and promotes glucose absorption by activating the PI3K/AKT and the AMPK pathways. The positive effects on lipid metabolism are related to the inhibition of the enzyme HMG-CoA reductase. Pomegranate reduces insulin resistance by activating the Akt-GSK3 β signaling pathway and decreases gluconeogenesis and enhances glycogen production by stimulating the PI3K/AKT signaling pathway. It also mitigates inflammation and oxidative stress by modulating the HMGB-1/TLR4/NF- κ B pathway. Pomegranate increases cellular viability through autophagy and anti-apoptotic mechanisms. It has been proven that these plants may be effective against the mentioned disease groups by regulating mitochondrial biogenesis-related pathways and by antioxidant, anti-inflammatory, and antiapoptotic mechanisms of action. It is extremely important that the results obtained are supported by randomized, controlled clinical trials in large populations with standardized, quality-controlled herbal products. In this regard, preclinical and clinical studies assessing the mechanisms of action of these herbal products should be increased.

ABBREVIATIONS

DM: Diabetes mellitus, HDL-c: high-density lipoprotein cholesterol, TG: triglyceride, LDL-c: low-density lipoprotein cholesterol, POMC: proopiomelanocortin, ObR: leptin receptor, β 3-ADR: β 3-adrenergic receptor, PPAR- γ : peroxisome proliferator-activator receptor- γ , LPL: lipoprotein lipase, HSL: hormone-sensitive lipase, InsR: insulin receptor, GLUT-4: glucose transporter type-4, UCP-1: uncoupling protein-1, IL: interleukins, TNF- α : tumor necrosis factor-alpha, iNOS: inducible nitric oxide synthase, ABCA1: ATP-binding cassette transporter A1, HMG-CR: 3-hydroxy-3-methylglutaryl-CoA reductase, CYP46A1: cholesterol-24 hydroxylase, SREBP-2: sterol regulatory element binding protein-2, ACAT-2: acetyl-CoA acetyltransferase-2, HMG-CoA: β -hydroxy β -methylglutaryl-CoA, STZ: streptozotocin, HFD: high-fat diet, MyHC: myosin heavy chain, PGC-1 α : peroxisome proliferator-activated receptor- γ coactivator α , AMPK: AMP-activated protein kinase, Nrf-1: nuclear respiratory factor-1, Tfam: mitochondrial transcription factor A, ACC: acetyl-CoA carboxylase, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, SREBF1: sterol regulatory element-binding transcription factor 1, LDLR: low-density lipoprotein receptor, NR2F2: nuclear receptor subfamily 2 group F member 2, FOXO1: forkhead box transcription factor O1, MDA: malondialdehyde, TAC: total antioxidant capacity, LXR- α : liver X receptor- α , ACLY: ATP-citrate lyase, FAS: fatty acid synthase, IRS-1: insulin receptor substrate-1, HO-1: heme oxygenase-1, BDNF: brain-derived neurotrophic factor, CARTPT: cocaine and amphetamine-related transcript, CCK: cholecystokinin, C/EBP- α : CCAAT/enhancer-binding protein, DLK-1: delta-like 1 homolog, PNPLA2: patatin phospholipase domain containing 2, MGLL: monoglyceride lipase, BMP4: bone morphogenetic protein 4, PRDM16: PR domain containing 16, COX: cyclooxygenase, MCP1: monocyte chemotactic protein 1, ATGL: adipose triglyceride lipase, ACOX1: acyl-coenzyme A oxidase 1, CPT1: carnitine palmitoyltransferase 1, GSK 3 β : glycogen synthase kinase 3 β , PEPCK: phosphoe-

acetyl-CoA carboxylase, TGF- β : transforming growth factor- β , FGF15: fibroblast growth factor 15, PKC θ : protein kinase theta, FDPS: farnesyl diphosphate synthetase, DHCR24: 24-dehydrocholesterol reductase, DHCR7: 7-dehydrocholesterol reductase, LSS: lanosterol synthase, MVK: mevalonate kinase, Sc4mol: sterol-C4-methyl oxidase-like, SS: squalene synthase, SCD-1: stearoyl-coenzyme A desaturase, GPAT: glycerol-3-phosphate acyltransferase, SOCS-3: suppressor of cytokine signaling-3, FXR: farnesoid X receptor, CYP8B1: sterol 12- α -hydroxylase, XBP1-s: X box-binding protein1-s, SCARB1: scavenger receptor class B type I, HFD: high-fat diet, ADP: adinopectin, PUFA: polyunsaturated fatty acid, FNDC5: fibronectin type III domain containing 5, CYP7A1: cholesterol 7 α -hydroxylase, PTP-1B: protein tyrosine phosphatase 1B, JNK: phospho-c-Jun N-terminal kinase

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AUTHOR CONTRIBUTION STATEMENT

Concept: GR; Data Collection or Processing: GR, İG; Analysis: İG; Interpretation: GR, Literature Search: GR, İG; Writing: GR, İG; Proofreading: GR

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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The New Generation E-Cigarette Pandemic Threatening Youth: Puff Bars

İsmet ÇOK[°], Pınar UĞURLU^{**}

The New Generation E-Cigarette Pandemic Threatening Youth: Puff Bars

Gençleri Tehdit Eden Yeni Nesil E-Sigara Pandemisi: Puff Barlar

SUMMARY

Disposable e-cigarettes, which come under many different brands and varieties, are generally known under the brand name Puff Bar, which was the first product released. These disposable products contain high levels of flavors and synthetic nicotine and now play an important role in introducing young people to tobacco products and in the spread of nicotine-type addiction in society. This review examines disposable e-cigarettes and the factors influencing their popularity among young people, based on existing literature. Articles regarding disposable e-cigarette usage among adolescents and young adults were searched and reviewed using PubMed, ScienceDirect, and Google Scholar. Their flavors, low cost, vibrant colors, and easy availability make Puff Bars and other disposable e-cigarette products attractive to young people. These types of Electronic Nicotine Delivery Systems (ENDS) products contribute significantly to the use of nicotine-containing products by young people who have never smoked and to the development of nicotine addiction. This review provides detailed information about the reasons for new generation e-cigarette use in light of findings from various studies conducted with young people and emphasizes the importance and necessity of new toxicity studies on disposable e-cigarettes, synthetic nicotine, and flavors.

Keywords: Puff Bar, Disposable e-cigarette, Young, E-cigarette, Flavorings.

ÖZ

Birçok farklı marka ve çeşidi bulunan tek kullanımlık e-sigara, genellikle piyasaya sürülen ilk ürün olan Puff Bar marka adı altında bilinmektedirler. Tek kullanımlık bu ürünler yüksek düzeyde aroma ve sentetik nikotin içermektedirler ve gençleri tütün ürünleriyle tanıştırmada ve toplumda nikotin tipi bağımlılığın yayılmasında önemli bir rol oynamaktadır. Bu derlemede, tek kullanımlık e-sigara ve gençlerin tek kullanımlık e-sigara tercih etmesine neden olan faktörler mevcut literatür ışığında değerlendirilmiştir. Ergenler ve genç yetişkinler arasında tek kullanımlık e-sigara kullanımına ilişkin makaleler PubMed, ScienceDirect ve Google Scholar kullanılarak aranmış ve incelenmiştir. Aromaları, düşük maliyetleri, canlı renkleri ve kolay bulunabilmeleri Puff Bar'ları ve diğer tek kullanımlık e-sigara ürünlerini gençler için çekici hale getirmektedir. Bu tür Elektronik Nikotin Taşıyıcı Sistem (ENDS) ürünleri, hiç sigara içmemiş gençler tarafından nikotin içeren ürünlerin kullanılmasına ve nikotin bağımlılığının gelişmesine önemli ölçüde katkıda bulunmaktadır. Bu derlemede, gençler üzerinde yapılan çeşitli çalışmalardan elde edilen bulgular ışığında yeni nesil e-sigara kullanım nedenleri hakkında detaylı bilgi verilmekte ve tek kullanımlık e-sigara, sentetik nikotin ve aromalar üzerinde yeni toksisite çalışmalarının önemi ve gerekliliği vurgulanmaktadır.

Anahtar Kelimeler: Puff Bar, Tek kullanımlık e-sigara, Gençlik, E-sigara, Aroma vericiler.

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[°] ORCID: 0000-0003-3128-677X, Gazi University, Faculty of Pharmacy, Department of Toxicology, 06330, Hipodrom, Ankara, Turkey

^{**} ORCID: 0009-0002-6687-5304, Gazi University, Faculty of Pharmacy, 06330, Hipodrom, Ankara, Turkey

INTRODUCTION

The increase in nicotine consumption, which began with the invention of the tobacco rolling machine in the 1880s, is now successfully adapting to the new world order with the use of electronic systems. Jerzyński and colleagues estimated that between 2012 and 2023, the number of e-cigarette users worldwide would quadruple from 21.3 million to 86.1 million. When this figure was determined, products called “Puff Bars,” currently the most popular e-cigarette product in all societies, were not yet on the market (Jerzyński et al., 2021).

E-cigarettes are the most important member of the new electronic nicotine delivery systems (ENDS) and cover a wide range of products currently on the market. Today, e-cigarette types can be grouped into four basic categories (Mercier et al., 2024) from those that look like cigarettes, pens, or USB drives to somewhat larger products like “personal vaporizers” and “tank systems” (pods). Additionally, the ENDS are sold in many flavors. For these reasons, the product range of e-cigarettes is diversifying and changing constantly.

Puff Bars, launched in 2019, is an e-cigarette brand whose cartridges cannot be replaced, unlike other fourth-generation pod products such as JULL (non-disposable cartridge-based ENDS) (Majmundar et al., 2023). These disposable e-cigarette products are increasing in usage very rapidly, especially among young people in every corner of the world, and contain various flavors. The brand name ‘Puff’ and the cloud logo appear on flashy and attractive devices of different sizes, containing e-liquid volumes with different amounts of nicotine. The company that produces the Puff Bar brand has many varieties, such as “Puff Bar, Puff Mini, Puff Plus, Puff XXL, Puff Krush, Puff Stig, and Airis Puff”. Many disposable e-cigarette brands produced by multiple purveyors (such as Vuse Go, Elf Bar, Lost Mary, SKE Crystal, Saltica S-Phone, BLVK, Cloud Nurdz, and Syn Bar) have begun to appear on websites. For example, in the U.S., according to the

2023 National Youth Tobacco Survey (NYTS), the most frequently reported brands by student e-cigarette users were Elf Bar (56.7%), Esco Bars (21.6%), Vuse (20.7%), JUUL (16.5%), and Mr. Fog (13.6%) (Birdsey et al., 2023). Disposable e-cigarettes are available on the market in many different flavors, including fruity, fruity-menthol, menthol, tobacco, and candy (Dai & Hao, 2022; Ramamurthi et al., 2023). All of these varieties contain strong nicotine salts at doses of 50 mg/ml or higher. Most non-refillable e-cigarettes are made in China (Ramamurthi et al., 2023). An average Puff Bar, which was launched two years ago, is no longer usable after approximately 400 puffs (about 40 cigarettes), while some recently produced disposable e-cigarettes last up to 100,000 puffs.

On December 18, 2018, the U.S. Surgeon General declared an “e-cigarette epidemic” because e-cigarette use among American high school students increased from 1.5% to 27.5% from 2011 to 2019. As a result of the 2019 NYTS and other reports on youth tobacco use, the FDA took action to end this epidemic by raising the federal minimum age for the sale of tobacco products (including e-cigarettes) from 18 to 21 in December 2019 and prioritizing enforcement against illegal flavored (e.g. fruit) e-cigarettes (FDA, 2021). The FDA later banned flavored (except menthol and tobacco flavors) and cartridge e-cigarettes in February 2020. On the other hand, the company that produces Puff Bar products announced that it voluntarily withdrew from the online sale and distribution of its products. Due to these effective regulations, e-cigarette sales, excluding non-refillable e-cigarettes, decreased between August 2019 and May 2020. The company that produces Puff Bars re-launched its Puff Bar products in February 2021, claiming that they use “tobacco-free nicotine” in their products, that is, “nicotine not derived from tobacco”, and that their products do not contain tobacco or any substances derived from tobacco. Thus, non-refillable e-cigarettes like the Puff Bar have come to the fore after the ban on cartridge and flavored e-cigarettes.

In April 2021, Puff Bar became the most popular disposable e-cigarette brand in the U.S., taking nearly half of the e-cigarette market share in the U.S. (51.3%) (Truth Initiative, 2021).

While 2019 U.S. NYTS data showed that the most frequently used ENDS brand by youth was JUUL (Anic et al., 2021), in 2020, 26.1% of high school students and 30.3% of middle school students reported that the e-cigarette brand they used was Puff Bar. It was determined that JUUL use declined among students quickly (5.7% of high school students and 12.5% of middle school students). Of the 82% (2.53 million) of American high school students who used e-cigarettes in 2020, most used fruity, menthol, and sugary flavors. According to the 2023 U.S. NYTS, disposable e-cigarettes are the most popular device type among e-cigarette users (60.7%), followed by pre-filled or refillable cartridges or pods (16.1%), and mod systems or tanks were the last choice of youth at 5.9% (Birdsey et al., 2023). According to the latest 2024 U.S. Youth Survey of middle and high school students, e-cigarettes are the most commonly used tobacco product among U.S. youth (5.9%). Between 2023 and 2024, e-cigarette use decreased among all students (from 2.13 million to 1.63 million students). This decrease was most notable among high school students, while there was no change in the rate of e-cigarette use among middle school students (Jamal et al., 2024). On the other hand, it is estimated that sales of disposable e-cigarettes in Switzerland may have increased by 2200% in 2022 (Ruggia, 2022). It is difficult to access statistical information on the rates of disposable e-cigarette use in countries around the world due to the lack of a sufficient number of studies.

METHOD

Words such as Puff Bar, disposable e-cigarette, young, e-cigarette were used to search for articles via search engines (ScienceDirect, PubMed, and Google Scholar). Using these keywords, research articles and letters to the editor published especially in the last decade were identified and examined.

RESULTS

Why are youth using e-cigarettes?

The pleasant smell of e-cigarette vapor:

Flavoring agents used in e-cigarettes today play an important role in increasing Puff Bars use, especially among adolescents. The popularity of these products is increasing due to their wide range of flavors and options that appeal to many different tastes. In fact, young people who use e-cigarettes usually associate their starting/trying e-cigarettes with the thousands of “flavors” they contain, and they cite the attractive flavors of e-cigarettes as the primary reason they began (Ambrose et al., 2015).

One of the expected results of tobacco addiction is that young people who start using tobacco products by trying a flavored product will most likely switch to other tobacco products. Young adults use e-cigarettes such as Puff Bars because e-cigarettes contain various flavorings, and these flavored products are more addictive than tobacco flavors. According to a 2016 report from the Behavioral Risk Factor Surveillance System survey, 44.3% of young adult (18-24 years old) e-cigarette users had never smoked before trying e-cigarettes in the U.S. (Mirbolouk et al., 2018).

Flavorings and synthetic coolants are used in ENDS products at higher levels compared to other consumer products, such as in edibles (e.g., beverages, chewing gum, and baked goods) and cosmetics (e.g., soaps, lotions, and perfumes) (Omaiye et al., 2019; 2022). For example, the concentration of ethyl maltol, which imparts a sweet flavor, in Puff liquids ranges from 0.007% to 0.99%, exceeding ingestible concentrations in 77% of products tested (Behar et al., 2021; Omaiye, 2022; Tierney et al., 2016).

Product manufacturers are increasing the amount of synthetic flavorings and menthol in ENDS to appeal to young people, while completely ignoring the potential health risks of inhaling these substances. On the other side, our knowledge of the chemical structures and toxicities of new compounds that may be formed, especially when flavorings are heated, is in

its infancy (Cok, 2023). Flavored e-cigarette users also have lower intentions to quit e-cigarette or tobacco use (Dai & Hao, 2016). Therefore, we need more information about which chemicals from ENDS use pose a threat to youth and how they can be controlled.

Presenting products in attractive shapes and colors, and product accessibility: Puff Bars primarily attract the attention of young people due to their colorful and eye-catching packaging. New products that are released are one step ahead of previously released products with their eye-catching appearances. The striking colors of these products are one of the prominent features that make them noticeable to young people. These products are also gaining the attention of young people in terms of incorporating technological features such as digital displays.

The low cost of Puff Bars makes them affordable. Depending on the user's usage habits, Puff Bars can be used for a few days to a few weeks, which also makes the products affordable and effective, thus appealing to young people. Puff Bars can often be purchased for a low price online from e-cigarette shops, gas stations, etc., and used for a few days and then thrown away (Rosenthal et al., 2022). Considering that young people have lower budgets, the price ranges of these products are attractive to them. The widespread sales of these products over the internet make it easier for teenagers to purchase these products, as it is difficult to check the age of minors online (Josseran et al., 2023).

Convenience and perceived privacy of vaping and using: Puff Bars are pre-charged and pre-filled e-cigarettes. This provides simplicity and ease of use for users, which is one of the main reasons why Puff Bars and other disposable vapes are preferred by young people.

Unlike JUUL, Puff Bars are not refillable and run out quickly, which is why teens tend to throw them away after use. These devices are similar to USB flash drives; they do not take up much space in the pocket.

In addition, due to their small size, adolescents can easily hide their e-cigarette usage habits from their parents, families, and those around them (Rosenthal et al., 2022; Ruggia, 2022).

Inadequate recognition of risks: Another important reason for the increasing interest in Puff Bars among young people is that they think that disposable e-cigarettes are healthier than regular cigarettes. Young people think that e-cigarettes are a way to get rid of a nicotine addiction. This situation is valid not only for young people but also for many adults who use e-cigarettes. Using e-cigarettes to get rid of nicotine addiction is not one of the treatment methods recommended by any health authority. On the contrary, it is an important use that plays a role in keeping nicotine addiction alive. For this reason, and for young people to think in this way, they are at a greater risk of e-cigarette addiction.

Disposable e-cigarettes often contain higher levels of nicotine compared to other e-cigarette products, such as JUUL (Tan and Weinreich, 2020). High nicotine levels play an important role in the development of nicotine-type addiction. Thus, it is very likely that a nicotine-type addiction that begins with these products will turn into other tobacco product use and expand to different dimensions. It is also stated that young adults have difficulty adequately understanding the potential risks of e-cigarettes.

Nicotine exposure during adolescence through smoking or using e-cigarettes may be detrimental to brain development, affecting neural connectivity in the central nervous system, affecting information processing, and impairing cognitive function, attention, and short-term memory (Dubrosa et al., 2023). Although Puff Bars contain synthetic nicotine, this type of nicotine has the same chemical and biological properties as tobacco-derived nicotine. Adolescent nicotine use may increase the risk of addiction to other drugs, including increasing the risk of marijuana use and alcohol dependence (Audrain-McGovern et al., 2018). Because nicotine is

as addictive as heroin and cocaine (Choi et al., 2021), the earlier a person starts using nicotine, the harder it is to quit later in life, as these people are more vulnerable to nicotine addiction. This also applies to e-cigarette users under the age of 25 because their nervous systems and brains are still developing. Nicotine is also considered a gateway drug for illicit drugs, including methamphetamine and cocaine (Ren & Lotfipour, 2019). Additionally, e-cigarette use in adolescents has been associated with an increased risk of initiating conventional cigarette smoking (Soneji et al., 2017).

High-volume CBD vaping products in commerce: ENDS have been modified for nearly a decade to deliver illicit substances beyond nicotine, such as Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), and certain drugs. CBD is the main cannabinoid component in hemp. The market for CBD-based products has been growing rapidly, especially in recent years. CBD is a non-psychoactive cannabis active ingredient approved for a variety of ailments, including pain management and epilepsy (Epidiolex) via oral administration. Smoking CBD-dominant cannabis strains or vaporizing CBD oil is the fastest way to experience the effects. Vaporizing CBD via e-cigarettes and its effects are unregulated and insufficiently researched by health authorities. The prevalence of CBD vaping is increasing globally, especially in the U.S. and Europe. In fact, the market size of CBD e-liquids is expected to reach \$74 billion by 2030, up from \$3 billion in 2022 (Data Bridge Market Research, 2023). Flavored or unflavored CBD e-liquids are sold in online stores with a wide range of cannabidiol-infused CBD vaporizer options in various strengths and sizes. This provides an extremely suitable environment for adolescents to try CBD-containing products and expand their experience. In other words, e-liquids containing THC and CBD play an important role in the spread of e-cigarette usage. In the U.S., as of 2022, approximately 19% of adult e-cigarette users (Dai et al., 2023) and 21% of adolescent (11-18 years old) e-cigarette users (Goodman et al., 2022) are also

vaping CBD. Recent studies have shown that vaping CBD causes a proinflammatory response in the lungs of mice, creating pathologies similar to those seen in smoking-related diseases (Bhat et al., 2023). In August 2019, the most significant e-cigarette or vaping product use-associated lung injury (EVALI) in the U.S. occurred affecting 2,807 people, primarily adolescents and young adults. It caused 68 deaths and was associated with vaping e-liquids containing THC, vitamin E acetate, and medium-chain triglycerides (CDC, 2021). A very recent study showed increased toxic emissions of formaldehyde, acetaldehyde, acrolein, diacetyl, and methylglyoxal in CBD vapor emissions (Robertson et al., 2024).

Marketing models and programs aimed at youth: One of the reasons for the increasing popularity of Puff Bars is that young people interact on social media platforms like TikTok, where one-third of the 49 million users in the U.S. are under the age of 14. Although minors are prohibited from using alcohol, drugs, or tobacco on social media platforms like *TikTok* or *Instagram*, Puff Bars are being popularized by platform users and targeted advertising. According to a study, when young people are exposed to e-cigarette-related posts shared by their peers on social media, their tendency to use e-cigarettes increases.

Adolescents and young adults in particular tend to perceive social media influencers as role models. This can result in more positive perceptions and intentions to purchase or use products promoted by social media influencers. Young people believe that they become COOL by purchasing COOL products (Lee et al., 2024). When following social media influencers with e-cigarettes, adolescents prefer *TikTok* and *Pinterest* more frequently, while young adults use *Twitter* more frequently.

Less perceived health risk due to lower temperature evaporation process: While the variety of chemicals in e-cigarette aerosols is being determined by an increasing number of studies, it is also revealed

that there is inevitably exposure to carcinogenic chemicals from these products. It has been reported that e-cigarette use results in lower levels of exposure to toxic compounds such as tar, carbon monoxide (CO), benzene, lead (Pb), nickel (Ni), and arsenic (As) than those encountered in traditional cigarette use (Eshraghian & Al-Delaimy, 2021; Gray et al., 2022; Lu et al., 2021). On the other hand, e-cigarette users have higher biomarker concentrations of some metals (e.g., cadmium and selenium), volatile organic compounds (e.g., acrolein, acrylamide, and acrylonitrile), and propylene glycol compared to non-users. Exposure to carcinogenic chemicals (e.g., benzene and chromium) may also be higher in e-cigarette smokers than in non-smokers (Raj et al., 2020; Szukalska et al., 2020). It should also be noted that some carbonyl compounds, such as acetaldehyde, formaldehyde, and acrolein in e-cigarette aerosols are also produced by thermal decomposition of e-cigarette cartridge content and are classified as carcinogenic (IARC, 2021; Li et al., 2021). We also have little information on the toxicity profiles of new chemicals originating from flavoring additives in e-liquids.

Exposure to such chemical compounds can lead to various health risks, especially cancer. For example, inhalation of e-cigarette aerosols can cause toxicity in oral cells, the first exposure site, and may have the potential to induce oral cancer. It is suggested that exposure to e-cigarettes can cause adverse effects on oral health, including changes in the oral microbiome, inflammation, dysbiosis, periodontal diseases, and deterioration of teeth and gum health. Exposure to e-cigarettes has also been found to have toxic effects on head-neck and mouth cells in terms of abnormal morphology, decreased viability, oxidative stress, delayed fibroblast migration, cytotoxicity, and genotoxicity (Szukalska et al., 2020; Wilson et al., 2022). These health risks posed by e-cigarettes carry similar potential risks for Puff Bars.

Puff bar products, the most important family of 4th-generation e-cigarette products, have rapidly spread from their initial launch in the United States

to various continents and countries around the world, finding increasing numbers of new users in various regions such as Canada, the United Kingdom (Hammond et al., 2025), Australia (Jongeneelis, 2023), Switzerland (Ruggia, 2022), and China (Ramamurthi et al., 2023). These products have rapidly reduced the use of their predecessors, the 3rd and 4th generation e-cigarettes, and have gained a leading position among ENDS. For example, Puff bar use among vapers in the United Kingdom reached 83% by 2023, 59% in Canada, and 67% in the United States (Hammond et al., 2024). Furthermore, recent epidemiological findings in Switzerland indicate that more than half of young people aged 14-25 have tried these products (Trovato-Abdellali et al., 2024). In France, 13% of middle and high school students reported having previously used puff bars (Josseran et al., 2023). There is virtually no scientific data on the use rates and potential health risks of these products, whose imports are banned in Türkiye.

CONCLUSION

While smoking is decreasing in the world, the use of new generation tobacco products is increasing in all societies. The reason for this is that the tobacco industry navigates every legal gap very well and creates a new target audience for itself. The real target of this public health problem is the youth. The rise in disposable e-cigarette use poses a public health threat and exacerbates the youth vaping epidemic, as most youth who use e-cigarettes report that they first started using a flavored variety. Starting e-cigarettes in adolescence or young adulthood increases the likelihood of later smoking. Flavors are the most important factor in the increase in ENDS use, especially disposable e-cigarettes. Banning the use of flavors or restricting the concentrations used can reduce e-cigarette use and potentially reduce exposure to toxic e-liquid components. The legal regulations introduced for cigarettes, especially flavors, need to be re-established or reviewed for e-cigarettes. It is necessary to increase social and scientific activities in all societies to inform the youth about e-cigarette use and its risks.

AUTHOR CONTRIBUTION STATEMENT

Concept and Design (İÇ), Literature Screening (PU), Interpretation (İÇ), Writing (İÇ, PU).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Novel Nanocarriers for Rosuvastatin Calcium

Kadir AYKAÇ[°], Müzeyyen DEMİREL^{**}

Novel Nanocarriers for Rosuvastatin Calcium

SUMMARY

Rosuvastatin calcium (RC), a statin drug, is commonly prescribed for the treatment of hyperlipidemia by lowering levels of low-density lipoprotein (LDL) cholesterol, triglycerides, and apolipoprotein B, while simultaneously increasing high-density lipoprotein (HDL) cholesterol. It has also shown effectiveness in managing conditions such as familial hypercholesterolemia, dyslipidemia, osteoporosis, atherosclerosis, benign prostatic hyperplasia, and Alzheimer's disease. However, its therapeutic potential is limited due to challenges like poor aqueous solubility, low intestinal absorption, reduced oral bioavailability, and significant first-pass metabolism. One strategy to overcome these issues is the reduction of drug particle size to the nanoscale, which enhances the dissolution rate of poorly water-soluble compounds. Numerous advanced formulation strategies have been explored to improve the solubility and dissolution of such drugs. These include nanocarrier systems, hydrogels, nanogels, amorphous solid dispersions, co-crystals, nanocrystals, liposomes, micronization, polymer-based nanoparticles, solid lipid nanoparticles, supercritical fluid techniques, nanosuspensions, nanoemulsions, surfactants, micellar solubilization, pH adjustment, co-solvents, hydrotropic agents, and cyclodextrin complexes. This review focuses on the development and application of nanocarrier-based systems to improve the solubility, dissolution rate, and overall bioavailability of RC. Furthermore, the physicochemical characteristics and pharmacological potential of RC in the context of nanocarrier-mediated delivery are discussed.

Keywords: Rosuvastatin calcium, nanocarriers, oral drug delivery, bioavailability enhancement.

Rosuvastatin Kalsiyum İçeren Yeni Nanotaşıyıcılar

ÖZ

Statin ilaç sınıfına ait olan rosuvastatin kalsiyum (RC), düşük yoğunluklu lipoprotein (LDL) kolesterol, trigliserit ve apolipoprotein B düzeylerini düşürürken aynı anda yüksek yoğunluklu lipoprotein (HDL) kolesterolü artırarak hiperlipidemi tedavisinde yaygın olarak reçete edilir. Ayrıca ailevi hiperkolesterolemi, dislipidemi, osteoporoz, ateroskleroz, iyi huylu prostat hiperplazisi ve Alzheimer hastalığı gibi durumların yönetiminde de etkili olduğu gösterilmiştir. Ancak, zayıf suda çözünürlük, düşük bağırsak emilimi, azalmış oral biyoyararlanım ve önemli ilk geçiş metabolizması gibi zorluklar nedeniyle terapötik potansiyeli sınırlıdır. Bu sorunların üstesinden gelmek için bir strateji, ilaç partikül boyutunu nanometre ölçeğine indirmek; bu, suda az çözünen bileşiklerin çözünme hızını artırır. Bu tür ilaçların çözünürlüğünü ve çözünmesini iyileştirmek için çok sayıda gelişmiş formülasyon stratejisi araştırılmıştır. Bunlar arasında nanotaşıyıcı sistemler, hidrojeller, nanojeller, amorf katı dispersiyonlar, ko-kristaller, nanokristaller, lipozomlar, mikronizasyon, polimer bazlı nanopartiküller, katı lipid nanopartiküller, süperkritik akışkan teknikleri, nanosüspansiyonlar, nanoemülsiyonlar, yüzey aktif maddeler, misel çözünürlüştürme, pH ayarlaması, ko-çözücüler, hidrotropik ajanlar ve siklodekstrin kompleksleri yer almaktadır. Bu derleme, RC'nin çözünürlüğünü, çözünme hızını ve genel biyoyararlanımını iyileştirmek için nanotaşıyıcı sistemlerin geliştirilmesi ve uygulanmasına odaklanmaktadır. Ayrıca, nanotaşıyıcı aracılı uygulama bağlamında RC'nin fizikokimyasal özellikleri ve farmakolojik potansiyeli tartışılmaktadır.

Anahtar Kelimeler: Rosuvastatin kalsiyum, nanotaşıyıcılar, oral ilaç uygulaması, biyoyararlanım artışı.

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[°] ORCID: 0000-0001-7426-1332, Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskişehir, Türkiye.

^{**} ORCID: 0000-0001-9927-683X, Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskişehir, Türkiye.

INTRODUCTION

Recent advancements in nanotechnology have significantly impacted the field of medicine, particularly in improving the diagnosis and treatment of various diseases. Nanomaterials possess unique physical and chemical properties due to their increased surface area at the nanoscale. In pharmaceutical sciences, one of the most promising applications of nanotechnology is the formulation of nanoscale drug delivery systems. These systems can enhance drug solubility and bioavailability, prolong drug release, and minimize adverse effects. Additional benefits include increased local drug concentration, reduced variability in gastrointestinal transit, minimized interpatient variability, prevention of dose dumping, and suitability for various routes of administration (oral, parenteral, inhalation). Furthermore, they are capable of encapsulating both hydrophilic and lipophilic drug molecules (Çetin & Şahin, 2016; Luo et al., 2021). In recent years, considerable attention has been devoted to the development of innovative delivery platforms for RC, particularly using nanoformulations such as solid dispersions (SDs), self-nanoemulsifying drug delivery systems (SNEDDS), nanoemulsions, and solid lipid nanoparticles (SLNs) (Balakumar et al., 2013).

Statins, first introduced in the 1970s as lipid-lowering agents, have become the most commonly prescribed class of antihyperlipidemic drugs worldwide (Al-Heibshy et al., 2019). In addition to lowering cholesterol, statins are also effective in reducing elevated triglyceride levels and increasing HDL cholesterol factors critical in reducing cardiovascular disease risk. Compared to other statins (e.g., lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, and cerivastatin), RC has a comparatively lower potential for drug-drug interactions. Rosuvastatin, marketed as Crestor® by AstraZeneca, was the seventh statin to be approved by the U.S. FDA in August 2003 for treating primary hypercholesterolemia at doses up to 40 mg/day (Quirk et al., 2003; FDA, 2025). It was first launched in the Netherlands in November 2002,

followed by Canada in February 2003. As of today, the drug has received approval in over 90 countries and has accumulated over 4 million patient-years of post-marketing use (FDA, 2025).

RC is widely used to manage hyperlipidemia and homozygous familial hypercholesterolemia by reducing LDL, triglycerides, and apolipoprotein B while increasing HDL levels. It is also prescribed to slow the progression of atherosclerosis and to prevent cardiovascular events (Al-Heibshy et al., 2020a; Vishwakarma et al., 2016). Among statins, it is considered one of the most potent in its class and is often referred to as a “super statin” due to its high efficacy (Ahmed, 2020; Zhou et al., 2013). Beyond its lipid-lowering capabilities, RC has been investigated for potential benefits in treating osteoporosis, benign prostatic hyperplasia, and Alzheimer’s disease (Sarfraz et al., 2017; Zalak et al., 2012).

Although RC demonstrates strong therapeutic performance, its clinical effectiveness is limited by several unfavorable biopharmaceutical properties. These include low solubility in water, limited permeability through the intestinal wall, reduced oral bioavailability, and a substantial hepatic first-pass effect (Arun et al., 2020). As a Biopharmaceutics Classification System (BCS) Class II drug, RC typically shows around 20% bioavailability, primarily attributed to extensive hepatic metabolism and significant plasma protein binding (~88%). Furthermore, its crystalline structure further restricts its solubility in aqueous environments (Al-Heibshy et al., 2020b; Al-Shdefat et al., 2020; Martin et al., 2003). To address these challenges, innovative drug delivery platforms are necessary to enhance absorption, improve pharmacokinetic behavior, and minimize side effects. Approaches such as improving solubility and avoiding extensive hepatic metabolism are being explored to unlock RC’s full therapeutic potential (Ashraf et al., 2023; Balakumar et al., 2013; Zalak et al., 2012; Zhou et al., 2013).

Particle size reduction at the nanoscale level has emerged as a promising strategy to improve solubility,

dissolution rate, and ultimately, bioavailability. This review investigates nanoformulation-based strategies designed to improve RC's biopharmaceutical profile and examines its relevant physicochemical and pharmacological attributes.

Physicochemical properties

RC is a synthetic statin with lipid-lowering effects and a strong affinity for hepatocytes. Chemically, it is defined as (3R,5S,6E)- 7- {4- (4- fluorophenyl)- 6- (1-methylethyl)- 2- [methyl (methylsulfonyl) amino] pyrimidin- 5- yl}- 3,5- dihydroxyhept- 6-enoic acid calcium salt (2:1) (Figure 1) (Cada et al., 2004; Sarfraz et al., 2018; USP 44-NF39, 2022).

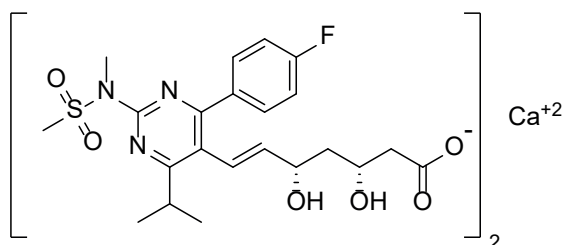


Figure 1. Chemical structure of RC (USP 44-NF39, 2022).

In its physical form, RC appears as a white to off-white hygroscopic powder with a molecular weight of approximately 1001 g/mol (Türk Farmakopesi, 2016). It exhibits limited solubility in water and methanol, is more readily soluble in methylene chloride, and is practically insoluble in anhydrous ethanol. Proper storage conditions include well-sealed containers, protected from light and moisture, at a controlled temperature of 20-25°C (Türk Farmakopesi, 2016; USP 44-NF39, 2022). Solubility of RC decreases at lower pH levels, and as a BCS Class II drug, it is characterized by poor aqueous solubility and high intestinal permeability (Alshora et al., 2019; Patil-Gadhe & Pokharkar, 2016; Ponnuraj et al., 2015; Sarfraz et al., 2017; Shoukat et al., 2024). It has a lipophilic nature with a log P value of 2.4 and low water solubility (0.00936 mg/mL) (Ahsan & Verma, 2017; Oza et al., 2020). Moreover, its partition coefficient in octanol/

water is -0.33 at pH 7.4, further confirming its limited absorption and poor bioavailability in conventional oral forms (~20%) (Ahmed, 2020; Carswell et al., 2002; Thenmozhi et al., 2023).

Adverse effects

Statins, including RC, have been reported to cause mild elevations in liver enzyme levels (transaminases), although the clinical significance of these elevations remains uncertain. In general, minor increases do not suggest a substantial risk of hepatic injury during treatment (FDA, 2025; McKenney et al., 2006). Muscular symptoms such as soreness, pain, weakness, flu-like symptoms, headaches, and myalgia are also commonly reported, while serious conditions like rhabdomyolysis are rare (Carswell et al., 2002; McKenney et al., 2006). Clinical studies have shown that high doses of RC (80 mg/day) resulted in incidences of myopathy and rhabdomyolysis in 1.0% and 0.4% of patients, respectively (Thenmozhi et al., 2023). Due to these adverse outcomes, this dose was not approved for clinical use (FDA, 2025). Additionally, proteinuria was observed in approximately 12% of patients receiving the 80 mg dosage, leading to its exclusion from the market (Jones et al., 2003). Although proteinuria has been associated with other statins as well, its prevalence may be higher with more potent agents like RC (Bays, 2006). However, the FDA concluded in 2005 that proteinuria associated with statin therapy does not indicate renal damage or increased risk of kidney failure (FDA, 2025).

Pharmacokinetics properties

RC has an absolute bioavailability of around 20% (90% CI: 17.2–23.4), limited primarily by its poor solubility, low permeability, and significant hepatic metabolism, which includes processes like oxidation, glucuronidation, lactonization, and direct biliary secretion (Al-Heibshy et al., 2016; Thenmozhi et al., 2023; White, 2002). These pharmacokinetic drawbacks necessitate frequent (often thrice daily) dosing, increasing the risk of adverse effects and reducing patient adherence (Elsayed et al., 2020).

After oral administration, peak plasma concentrations ($C_{\max}$) are typically reached within 3-5 hours. For instance, a median $t_{\max}$ of 5 hours has been reported (range: 0.5-6 hours), with $C_{\max}$ and area under the plasma concentration-time curve (AUC) values increasing in a dose-proportional manner over the 5-80 mg range (Culhane et al., 2005; Kanukula et al., 2021). Geometric mean $C_{\max}$ values for 20 mg, 40 mg, and 80 mg doses were found to be 9.7 ng/mL, 37 ng/mL, and 46.2 ng/mL, respectively. Corresponding AUC values were 81.7, 255.9, and 329 ng·h/mL, respectively (Warwick et al., 2000).

The AUC_{0-24} value on day 14, compared to a single dose, was 76%, suggesting mild accumulation over time (Kanukula et al., 2021). While food intake reduces the $C_{\max}$ by roughly 20%, it does not significantly alter the total drug absorption (White, 2002). RC's lipid-lowering efficacy remains unaffected by food or dosing time (morning vs. evening) (Culhane et al., 2005; Martin et al., 2002).

Following a single 20 mg dose, the $C_{\max}$ is approximately 6.1 ng/mL, achieved around 5 hours ($t_{\max}$). At steady state, the $C_{\max}$ increases to about 9.7 ng/mL at 3 hours post-dose. Both $C_{\max}$ and AUC show a linear relationship across the dosage range of 5-80 mg, under both single and repeated dosing. The steady-state $t_{\max}$ ranges from 3 to 5 hours, which is longer than for many other statins that typically reach $t_{\max}$ in ≤ 3 hours (White, 2002). In three randomized, double-blind, placebo-controlled clinical trials conducted in healthy male participants, single oral doses of RC ranging from 20 to 80 mg exhibited a near-linear increase in both $C_{\max}$ and the average AUC over a 24-hour period (AUC_{0-24}) (Carswell et al., 2002). After daily administration of 40 mg RC for 7 consecutive days, steady-state levels revealed a $C_{\max}$ of 37.0 µg/L, with the time to reach this peak ($t_{\max}$) recorded at 3 hours. The drug's mean elimination half-life was approximately 20.8 hours, and the AUC_{0-24} reached 256 µg·h/L (Carswell et al., 2002).

A bioequivalence study, conducted under fasting conditions in healthy adult subjects, assessed the pharmacokinetic comparability between two RC 20 mg tablet formulations (reference and test). The 90% confidence intervals of the geometric mean ratios (GMRs) for $C_{\max}$ (95.01-112.66), AUC_{0-t} (93.38-111.67), and $AUC_{0-\infty}$ (93.65-111.29) fell within the standard bioequivalence acceptance limits of 80% - 125%, confirming the equivalence of the two formulations (Pena et al., 2024).

Following oral intake, RC undergoes significant first-pass hepatic metabolism and demonstrates a high plasma protein binding rate of approximately 88% (Al-Heibshy et al., 2019; Al-Heibshy et al., 2020b). At steady-state, the drug distributes extensively, with an average volume of distribution around 134 liters, and has a terminal half-life close to 19 hours. The primary route of elimination is via feces, with renal excretion contributing minimally. Unlike many other statins, RC is not extensively metabolized by the cytochrome P450 3A4 (CYP3A4) pathway. Instead, it is minimally metabolized by CYP2C9 and, to a lesser extent, by CYP2C19 (Culhane et al., 2005). Age and sex appear to have negligible influence on RC's pharmacokinetic behavior. In individuals with mild to moderate renal impairment, plasma concentrations remain stable after 14 days of 20 mg/day administration (Culhane et al., 2005). Using the Sheiner method, the average total body clearance (CL) following a single dose has been calculated to be 28.3 L/h (Kanukula et al., 2021).

Emerging evidence suggests that RC may have beneficial effects in pregnancies affected by fetal growth restriction, with no immediate adverse outcomes reported for neonates (Kasraeian et al., 2025). Current data do not support a definitive link between congenital anomalies and statin use during pregnancy. While findings to date suggest that statins are likely non-teratogenic, their use during pregnancy is still discouraged until further safety data becomes available (Karalis et al., 2016; Trakadis et al., 2009).

Hyperlipidemia and rosuvastatin calcium

RC is a highly effective lipid-lowering agent that works by inhibiting the activity of HMG-CoA reductase, the enzyme responsible for a critical step in cholesterol biosynthesis (Ahsan & Verma, 2017). Its primary therapeutic application is in managing hyperlipidemia, where it produces a strong, dose-dependent reduction in LDL cholesterol, while simultaneously increasing HDL cholesterol levels (Desavathu et al., 2020). It is also used in managing other lipid abnormalities, including familial hyperlipidemia, dyslipidemia, and elevated triglyceride levels. Additionally, RC has demonstrated potential benefits in conditions such as atherosclerosis, osteoporosis, benign prostatic hyperplasia, and neurodegenerative disorders like Alzheimer's disease (Balakumar et al., 2013; Karasulu et al., 2016; Shoukat et al., 2022).

Hyperlipidemia plays a major role in the development of cardiovascular disease, particularly acute coronary syndromes (ACS), which are a leading cause of morbidity and mortality worldwide (Darwish et al., 2022). Among lipids, LDL cholesterol is strongly linked with coronary artery disease risk, with numerous studies confirming a direct correlation between elevated LDL levels and increased cardiovascular events. As a result, LDL-C reduction remains a key therapeutic target in both the prevention and treatment of ACS (Al-Heibshy et al., 2020b).

Rosuvastatin calcium-loaded nanocarriers

Reducing drug particle size to the nanoscale significantly improves the dissolution rates of poorly soluble compounds, a principle supported by the Noyes-Whitney equation, which states that smaller particle sizes result in a greater surface area for dissolution (Rani et al., 2023; Song et al., 2021). Various formulation strategies have been devised to enhance solubility and bioavailability of drugs in BCS Class II and IV categories. These include the development of nanogels, hydrogels, nanocrystals, co-crystals, amorphous SDs, SLNs, liposomes, micronized forms, and supercritical fluids (SCFs) technologies. Additional

techniques include the use of surfactants, nanosuspensions (NSs), nanoemulsions, micellar systems, hydrotropic agents, pH modulators, co-solvents, and cyclodextrin inclusion complexes (Grimaudo et al., 2019; Joshi et al., 2023; Shoukat et al., 2024). Several novel delivery platforms have also been tested for RC to increase its solubility, dissolution rate, and overall bioavailability. These include microemulsions, liquid-solid systems, nanocrystals, SDs, inclusion complexes, both solid and liquid SNEDDS, SLNs and nano-sponge-based carriers (Alshora et al., 2019; Al-Heibshy et al., 2020b; Anwar et al., 2025; Beg et al., 2019).

Polymer-based nanoparticles

Polymeric nanoparticles are submicron colloidal carriers, typically between 1 and 1000 nm in size that can be biodegradable or non-biodegradable (Thenmozhi et al., 2023). Their application in drug delivery is widely studied due to several advantages: they can encapsulate both water-soluble and lipophilic drugs, offer high drug-loading capacity, and provide formulation stability, particularly when lyophilized or appropriately stored. They are also compatible with diverse administration routes, including oral, nasal, and parenteral methods (Çetin & Şahin, 2016). Depending on their structure and composition, nanoparticles can be categorized into various types such as polymeric, lipid-based, carbon-based, ceramic, metallic, and semiconductor nanoparticles (Darwish et al., 2022). Among these, polymeric nanoparticles have received significant attention for their customizable properties and potential for targeted and controlled drug delivery.

Thenmozhi et al. (2023) designed and assessed polymeric nanoparticles to enhance the oral delivery of RC. These nanoparticles were synthesized through nanoprecipitation using varying ratios of Eudragit L100 and S100 polymers. By controlling the formulation parameters, particle sizes ranging from 100 to 250 nm were achieved, with drug entrapment efficiencies (EE) between 28% and 79%. *In vitro* release testing exhibited a biphasic release pattern: an initial burst

within the first two hours, likely aiding in early absorption, followed by a prolonged release phase consistent with the Higuchi model of diffusion-controlled release. The RC-loaded nanoparticles approximately doubled the drug's aqueous solubility in comparison to its unformulated state. Furthermore, permeability studies across rat intestinal tissues showed significantly improved transport of RC when encapsulated in Eudragit-based nanoparticles versus the free drug. Transmission electron microscopy (TEM) confirmed the spherical morphology of the particles. Overall, the results suggest that Eudragit-based polymeric nanoparticles are a promising system for improving RC's oral bioavailability by enhancing solubility and intestinal absorption (Thenmozhi et al., 2023).

In another study, Alshora et al. (2019) utilized a planetary ball mill technique to prepare RC nanoparticles aimed at enhancing dissolution and systemic bioavailability. The optimized formulation showed a 1.3-fold increase in dissolution rate and a twofold rise in plasma concentration compared to raw RC ($P < 0.05$). Stability assessments conducted under long-term storage conditions (30°C and 60% relative humidity) indicated excellent formulation stability. Follow-up experiments involved refining both the formulation and stabilization strategies through wet milling with 0.1 mm milling media at 800 rpm for three 10-minute cycles. Among the resulting NSs, those stabilized with 10% polyvinylpyrrolidone (PVP) displayed the smallest particle size and highest stability. This optimized batch exhibited a particle size of 461.8 ± 16.68 nm and a zeta potential (ZP) of -31.8 ± 7.22 mV, compared to the unprocessed RC with a size of 618 μ m. Dissolution studies demonstrated that 72% of RC from the nanoparticle formulation dissolved within one hour, significantly outperforming the 58.25% from the pure drug ($P < 0.05$). *In vivo* pharmacokinetic evaluation confirmed a marked enhancement in drug absorption, with $C_{\max}$ increasing to 82.35 ng/mL at 2 hours post-administration, compared to just 9.2 ng/mL for the unformulated drug (Alshora et al., 2019).

Hirpara et al. (2018) formulated long-circulating PEGylated chitosan nanoparticles to improve RC's pharmacokinetic and therapeutic profile. PEGylation was achieved using a carbodiimide-mediated reaction involving PEG carboxylic acid derivatives. Two optimized formulations were produced, each with particle sizes under 200 nm and EE around 14%. *In vitro* release testing over 120 hours showed cumulative drug releases of $14.07 \pm 0.57\%$ and $22.02 \pm 0.81\%$, indicating a controlled and extended release behavior. TEM analysis revealed that the nanoparticles were predominantly spherical. In pharmacokinetic studies, the formulations provided a prolonged drug release profile lasting up to 72 hours. Additionally, pharmacodynamic assessments in hyperlipidemic rat models demonstrated significantly improved lipid-lowering activity of the PEGylated formulation compared to the free drug, underscoring the system's potential for enhanced therapeutic efficacy (Hirpara et al., 2018).

Parameswaran et al. (2022) developed RC-loaded chitosan nanoparticles via the solvent evaporation method, employing a 2^3 factorial design for optimization. The optimized formulation exhibited a particle size of 159.9 ± 16.1 nm, contributing to improved dissolution, permeability, and surface area. The formulation's ZP was 33.5 ± 1.54 mV, indicating favorable colloidal stability, while the polydispersity index (PDI) of 0.587 ± 0.16 suggested a relatively uniform particle distribution. EE and percentage yield were high, measured at $94.20 \pm 2.46\%$ and $96.80 \pm 2.08\%$, respectively. *In vitro* release studies showed a maximum drug release of $96.54 \pm 2.02\%$ within 24 hours, following a controlled zero-order release pattern and a non-Fickian diffusion mechanism. The release exponent ($n = 0.5-0.8$) from the Peppas model indicated drug transport was governed by both diffusion and polymer relaxation. *In vivo* pharmacokinetic studies showed a significant increase in $AUC_{0-\infty}$, confirming the enhanced bioavailability of RC from the chitosan nanoparticle system (Parameswaran et al., 2022).

Bhokare et al. (2020) successfully created sustained-release, biodegradable chitosan nanoparticles

loaded with the drug RC. They utilized a modified ionotropic gelation method, optimized through a 3² full factorial design, to prepare the nanoparticles. The *in vitro* release analysis indicated a two-phase pattern: an initial burst effect followed by a significantly slower drug release. This research confirms that the modified ionic gelation technique presents a promising strategy for the effective delivery hydrophobic drugs using chitosan nanoparticles (Bhokare et al., 2020).

In their 2020 research, Mujtaba and Alotaibi created chitosan-alginate nanoparticles for the drug RC. Their method involved the ionotropic pre-gelation of an alginate core, which was then followed by chitosan polyelectrolyte complexation. The researchers fine-tuned the formulation by optimizing the concentrations of the two biopolymers and the crosslinker. The resulting nanoparticles showed a two-stage drug release pattern: an initial fast release over the first two hours, succeeded by a slower, more gradual release spanning the full 24-hour period. Overall, the findings indicate that this novel chitosan-alginate nanoparticle system for RC is a promising approach for boosting the drug's solubility and, consequently, its therapeutic efficacy. The authors concluded that this new nanosystem also holds significant potential for the oral delivery of other hydrophobic compounds (Mujtaba & Alotaibi, 2020).

In a 2022 study, Saadallah and Hamid explored the creation, characteristics, *in vitro* release, and *ex vivo* permeability of RC-loaded Eudragit® L100 nanoparticles, which they produced using a nanoprecipitation method. Their *in vitro* release data showed that the Eudragit® L100 polymeric nanoparticles were capable of delivering RC with a sustained and pH-dependent release profile. Furthermore, the *ex vivo* permeability study revealed a 1.6-fold increase in the amount of RC that successfully penetrated the skin. These results suggest that Eudragit® L100 polymeric nanoparticles have a strong potential for successful use in both dermal and transdermal drug applications (Saadallah & Hamid, 2022).

Nanoparticle formulations were developed using Poloxamer 407 and lecithin via the thin-film hydration method. The particles had a size range of 167-408 nm, a PDI value between 0.15 and 0.37, and a ZP of -20 to -53 mV. An increase in the lecithin ratio was observed to decrease the release percentage. The optimized formulation was coated with silver, and its effect on wound healing was investigated. Histopathological studies and serum levels of tumor necrosis factor-alpha (TNF- α) and interleukin-1 β (IL-1 β) confirmed that the silver-coated RC nanoparticle-loaded gel formulation was more effective in wound healing compared to gentamicin ointment (Salem et al., 2021).

In a study, chitosan-coated silver nanoparticles were prepared using the solvent evaporation method with a 2³ factorial design. The nanoparticles, with a size range of 211-223 nm and a PDI value of 0.254, were measured to have a ZP of -35.3 mV. Mucoadhesion studies revealed that chitosan increased the binding of the nanoparticles to mucin proteins through hydrogen bonds and other interactions. Release studies were conducted in dissolution media with different pH values. For the ideal formulations, it was determined that the release kinetics followed the Higuchi model, regardless of the media's pH. Furthermore, a lower release rate was observed at lower pH values, which confirmed that the solubility of RC is directly proportional to the pH. In addition, cytotoxicity studies reported that the optimized formulation had the lowest IC₅₀ value and the strongest anticancer activity (Mahmoud et al., 2025).

In another study, RC-loaded Poloxamer® 188 nanoparticles were produced using the homogenization method. The nanoparticles, with a particle size of 183.4 nm, were compared to a commercially available tablet formulation. The nanoparticles, which had a PDI value of 0.241 and a ZP of -26.6 mV, demonstrated a faster drug release than the tablets. Within 5 minutes, while the pure RC suspension reached 13% and the commercial formulation reached 26% release, the Poloxamer nanoparticles achieved nearly 90% release. Furthermore, the half-life from the pharmacokinetic

parameters was determined to be 14.7 hours for the nanoparticles, compared to 8.9 hours for the commercial formulation. Pharmacodynamic studies also showed that the nanoparticle formulation was more effective (Dudhipala et al., 2021).

The effectiveness of any pharmaceutical compound is critically tied to its bioavailability. When the particle size of a drug is reduced, its dissolution rate in biological fluids and its overall bioavailability within the body are significantly improved. Najafi et al. (2021) employed the Gas Anti-Solvent (GAS) method to decrease the size of RC particles. Their analysis confirmed the success of this technique, showing that the RC particles produced by the GAS process (measuring 60.3 nm) were substantially smaller than the original particles (which measured 45.8 μm) (Najafi et al., 2021).

El-Salamouni et al. (2025) developed a sustained-release nanoformulation of RC co-precipitated with calcium carbonate, intended for local application directly into the bone of osteoporotic rats. The researchers created these nano-formulations using a coprecipitation method, systematically varying the concentrations of polyvinyl alcohol stabilizer (at 0.2, 0.4, or 0.6%) and equimolar ratios of calcium chloride and calcium carbonate (at 0.1, 0.3, or 0.5 M). The optimized formula demonstrated a satisfactory nanosize range, good homogeneity, and remained stable throughout the six-month study period. Crucially, the local administration of these calcium carbonate nanoparticles enabled a prolonged release of RC, confirming it as an intelligent strategy for quickly promoting the healing of bone defects associated with osteoporosis (El-Salamouni et al., 2025).

Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)

SLNs, with sizes ranging between 1-1000 nm, are widely studied nanocarriers known for their biocompatibility, non-toxicity, and scalability (Beg et al., 2017a; Singh et al., 2018). Compared to polymeric carriers, SLNs offer advantages such as low cost, ease

of fabrication, high drug loading for both hydrophilic and lipophilic molecules, controlled release behavior, and good physical stability (Beg et al., 2017a). As a more advanced system, NLCs integrate liquid lipids into the solid lipid matrix, enhancing drug loading capacity and minimizing drug expulsion during storage (Amin & Ahmad, 2017). NLCs also display improved dispersibility, reduced water content in formulations, and enhanced biopharmaceutical performance (Darwish et al., 2022; Patil-Gadhe & Pokharkar, 2016).

Al-Heibshy et al. (2020) investigated SLNs for RC delivery, using stearic acid and tripalmitin as the lipid core materials. The SLNs were prepared through a hot homogenization process followed by ultrasonication. Particle sizes ranged from 134.37 ± 0.91 nm to 351.13 ± 1.39 nm, with narrow size distribution ($\text{PDI} < 0.5$), indicating uniform formulation. ZP measurements revealed negative surface charges (-17.03 ± 0.53 to -40.80 ± 1.2 mV), consistent with the presence of anionic lipids and suggestive of good dispersion stability. *In vitro* drug release studies showed that pure RC released ~75.8% of the drug within 2 hours, reaching 95.5% by 6 hours. In contrast, SLN formulations provided a more sustained release profile over 24 hours, with a noticeable initial burst phase in the first 6 hours. Permeation studies confirmed improved RC transport across biological membranes from SLNs compared to the unformulated drug. Pharmacokinetic analysis demonstrated significant enhancements in C_{max} (1.4-fold) and AUC_{last} (8.5-fold) for the SLN formulation, suggesting substantial improvement in systemic bioavailability (Al-Heibshy et al., 2020b).

Dudhipala and Veerabrahma (2017) formulated RC-loaded SLNs using different lipid matrices stearic acid, glyceryl behenate, and glyceryl trilaurate through a hot homogenization technique followed by ultrasonication. These formulations were evaluated for particle size, PDI, ZP, EE, drug content, and *in vitro* drug release. Among the tested lipids, glyceryl trilaurate produced the most favorable results, yielding nanoparticles with an average size of 67.21 ± 1.71 nm, a PDI of 0.25 ± 0.01 , ZP of -28.93 ± 0.84 mV,

and EE of $93.51 \pm 0.34\%$. The physical stability of the optimized SLNs was maintained over a 90-day period under both refrigerated and ambient conditions. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) analyses showed no significant drug–excipient interactions. Morphological assessment via scanning electron microscopy (SEM) and TEM revealed that the nanoparticles were nearly spherical. *In vitro* release testing indicated a slower release from SLNs (65–80%) over 24 hours, compared to nearly complete release (96%) from an RC suspension. Pharmacokinetic studies demonstrated a 4.6-fold increase in oral bioavailability for the SLNs. Additionally, pharmacodynamic evaluations in hyperlipidemic rat models showed prolonged lipid-lowering activity for 36 hours with the SLNs, while the drug suspension showed efficacy for only 24 hours (Dudhipala & Veerabrahma, 2017).

In a separate investigation, Singh et al. (2018) developed RC-loaded SLNs using stearic acid through a modified solvent emulsification-diffusion technique. The nanoparticles were characterized for their size, surface charge, drug loading, and release behavior. The optimized formulation exhibited a particle size of 115.49 ± 2.97 nm, PDI of 0.456, ZP of -18.40 mV, and an EE of 97.16%, with drug loading reaching 60.34%. *In vitro* drug release followed a sustained pattern, with $88.70 \pm 3.59\%$ of the drug released within 12 hours, conforming to the Higuchi model ($R^2 = 0.9905$). TEM analysis showed spherical particle morphology. The pharmacokinetic profile indicated a 4.44-fold enhancement in bioavailability over a plain RC suspension. Stability testing over 180 days at $25 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ RH confirmed long-term stability of the formulation (Singh et al., 2018).

Darwish et al. (2022) explored the comparative performance of SLNs and NLCs for improving the solubility and oral bioavailability of RC. The nanoparticles were prepared using Apifil (solid lipid) and Maisine (liquid lipid) through high-speed homogenization combined with ultrasonication. The resulting nanoparticles were lyophilized and encapsulated into

oral dosage forms. Particle sizes reached up to 180.3 nm, and EE values varied from 40.77% to 91.67%, depending on formulation parameters. Clinical testing in six healthy volunteers revealed that NLC-based formulations demonstrated superior pharmacokinetic performance. Specifically, the NLC-loaded RC achieved a C_{max} of 8.92 ng/mL, compared to 2.56 ng/mL for the marketed RC product, translating to a ~349% relative increase in bioavailability. Additionally, NLC capsules exhibited better formulation stability than their SLN counterparts (Darwish et al., 2022).

In another study, an NLC formulation of RC was developed using melt emulsification followed by ultrasonication, optimized via a Box–Behnken statistical design. The lipid matrix consisted of 3% w/v glyceryl monostearate (solid lipid) and capmul MCM EP (liquid lipid), with poloxamer 188 and Tween 80 as surfactants. The final optimized formulation had a particle size of 150.3 ± 4.67 nm, a PDI of 0.175 ± 0.022 , ZP of -32.9 ± 1.36 mV, and an EE of $84.95 \pm 5.63\%$. *In vitro* release studies in simulated intestinal fluid (pH 6.8) indicated that the NLCs released the drug more effectively than a suspension of the active pharmaceutical ingredient (API). Confocal laser scanning microscopy showed enhanced penetration into intestinal tissues relative to a rhodamine B dye control. Pharmacokinetic studies in female Wistar rats revealed a 5.4-fold improvement in relative bioavailability for the NLCs. Furthermore, lipid-lowering efficacy was significantly improved in hyperlipidemic rats ($p < 0.01$), highlighting the therapeutic potential of the optimized NLC system (Amin & Ahmad, 2017).

Patil-Gadhe and Pokharkar formulated a dry powder inhaler (DPI) system containing RC-loaded NLCs aimed at targeted pulmonary delivery. The NLCs were lyophilized using 5% mannitol as a cryoprotectant and carrier. Particle deposition and aerodynamic properties were evaluated using an 8-stage cascade impactor, revealing a mass median aerodynamic diameter (MMAD) below $3\ \mu\text{m}$ and a fine particle fraction (FPF) exceeding 90% at a 60 L/min flow rate. Use of L-leucine as an anti-static agent further improved

dispersibility and aerosolization. *In vivo* pulmokinet-ic studies in Wistar rats showed enhanced pharmacokinetic outcomes, including a 1.14-fold increase in $C_{\max}$, a fivefold extension in half-life, and a dramatic 35-fold rise in AUC_{0-1} , suggesting a substantial increase in systemic exposure. The combination of lipid-based composition and small particle size likely facilitated macrophage evasion, resulting in improved lung targeting. This delivery platform holds promise for managing pulmonary conditions such as chronic obstructive pulmonary disease (COPD) (Patil-Gadhe & Pokharkar, 2016).

Self-nanoemulsifying drug delivery systems (SNEDDS)

Given RC's extensive hepatic metabolism through oxidation, lactonization, and glucuronidation pathways, improving oral bioavailability requires strategies that increase solubility and reduce first-pass metabolism (Phan et al., 2024). SNEDDS have emerged as a promising approach to address these challenges (Desavathu et al., 2020). SNEDDS are isotropic mixtures comprising lipids, surfactants, and cosurfactants, forming nano-sized oil-in-water emulsions (typically <250 nm) upon contact with gastrointestinal fluids (Kulkarni et al., 2015). These systems improve bioavailability by enhancing solubility, promoting membrane permeation, and facilitating lymphatic transport, thereby bypassing hepatic metabolism (Vishwakarma et al., 2016). Their small droplet size and lipidic composition contribute to improved absorption and more predictable plasma drug profiles (Karasulu et al., 2018). However, liquid SNEDDS are often thermodynamically unstable, leading to the development of solid SNEDDS formulations to enhance stability and practicality for pharmaceutical applications (Arun et al., 2020; Kulkarni et al., 2015).

Phan et al. (2024) developed a SNEDDS formulation of RC using Capryol 90, Cremophor RH40, and PEG 400 as the oil, surfactant, and co-solvent, respectively. The primary aims of the study were to compare the oral absorption of the RC-SNEDDS with the pure

drug and to establish a Level A *in vitro*–*in vivo* correlation (IVIVC). The pharmacokinetics of both formulations were assessed in beagle dogs following oral administration. Results indicated that the RC-SNEDDS improved oral bioavailability by 1.7 times and $C_{\max}$ by 2.1 times compared to the raw drug. A robust IVIVC was demonstrated by correlating the *in vitro* fraction of drug remaining to dissolve with the *in vivo* fraction of drug remaining to be absorbed. Optimal dissolution conditions were determined to be at pH 6.6 in a 900 mL medium, yielding the strongest linear relationship ($R^2 = 0.996$) and the lowest Akaike Information Criterion (AIC) value (506.9), supporting the predictability of the IVIVC model (Phan et al., 2024).

Using an optimized experimental design, Arun et al. (2020) formulated several RC-SNEDDS and evaluated them for emulsification time, ZP, globule size, PDI, and *in vitro* release. The optimized formulation contained 15% isopropyl myristate (lipid), 75% Tween 20 (emulsifier), and 10% ethanol (co-solvent). This composition enabled rapid emulsification in 150 seconds and yielded globules of 68 nm with a ZP of +27 mV. More than 85% of the drug was released within 30 minutes. *In vivo* pharmacokinetic studies in rats showed substantial enhancement in systemic exposure, with approximately 4.89- and 4.45-fold increases in $C_{\max}$ and AUC_{0-24h} , respectively ($p < 0.0001$), and a reduction in $t_{\max}$ by 0.98-fold ($p < 0.05$). These results suggest that the rapid drug release and enhanced intestinal absorption contributed to bypassing hepatic first-pass metabolism, thus improving bioavailability (Arun et al., 2020).

Desavathu et al. (2020) developed and characterized SNEDDS formulations using the aqueous titration method. The selected system, comprising cinnamon oil (oil), Cremophor RH40 (surfactant), and Transcutol P (co-surfactant) in a 1:5 oil-to-Smix ratio, yielded the smallest droplet sizes and demonstrated high thermodynamic stability. The SNEDDS formulations were encapsulated in hard gelatin capsules, making them suitable for drugs with low dose or high solubility requirements. Physical characteriza-

tion confirmed uniform droplet size distribution and stability based on ZP analysis. *In vitro* drug release studies demonstrated significantly enhanced dissolution rates compared to a marketed RC product. The optimized SNEDDS achieved a cumulative release of $97.70 \pm 0.25\%$, significantly surpassing the marketed formulation's release of $63.5 \pm 0.28\%$. These outcomes confirm the potential of SNEDDS in enhancing RC solubility and bioavailability (Desavathu et al., 2020).

Oza et al. (2020) formulated SNEDDS for RC using a simplex lattice design approach. Formulations were prepared using Peceol and ethyl oleate as oil components (in a 1:1 ratio), along with Labrasol and Cremophor EL as surfactants. The optimal SNEDDS contained 15% oil, 50% Labrasol, and 35% Cremophor EL. Physicochemical characterization showed globule sizes ranging from 22.90 ± 1.50 nm to 43.90 ± 1.40 nm, with no significant changes after 3 months of stability testing. High transparency ($95.40 \pm 1.40\%$ to $99.50 \pm 1.10\%$) and rapid drug diffusion ($63.65 \pm 1.51\%$ to $93.72 \pm 1.46\%$ within 10 minutes) were observed. These data highlight the potential of SNEDDS to significantly improve the aqueous solubility and delivery efficiency of RC (Oza et al., 2020).

Karasulu et al. (2018) designed an RC-SNEDDS consisting of 12.8% oleic acid, 11% Labrafil M, 3.3% Labrasol, and 4.4% Transcutol HP. The resultant nanoemulsion had droplet sizes in the 200–250 nm range and showed excellent physical stability. Permeation studies demonstrated a fourfold increase in drug transport compared to the marketed Crestor® 20 mg tablet. Pharmacokinetic analysis in Yorkshire pigs confirmed higher bioavailability for the SNEDDS formulation. Correspondingly, pharmacodynamic studies revealed superior lipid-lowering effects: triglycerides and total cholesterol were reduced by 37% and 19%, respectively, compared to only 6% and 2% reductions with the commercial formulation. These findings emphasize the formulation's potential to enhance therapeutic outcomes by improving both absorption and efficacy (Karasulu et al., 2018).

To improve the dissolution and antihyperlipidemic activity of RC, Ahsan and Verma (2017) formulated a solid SNEDDS (S-SNEDDS). The solid form demonstrated good flow characteristics, with compatibility studies confirming the absence of interactions between drug and excipients. Electron microscopy revealed spherical, nanosized droplets (~100 nm) without aggregation. Dissolution testing showed a significant improvement: S-SNEDDS achieved 95% release within 60 minutes, compared to 51.89% from the raw drug. *In vivo* evaluations over 14 days indicated notable lipid-lowering benefits, with cholesterol and triglycerides reduced by 33.47% and 40.77%, respectively, and atherogenic index decreased by 81.28%. Additionally, HDL levels increased by 118.43%. These results suggest that the S-SNEDDS platform can substantially improve both the dissolution rate and therapeutic effectiveness of RC (Ahsan & Verma, 2017).

A Quality by Design (QbD) approach was employed to develop liquid SNEDDS (L-SNEDDS) of RC using long-chain lipophilic excipients. Solubility screening revealed Peceol (lipid), Tween 80 (surfactant), and Transcutol HP (co-solvent) as optimal components. Pseudoternary phase diagrams indicated the most extensive nanoemulsion region at a 1:1 S_{mix} ratio. Using a D-optimal mixture design, the optimized L-SNEDDS achieved rapid emulsification (~131 seconds), sub-100 nm droplet size, and >80% drug release within 15 minutes. Permeability studies showed significant enhancement: over 70% drug permeation across rat intestinal tissue *ex vivo* and 1.8- to 2.1-fold increases in *in situ* permeability and absorptivity versus plain drug suspension. *In vivo* pharmacokinetics demonstrated 1.8-fold and 5.7-fold increases in AUC_{0-t} and C_{max} , respectively, along with a 66% reduction in t_{max} . Over 21 days, the optimized L-SNEDDS led to substantial lipid-lowering effects: reductions in total cholesterol (52%), LDL (45%), and triglycerides (35%), outperforming both the marketed formulation and pure drug. Notably, HDL levels rose by 128% ($p < 0.0001$) (Beg et al., 2017b).

Kulkarni et al. (2015) developed S-SNEDDS of RC using a lipid blend of LAS/Capryol 90 and Maisine 35-1, combined with a S_{mix} of Tween 20 and Lutrol E400. The liquid formulation was solidified using Aerosil 200 (1:0.25 w/w ratio). Characterization revealed nano-sized droplets (119.8–228.9 nm), rapid self-emulsification within ~60 seconds, and high transmittance (~100%). *In vitro* release demonstrated a fourfold increase in dissolution within 10 minutes compared to raw RC. Pharmacodynamic studies using a Triton-induced hyperlipidemia model showed improved bioavailability and therapeutic response (Kulkarni et al., 2015).

Another investigation developed RC-loaded S-SNEDDS using Capmul MCM (oil), Tween 20 (surfactant), and PEG 200 (co-surfactant). The optimized liquid SNEDDS was adsorbed onto porous polystyrene spheres, yielding a solid system with good flowability and preserved droplet size post-reconstitution. Dissolution testing showed 98.92% drug release within 60 minutes, significantly higher than the 38.6% release from raw RC. Pharmacokinetic assessments showed a remarkable ~8-fold increase in both C_{max} and AUC_{0-t} over the pure drug, validating the effectiveness of S-SNEDDS in enhancing bioavailability (Panner Selvam et al., 2015).

Rokad et al. (2014) formulated S-SNEDDS using Capmul MCM, Cremophor ELP, and propylene glycol. The optimized system, encapsulated in hard gelatin capsules, showed a ZP of -22.11 mV, particle size of 10.59 nm, and rapid self-emulsification. Drug release was highly efficient, reaching 97.7%, exceeding even the marketed RC product. This study emphasized the benefits of transitioning from liquid to solid SNEDDS for enhanced solubility, stability, and reduced drug precipitation (Rokad et al., 2014).

Balakumar et al. evaluated SNEDDS containing cinnamon oil (30%), Labrasol (60%), and Capmul MCM C8 (10%). The formulation showed consistent droplet size (<200 nm), robust self-emulsification, and high stability across various pH levels. It exhib-

ited a ZP of -29.5 ± 0.63 mV and an average particle size of 122 nm. *In vitro* studies showed improved dissolution, while *in vivo* analysis indicated a 2.45-fold enhancement in bioavailability compared to the plain suspension. The pharmacokinetics followed a one-compartment model, as analyzed using PKSolver 2.0. (Balakumar et al., 2013).

Vesicular nanocarriers

Vesicular carriers including liposomes, niosomes, and chitosomes consist of lipid bilayers or surfactant-based membranes encapsulating drugs. These systems enable targeted delivery and sustained release, improving both pharmacokinetics and pharmacodynamics.

Niosomes

Niosomes are bilayer vesicles composed of non-ionic surfactants and cholesterol. Unlike liposomes, they avoid phospholipids and exhibit greater physical stability (Shrivastava et al., 2025). Salih et al. formulated RC-loaded niosomes using the thin-film hydration method. Span 60-based formulations demonstrated higher drug entrapment and sustained release over 7 hours. TEM showed spherical vesicles ~150 nm in size (Salih et al., 2013).

Chitosomes and liposomal nanoparticles

Ahmed (2020) compared chitosan-coated lipid vesicles (chitosomes) with negatively charged liposomal nanoparticles. Chitosomes demonstrated enhanced cytotoxicity (lower IC_{50}) and broader therapeutic indices, while liposomal formulations showed higher intestinal cell compatibility. These findings suggest chitosomes may be more suitable for targeted delivery in hyperlipidemia, while liposomes are advantageous for cardiovascular therapeutic applications (Ahmed, 2020).

Other nanocarriers

RC, a BCS Class II compound, suffers from low aqueous solubility, which significantly limits its oral bioavailability (Shoukat et al., 2024). To address this challenge, numerous advanced drug delivery systems have been explored to enhance its solubility and dis-

solution. These include nanogels (NGs), nanocrystals (NCs), inclusion complexes, NSs, SDs, and vesicular systems. Below is a summary of key strategies, excluding emulsion-based carriers.

Nanogels (NGs)

NGs are nanoscale hydrogel systems (10–500 nm) that combine high water content with the responsiveness of polymer networks. Their ability to swell, retain water, and provide sustained release makes them suitable for enhancing the solubility of hydrophobic drugs like RC (Gallo et al., 2025; Shoukat et al., 2022). A recent study fabricated interpenetrating polymer network (IPN) NGs using chitosan (CS) and beta-cyclodextrin (β -CD). These NGs significantly improved RC solubility and release compared to conventional formulations. In hyperlipidemic rabbit models, RC-loaded NGs demonstrated marked reductions in LDL-C and increases in HDL, confirming superior therapeutic performance (Shoukat et al., 2022; Shoukat et al., 2024).

Nanocrystals (NCs)

NCs, existing between crystalline and amorphous states, enhance solubility by increasing surface area and reducing particle size. This transformation often involves partial amorphization, increasing surface energy and dissolution rate (Wang et al., 2012). Palani, et al. (2015) developed RC NCs via top-down and bottom-up techniques. The resulting tablets showed a 36% improvement in dissolution and 1.87-fold increase in bioavailability over micronized RC formulations (Palani et al., 2015).

Inclusion complexes with cyclodextrins

Cyclodextrins (CDs) form host-guest inclusion complexes with hydrophobic drugs, thereby enhancing aqueous solubility. Their hydrophobic interior accommodates drug molecules, while the hydrophilic exterior interacts with water (Anjani et al., 2022). Al-Heibshy et al. (2020) prepared RC inclusion complexes using methyl- β -cyclodextrin (M- β -CD) and sulfobutylether- β -cyclodextrin (SBE- β -CD; Captisol®) via kneading and lyophilization. These complex-

es achieved drug EE up to 105%, 3.7–4.1-fold solubility enhancement, and comparable dissolution to pure RC. Caco-2 cell assays confirmed biocompatibility, and the apparent permeability coefficient (Papp) reached 3.08×10^{-7} cm/s, indicating enhanced intestinal permeation (Al-Heibshy et al. 2020a).

Nanosuspensions (NSs)

NSs consist of pure drug particles dispersed in a liquid medium and stabilized by surfactants or polymers. Particle size reduction into the nanometer range drastically increases surface area and dissolution rate (Jacob et al., 2025). Rani et al. (2023) formulated an RC-NSs using PVP K-30 and Tween 80 via high-pressure homogenization. A 3-factor factorial design optimized particle size (92.79 nm), and PDI (0.201). *In vitro* studies showed significantly improved dissolution compared to pure RC (Rani et al., 2023). Joshi et al. (2023) successfully developed and evaluated transdermal patches loaded with an RC NSs. To enhance the drug's dissolution profile, a NSs was prepared using a precipitation-ultrasonication technique. This method involved blending various water-soluble, film-forming polymers, specifically hydroxyl propyl methyl cellulose (HPMC K4M) and Eudragit. The researchers concluded that these transdermal patches are potentially suitable for sustained drug release, thereby improving patient compliance (Joshi et al., 2023).

Solid dispersions (SDs)

SDs distribute drug molecules within inert hydrophilic carriers in the solid state, usually via melting or solvent techniques (Mhaske et al., 2025). Arjun et al. (2020) prepared RC-SDs via melt fusion and NSs using high-shear homogenization. Tablets made from both forms exhibited enhanced drug release in pH 6.8 buffer within 60 minutes, outperforming both the control and a marketed RC tablet (Arjun et al., 2020).

Nanosponges (NSPs)

NSPs are hyper-cross-linked polymeric nanoparticles characterized by colloidal size and nanoscale porous cavities. β -CD-based NSPs are particularly

attractive for drug delivery due to their ability to sustain drug release, enhance stability, achieve high drug loading, and encapsulate both hydrophilic and hydrophobic agents. These features contribute to improved bioavailability through modulation of pharmacokinetic profiles (Sampathi et al., 2025). Satya Lakshmi et al. (2021) prepared β -CD-based RC-NSPs using the emulsion solvent diffusion method. The resulting RC-loaded NSPs were formulated into extended-release tablets. Release kinetics predominantly followed first-order models, while some formulations aligned with Higuchi or Korsmeyer-Peppas models. Notably, erosion was identified as the dominant mechanism driving drug release in most formulations, rather than diffusion (Satya Lakshmi et al., 2021).

Nanofibers (NFs)

NFs are solid fibers with diameters typically below 100 nm, offering high surface area, exceptional porosity, flexibility, and favorable mechanical properties. These characteristics make nanofibers ideal drug delivery systems for precisely controlling release rate, duration, and site, thereby enhancing therapeutic efficacy and safety (Alijaniha et al., 2025). Mohammed & Al-Gawhari (2024) developed and optimized RC nanofibers and cyclodextrin inclusion complex nanofibers via electrospinning. Pharmacokinetic studies revealed significantly enhanced drug absorption, faster release, and superior bioavailability compared to pure RC and marketed formulations. The nanofibers demonstrated increased AUC and C_{max} values alongside reduced t_{max} , indicating more rapid and efficient systemic uptake (Mohammed & Al-Gawhari, 2024). Additionally, orodispersible films co-loaded with amlodipine besylate and RC were fabricated using electrospinning, achieving rapid dissolution (>90% release within 5 minutes). This method notably improved RC solubility and shows promise for scalable industrial production of fixed-dose combination films (Muratoğlu et al., 2024).

CONCLUSION

Statins, first discovered in the 1970s, remain the

most widely prescribed class of antihyperlipidemic agents worldwide. RC, often termed the “super-statin,” is distinguished by its potent lipid-lowering efficacy and broad global approval. However, RC’s low aqueous solubility and limited permeability result in modest oral bioavailability (~20%) when administered via conventional dosage forms. To overcome these challenges, diverse nanocarrier-based delivery systems have been developed, including polymeric nanoparticles, SLNs, NLCs, SNEDDS, NGs, NCs, NSs, niosomes, NSPs, and NFs. Collectively, these approaches have significantly enhanced RC’s solubility, dissolution rate, and pharmacokinetic profile, leading to improved oral bioavailability and therapeutic outcomes. These advancements underscore the considerable potential of nanotechnology-driven delivery platforms as promising strategies for optimizing the oral delivery of RC.

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AUTHOR CONTRIBUTION STATEMENT

Conception (MD), Literature research and writing (KA, MD), Reviewing the text (KA, MD), Supervision (MD).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Integrating Artificial Intelligence Across the Drug-Discovery Pipeline: From Targets to Preclinical Proof

Dilhan NAMLI*, Nesrin GÖKHAN KELEKÇİ***

Integrating Artificial Intelligence Across the Drug-Discovery Pipeline: From Targets to Preclinical Proof

İlaç Keşfi Sürecinde Yapay Zekâ Entegrasyonu: Hedeflerden Preklinik Kanıt

SUMMARY

Artificial intelligence (AI)-based approaches have drawn significant attention for their potential to address major challenges in drug discovery processes, such as time constraints, high costs, and low success rates. Specifically, machine learning (ML) and deep learning (DL) algorithms are effectively utilized in various stages of drug development, including target identification, molecular screening, lead compound selection, optimization, and ADMET prediction. In this study, the integration of current AI models into pharmaceutical R&D processes is examined from an interdisciplinary perspective, and their application domains are evaluated through relevant case studies in the literature. It has been observed that ML-based methods can yield successful results even with limited data, while DL architectures offer advantages in modeling complex molecular relationships. Furthermore, the architectural frameworks, training strategies, and diversity of ML and DL algorithms are comprehensively discussed within the scope of the study. It is demonstrated that traditionally experience-based decision processes such as retrosynthetic planning and formulation development can be accelerated and made more sustainable through data-driven systems. Additionally, AI-assisted predictions are shown to reduce the experimental burden and enhance research efficiency in preclinical and clinical stages. These evaluations suggest that AI technologies are not merely supportive tools but also strategic components at the core of innovative drug discovery approaches.

Keywords: Artificial intelligence, drug design, predictive modeling, computational drug discovery, virtual screening.

ÖZ

Yapay zekâ tabanlı yöntemler, ilaç keşfi süreçlerinde karşılaşılan zaman, maliyet ve başarı oranı gibi temel sorunlara çözüm üretme potansiyeliyle dikkat çekmektedir. Özellikle makine öğrenmesi ve derin öğrenme algoritmaları, ilaç geliştirme sürecinin hedef belirleme, molekül tarama, öncü bileşik seçimi, optimizasyon ve ADMET tahmini gibi aşamalarında etkin biçimde kullanılmaktadır. Bu çalışmada, güncel yapay zekâ modellerinin farmasötik Ar-Ge süreçlerine entegrasyonu disiplinlerarası bir yaklaşımla ele alınmış; literatürde yer alan vaka örnekleriyle bu modellerin uygulama alanları değerlendirilmiştir. Makine öğrenmesi tabanlı yöntemlerin sınırlı veriyle dahi başarılı sonuçlar üretebildiği, derin öğrenme mimarilerinin ise karmaşık moleküler ilişkileri tanımlamada avantaj sağladığı gözlemlenmiştir. Ayrıca çalışma kapsamında, makine öğrenmesi ve derin öğrenme algoritmalarının mimari yapıları, eğitim stratejileri ve model çeşitliliği detaylı olarak ele alınmıştır. Retrosentez planlaması ve formülasyon geliştirme gibi geleneksel olarak deneyime dayanan karar süreçlerinin, veri odaklı sistemlerle desteklenerek daha hızlı ve sürdürülebilir hâle getirilebileceği ortaya konmuştur. Preklinik ve klinik aşamalarda yapay zekâ destekli tahminlerin deney yükünü azalttığı ve araştırma verimliliğini artırdığı vurgulanmıştır. Bu kapsamda yapılan değerlendirmeler, yapay zekâ teknolojilerinin yalnızca destekleyici bir araç değil, aynı zamanda yenilikçi ilaç keşif yaklaşımlarının merkezinde yer alan stratejik bir unsur olduğunu göstermektedir.

Anahtar Kelimeler: Yapay zekâ, ilaç tasarımı, öngörülse modelleme, hesaplamalı ilaç keşfi, sanal tarama.

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* ORCID: 0009-0004-9682-5556, R&D Formulation Department, GEN İlaç ve Sağlık Ürünleri San. ve Tic. A.Ş., Ankara, Turkey

** ORCID: 0000-0001-7383-8443, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

INTRODUCTION

In recent years, artificial intelligence (AI) has revolutionized many disciplines, particularly the health sciences, by simulating cognitive functions such as learning and decision-making. The integration of this technology into drug discovery processes marks the beginning of a new era by aiming to overcome the limitations of traditional methods and resolve the growing complexity of biomedical research.

Conventional drug discovery methods rely heavily on experimental procedures, which are both time-consuming and costly. Typically, only a few compounds among thousands progress to clinical evaluation, and even fewer reach the market. Developing a single drug may take an average of 10 to 15 years and cost between 1 to 2 billion USD. Furthermore, up to 90% of drug candidates fail during clinical trials. These high failure rates not only cause financial losses but also result in the inefficient use of human resources and experimental infrastructure.

As a result, the need for faster, more flexible, and more accurate systems that can transcend the limitations of traditional approaches has become increasingly urgent. At this point, AI steps in with its data-driven analytical capabilities and provides critical decision support tools from the early phases of drug discovery onward (Parvatikar et al., 2023; Wu et al., 2024b; Yang, Wang, Byrne, Schneider, & Yang, 2019a).

AI-based methods are being integrated into various stages of the drug development pipeline in order to overcome these challenges. By leveraging machine learning (ML) and its subfield, deep learning (DL), accurate predictions can be made on large-scale biomedical datasets. These technologies enable the development of decision support mechanisms across a wide range of processes—from target identification

and molecular design to synthesis planning and pre-clinical or clinical studies.

There exists a hierarchical structure between the concepts of artificial intelligence, machine learning, and deep learning: while AI is the overarching concept, ML represents a subfield that focuses on learning from data. Deep learning, in turn, is a specialized approach within ML that employs multi-layered neural network architectures for high-level pattern recognition (Janiesch, Zschech, & Heinrich, 2021). These interrelated disciplines have the potential to reshape the future of pharmaceutical innovation.

This structural relationship, along with the integration of these approaches into various stages of drug discovery and development, is schematically illustrated in Figure 1.

In this study, the historical development of artificial intelligence and its role in the healthcare sector are first examined. Subsequently, AI-driven drug discovery processes are analyzed in detail under the headings of target identification, lead compound discovery, molecular optimization, and synthesis planning. The following sections explore machine learning and deep learning models, followed by an evaluation of their effectiveness based on recent applications in the literature. Finally, the integration of AI into pharmaceutical development processes is discussed—ranging from preclinical stages to clinical research, and from formulation development to ethical and regulatory considerations. In light of all these sections, the positioning of AI technologies within pharmaceutical research is addressed through an interdisciplinary perspective.

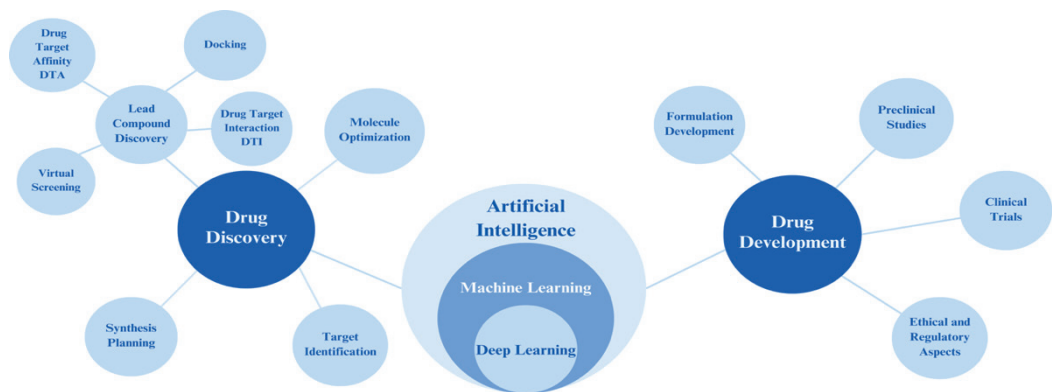


Figure 1. A schematic representation of the structural hierarchy among AI, ML, and DL, and their roles in drug discovery and development processes.

THE HISTORY OF AI & ITS PLACE IN THE HEALTHCARE SECTOR

The historical development of AI technologies provides a crucial foundation for understanding the evolution of their applications in healthcare and drug discovery (Figure 2.). This journey began in 1943 with the mathematical modeling of artificial neural networks by McCulloch and Pitts (McCulloch & Pitts, 1943) and gained conceptual depth with the introduction of the

Turing Test in 1950 (Turing, 1950). In the following decades, the advancement of approaches such as machine learning, deep learning, and big data analytics significantly accelerated progress. Since the 1980s, evolving neural network architectures have laid the groundwork for clinical decision support systems and drug discovery models, especially in areas like visual data processing and natural language processing (Gupta et al., 2021; Kaul, Enslin, & Gross, 2020; Niazi, 2023).

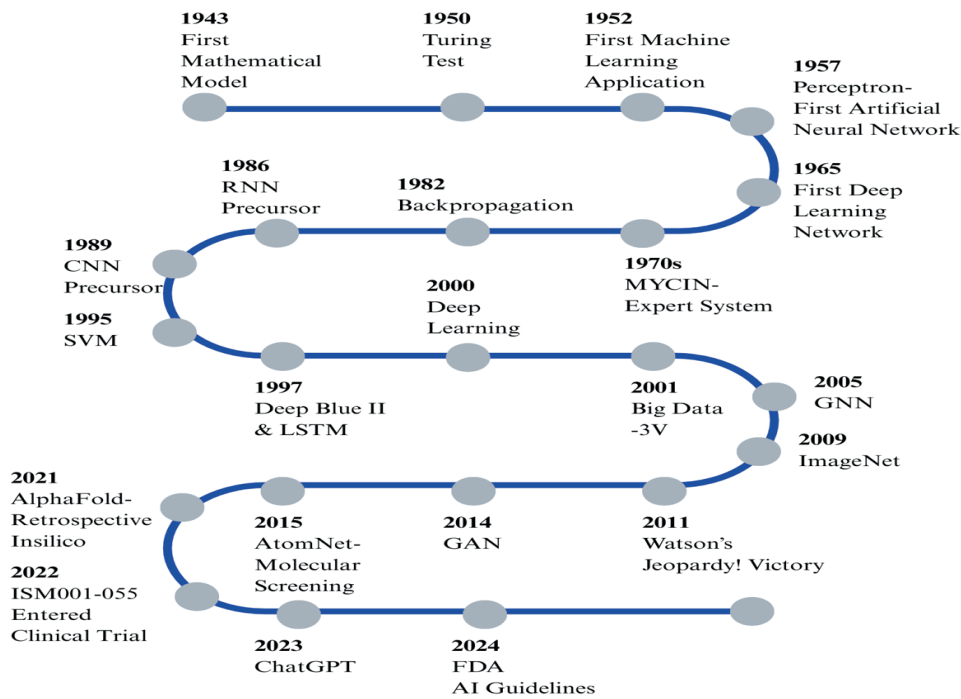


Figure 2. Timeline of historical developments in the field of artificial intelligence (1943–2024).

Visualized through a spiral timeline, these developments present the evolutionary trajectory of AI technologies in drug discovery within a temporal perspective—revealing a multilayered progression from early mathematical models to the widespread use of modern neural network architectures. This timeline not only illustrates a technical chronology, but also highlights scientific breakthroughs, technological leaps, and the stages at which AI integration gained momentum in pharmaceutical R&D processes. In this context, examples such as AtomNet (Wallach, Dzamba, & Heifets, 2015), AlphaFold (Jumper et al., 2021), and Insilico Medicine (Zhavoronkov et al., 2019) demonstrate that artificial intelligence is not only a theoretical concept, but also an effective tool for practical applications (Gupta et al., 2021; Insilico Medicine, 2022; Kaul et al., 2020; Kumar et al., 2022; Li et al., 2024; Niazi, 2023; Rai, Sahu, & Sawant, 2018).

AI-DRIVEN MOLECULAR DISCOVERY, OPTIMIZATION & SYNTHESIS PROCESSES

Target Identification and Lead Compound Discovery

Target Identification

Drug discovery relies on identifying biomolecular targets involved in disease mechanisms and discovering lead compounds that interact with them. In the era of personalized medicine, integrating molecular, environmental, and microbiome data has become

essential for defining clinically relevant targets. The growing regulatory focus on safety and ethics also demands more rigorous target validation studies.

Effectively managing this multidimensional process requires hybrid systems that combine human expertise with AI-based data processing. Human intuition enables recognition of complex biological patterns, whereas AI provides cognitive support in analyzing massive, multidimensional datasets. Such human-AI collaboration-empowered by cheminformatics and knowledge-based technologies-enhances the reliability and efficiency of target identification.

By enabling the integration and interpretation of large-scale biomedical data, systems biology and network-based computational models grounded in omics data have improved the reliability of target identification and promoted more holistic, data-driven strategies in drug discovery (Katsila, Spyrou, Patrinos, & Matsoukas, 2016). Integrating genetic and genomic evidence further improves target validation: clinical success rates can increase by up to 80% when strong genetic support exists for a given target (Wenteler et al., 2024).

Oprea and colleagues (2018) classified the human proteome into four categories -clinically known, chemically known, biologically known, and dark targets- collectively referred to as the “druggable genome,” as illustrated in Figure 3 (Oprea et al., 2018).

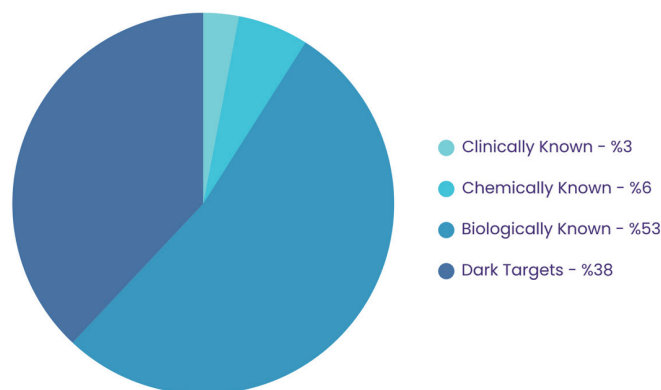


Figure 3. Classification of the human proteome according to Oprea et al., 2018.

This classification clearly underscores the vast potential that remains untapped in the field of drug discovery (Doytchinova, 2022). Accordingly, the Illuminating the Druggable Genome (IDG) initiative, launched by the U.S. National Institutes of Health (NIH) in 2014, aims to systematically elucidate the druggable genome. Open-access platforms such as Pharos and the Target Central Resource Database (TCRD) provide researchers with comprehensive data on over 20,000 human protein targets. These resources have significantly contributed to the understanding of underexplored protein targets and have facilitated the identification of novel therapeutic opportunities (Sheils et al., 2021).

Structural characterization of target macromolecules is essential for rational drug design. Techniques such as nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and more recently, cryo-electron microscopy (Cryo-EM) are used to determine protein structures, which are archived in the

Protein Data Bank (PDB). As of Q1 2025, the PDB contains structural data for 234,785 biomacromolecules, while PDBind includes 3D structures and binding affinities for 27,385 complexes. This wealth of data on protein-ligand interactions serves as a valuable foundation for AI-integrated models, guiding the identification of new therapeutic opportunities.

In conclusion, the integration of AI-powered *in silico* approaches with genetic data not only enhances the accuracy and clinical success rate of target identification but also enables the development of more time- and cost-efficient drug discovery strategies.

Lead Compound Discovery

Drug discovery has historically relied on various approaches, the earliest of which involved serendipitous findings and the chemical modification of existing bioactive molecules. Over time, these empirical methods evolved into systematic screening strategies based on chemical libraries, molecular targets and AI (Doytchinova, 2022) (Figure 4).

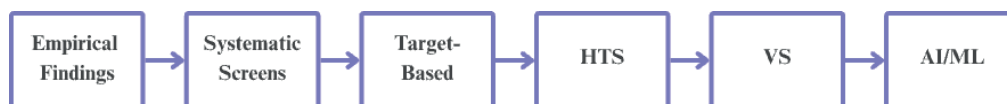


Figure 4. Evolution of lead discovery

With advances in combinatorial synthesis, automation, and genomic sequencing, the field shifted toward more target-oriented and efficient discovery pipelines. However, the exponential growth in compound diversity and target complexity has increasingly challenged conventional experimental methods (Wilkey, Haunso, Tudor, Webb, & Connick, 2017). The demand for systematic screening led to the development of high-throughput screening (HTS), which automates the testing of hundreds of thousands of molecules against biological targets. Despite its util-

ity, HTS is costly and resource-intensive, prompting a transition toward computational strategies such as virtual screening (VS). Integrating AI and ML algorithms has enabled VS to evolve from a computational extension of HTS into a core component of rational drug design (RDD) (Figure 5.). By virtually scanning large chemical libraries *in silico*, VS identifies potential “hit” compounds for experimental validation, thus accelerating early-stage drug discovery (Andricopulo & Ferreira, 2014).

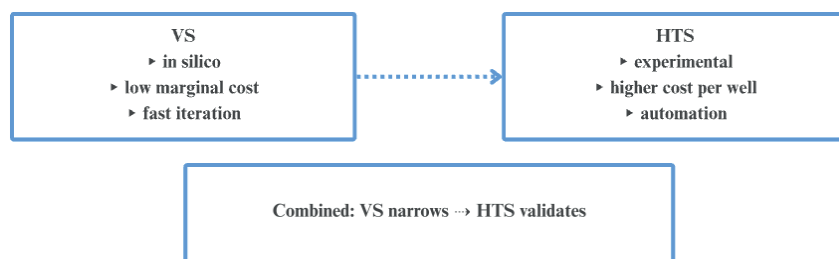


Figure 5. Virtual screening vs HTS

Rational drug design begins with the identification of a biological target associated with a specific disease and proceeds through the discovery and optimization of ligands that exhibit desired pharmacological activity (Doytchinova, 2022). In this context, two primary approaches form the foundation of rational drug design:

- *Ligand-Based Drug Design*

In many cases, the discovery of pharmacologically active new compounds can be achieved without requiring any information about the three-dimensional structure of the target biomolecule. In this context, LBDD strategies are widely used in the design and optimization of new ligands, based on the structural and physicochemical properties derived from previously validated bioactive molecules. In this method, the structural and activity data of inhibitors are analyzed to determine features associated with binding efficacy. Subsequently, large compound databases are screened to identify drug candidates that match these characteristics (Andricopulo & Ferreira, 2014; Wu et al., 2024a).

One of the prominent approaches within LBDD is the modeling of Quantitative Structure-Activity Relationships (QSAR). QSAR approaches aim to describe and quantitatively model the relationships between molecular features and biological activity. These relationships are modeled through mathematical formulas that allow the prediction of the potential activity of compounds that have not yet been synthesized. Another frequently employed method within LBDD strategies is pharmacophore modeling. This approach seeks to identify structural motifs that are commonly

found in a set of ligands and are assumed to play a critical role in binding to the target protein. The process involves analyzing the conformational space of molecules and aligning common features, ultimately leading to the generation of a three-dimensional pharmacophore hypothesis. This hypothesis can then be used to screen compound databases containing molecules with unknown activity (Andricopulo & Ferreira, 2014).

Another strategy within LBDD is ligand-based virtual screening (LBVS), which enables the identification of novel candidate molecules by relying solely on the structural and physicochemical properties of active compounds, without directly utilizing the three-dimensional structural information of the macromolecular target. In recent years, the integration of ML and deep learning (DL) algorithms into LBVS workflows has gained significant attention. These algorithms represent drug and target protein data using various encoding methods in vector or graph formats and extract meaningful features by processing this information. In this context, predictions of drug–target interaction (DTI) and drug–target affinity (DTA) have emerged as key steps within LBVS. This workflow is illustrated in Figure 6.

DTI prediction aims to computationally identify potential interactions between drug molecules and numerous possible targets in the organism. Compared to virtual screening, DTI has a narrower focus and can be considered a subcomponent of it. In this process, existing experimental data and known drug–target interactions are leveraged using ML and data mining techniques to predict novel potential associations. In

ML-based integrated models, DTI prediction is generally approached as a classification problem, here DTI tools produce binary outputs indicating the presence or absence of interaction between a molecule

and a target. In contrast, DTA prediction employs regression-based models to estimate quantitative values such as K_d , K_i , or IC_{50} that reflect the strength of the interaction (Wu et al., 2024a).

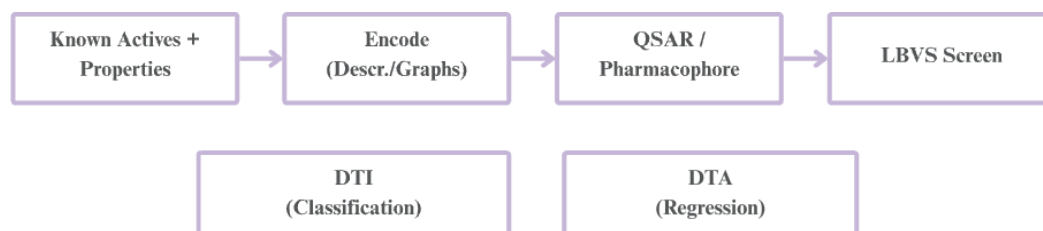


Figure 6. LBDD

• Structure-Based Drug Design – SBDD

Structure-based drug design is an approach that utilizes the known 3D structure of a target protein. More specifically, it is referred to as structure-based virtual screening (Ghislat, Rahman, & Ballester, 2021). Widely employed in solving various drug design challenges, Once the structure is resolved, an appropriate binding site on the target protein is identified, followed either by the optimization of existing ligands or the discovery of new ligands through *in silico* screening processes. Promising compounds resulting from this process may be synthesized or procured commercially and tested *in vitro* against the target protein.

Structure-Based Virtual Screening (SBVS) methods rely on the computational docking of large libraries of small molecules into the binding site of the target protein. Scoring functions (SFs) are employed to predict the binding affinity of ligands positioned within the binding site. these functions enable the ranking of compounds in chemical libraries based on their predicted affinity for the target protein. Compounds estimated to have higher binding affinity (i.e., lower K_d , K_i , or $\Delta G_{\text{binding}}$ values) are ranked at the top. These compounds are generally assumed to exhibit lower IC_{50} or EC_{50} values. Scoring functions are not limited to regression-based models; some also estimate class probabilities or directly classify molecules as active/inactive. Molecular docking is a widely used technique aimed at predicting the potential conformations of ligands that can fit into the

binding pocket of a biological receptor (Andricopulo & Ferreira, 2014; Salgin-Goksen et al., 2021; Tuncel et al., 2025). The identification of promising lead compounds is a complex process that requires the integrative application of various strategies. In this context, considering that target biomolecules may undergo conformational changes at different levels, SBVS strategies can be supported by molecular dynamics (MD) simulations. MD simulations play a fundamental role in predicting molecular motions and structural changes.

In certain cases, structural alterations in the target protein may be limited, allowing ligands to bind readily to well-defined binding pockets. However, it is also known that some proteins undergo significant conformational changes during the molecular recognition process. In such cases, a representative ensemble of multiple conformations of the target protein is generated, and incorporating these conformational models into SBVS analyses contributes to obtaining more meaningful and reliable results.

MD simulations have been successfully employed in elucidating molecular mechanisms supported by experimental data. Although they present some limitations in terms of system size and computational cost, when combined with medicinal chemistry approaches, they offer multifaceted contributions to drug design efforts (Andricopulo & Ferreira, 2014).

However, in cases where experimental structural data for the target protein are unavailable, struc-

ture-based screening processes can be supported by AI-based prediction methods. High-accuracy protein structure predictions can be achieved using advanced deep learning models such as AlphaFold2, while generative chemistry platforms like Chemistry42 can be used to design new molecules based on these pre-

dictions. Consequently, even for biomolecules with limited structural information, virtual screening and lead discovery can be effectively carried out (Niazi, Zamara, & Magoola, 2024). The AI-based SBDD workflow is illustrated in Figure 7.

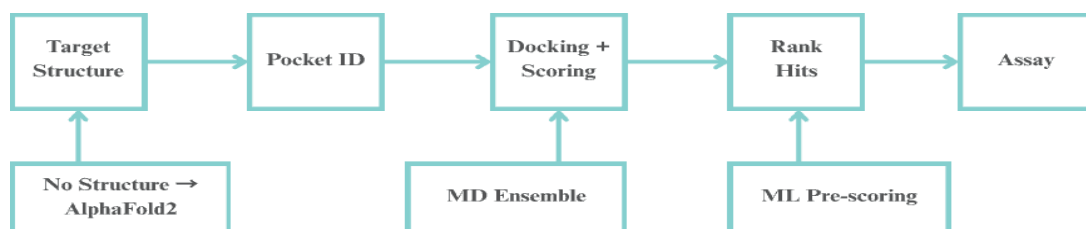


Figure 7. SBDD

ML, one of the most advanced fields of artificial intelligence, plays a significant role in drug design processes. In scenarios where target-specific ML-based scoring functions can be trained, various studies have reported higher SBVS accuracy compared to classical scoring functions. ML-based scoring functions developed within this context have been shown to process available target data effectively. Especially in the re-scoring of crystal structures, ML-based models have demonstrated high accuracy in predicting the binding affinity of ligands to target proteins. Similarly, successful results have been obtained for redocked li-

gand poses (Ghislat et al., 2021).

In recent years, studies that integrate MD simulations with ML approaches to improve the accuracy and efficiency of binding affinity predictions have drawn significant attention. These studies incorporate not only classical 3D structural descriptors but also dynamic information that reflects structural changes of the system over time, thereby generating more comprehensive 4D descriptors (Figure 8.). MD simulations are capable of modeling dynamic processes in biological systems across timescales ranging from picoseconds to milliseconds.

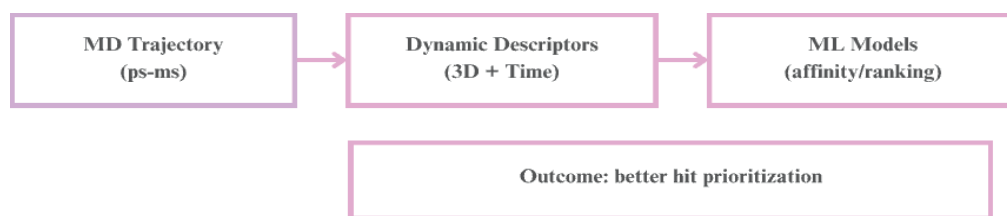


Figure 8. MD + ML Integration

In this context, a study by Jamal et al. developed ML-based models to predict biologically active compounds for the Caspase-8 target. During the training phase of these models, descriptors derived from MD simulations were employed. The study reported that models constructed with descriptors derived from longer simulations achieved the highest classification performance. These findings demonstrate that such models can be effectively utilized in the prioritization

and optimization of lead compounds (Jamal, Grover, & Grover, 2019).

Lead Optimization

Lead optimization is the systematic refinement of early hit/lead compounds to improve potency, selectivity, and pharmacokinetic/tox profiles (ADME), bridging hit discovery to preclinical/clinical development. Building on candidates from HTS and VS (see: Target Identification and Lead Discovery), this stage

enhances target interactions while improving properties such as solubility, stability, and bioavailability a critical inflection point in drug design.

Traditionally, teams iterate through design synthesis test cycles guided by empirical rules and expert intuition; this is effective but time-consuming. AI-driven methods accelerate the loop by learning from large structure–activity and experimental datasets to predict binding affinity and ADMET, prioritize modifications, and reduce the number of wet-lab iterations.

Recent platforms BIOVIA GTD, Query-based Molecule Optimization (QMO), Chemistry42, and ZairaChem apply generative models (e.g., genetic algorithms, neural networks) to propose potent, selective, and structurally novel variants (Bleicher et al., 2022). For example, in SYK inhibitor programs (entospletinib, lanraplenib), GTD re-identified and refined candidates by constraining chemical space and recombining features across series, improving lead quality (Bleicher et al., 2022; Loos et al., 2024).

Looking ahead, tighter integration of deep learning and reinforcement learning with established medicinal chemistry workflows and hybrid models that blend expert knowledge with data-centric systems should further improve multi-parameter optimization. Progress will depend on robust data infrastructures and close interdisciplinary collaboration (Niazi et al., 2024).

AI-Assisted Synthesis Planning and Synthetic Accessibility

The 1828 Wöhler urea synthesis launched total synthesis as a field. Today, discovery pipelines use ligand-based and structure-based virtual screening to

find binders, but these methods are limited to pre-existing molecules, constraining chemical space, patentability, and diversity. Hence the rise of *de novo* design yet synthetic accessibility remains the key bottleneck. Classical retrosynthesis plans routes by working backward from the target to purchasable precursors. Effective but manual, it scales poorly for complex structures and depends heavily on expert intuition motivating AI integration. AI-driven retrosynthesis couples large reaction databases with ML to propose feasible routes. Typical systems comprise:

1. Representations (SMILES, fingerprints, graph encodings),
2. Single-step prediction (template-based rules vs template-free bond disconnections),
3. Multi-step planning, and
4. Route evaluation.

Quality control includes round-trip evaluation (forward models regenerate the product), reaction diversity metrics (e.g., Jensen–Shannon divergence), and feasibility scores (SCScore) alongside ML predicted yields. Multi-step planning often mirrors reinforcement-learning-like decision sequences optimized for cost, step count, and overall yield (Jiang, Yu, Kong, Mei, & Luo, 2023). Data-driven approaches now often surpass rule-based systems. Wang et al. introduced RetroExplainer, combining the Retro*10 algorithm with commercial reagent databases to ensure synthesizability and avoid manual reactant selection; across 101 test cases, 86.9% of single-step predictions matched literature routes. The method also offered interpretable energy scores and reproduced a four-step route to protokylol, as shown in Figure 9, consistent with reported chemistry (Wang et al., 2023).

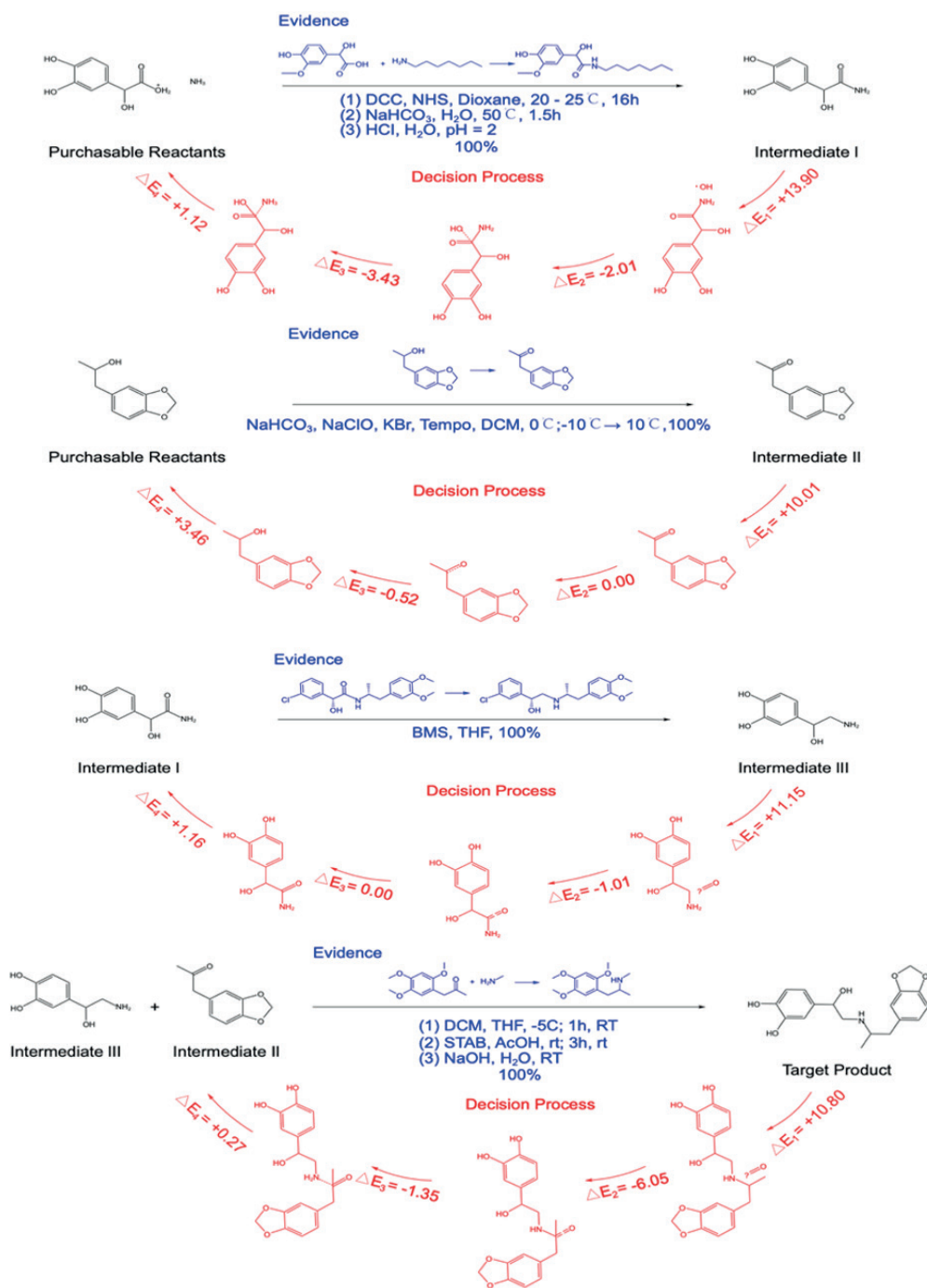


Figure 9. Retrosynthetic route for protokylol generated by RetroExplainer. The structures shown in grey represent intermediate compounds and the final target molecule along the synthetic pathway. At each retrosynthetic step, the blue-highlighted regions indicate literature or dataset examples provided by the model as supporting evidence. The red-highlighted structures and arrows represent the model's decision-making process, where the ΔE value displayed on each arrow corresponds to the energy score of the respective synthetic step. Lower ΔE scores indicate chemically more plausible transformations.

MACHINE LEARNING

AI is a field of science and engineering that aims to enable computers to imitate human behavior, replicate decision-making processes, and solve complex tasks autonomously. In its early stages, AI systems were built upon predefined rules and formal representations of knowledge, where decision-making mechanisms relied on these fixed rules. However, the dynamic nature of real-world problems has revealed the limitations of such rigid systems, highlighting the need for more flexible, data-driven learning capabilities.

In response to this need, ML methods have emerged, allowing computer programs to improve their performance on specific tasks through experience. ML automates analytical modeling processes and enables cognitive functions -such as classification, regression, and clustering- to be learned from data without explicit programming. With the increasing volume of data, advancements in computational

infrastructure, and the development of novel algorithms, ML has found widespread application across numerous industries.

Progress in ML has also led to the evolution of artificial neural networks (ANNs) into deeper and more complex architectures. DL, which utilizes deep neural networks (DNNs) comprising multiple layers, enables the learning of high-level representations from data. DL models can directly extract intricate patterns from raw data and deliver superior performance on large-scale datasets. Nevertheless, in scenarios with low data dimensionality or limited training data, traditional -or shallow- ML algorithms often outperform deep learning models, offering not only better accuracy but also higher interpretability of the results (Janiesch, Zschech, & Heinrich, 2021). A visual representation of this conceptual distinction is provided in Figure 10, which broadly illustrates the positions of shallow and deep learning models within the broader machine learning framework.

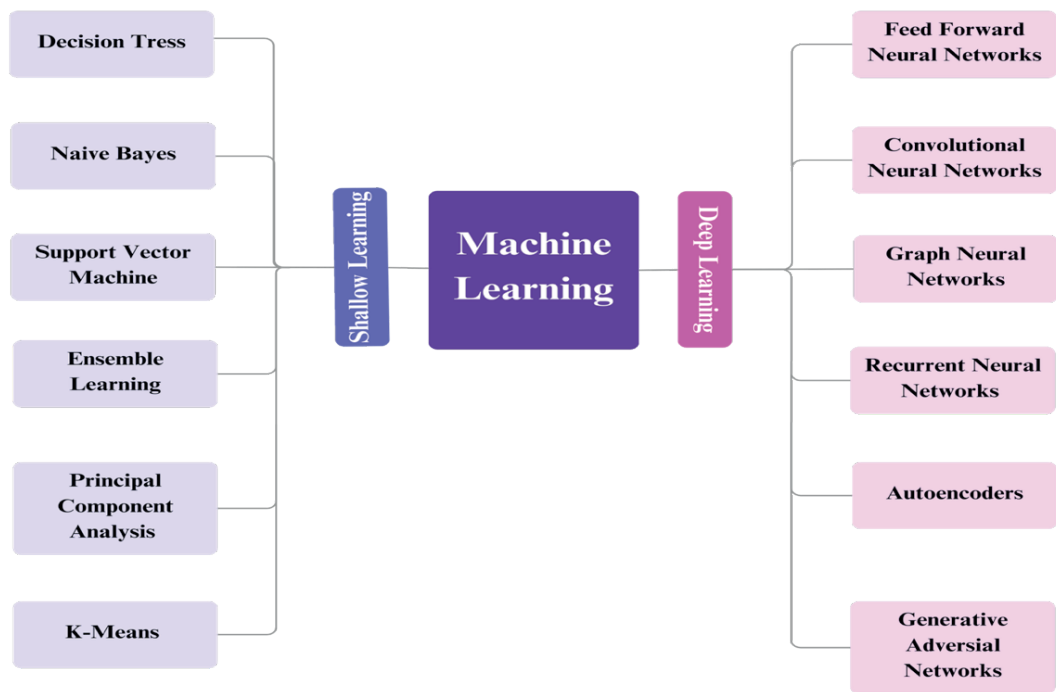


Figure 10. A schematic classification illustrating the positions of shallow and deep learning algorithms within the broader framework of machine learning.

Ultimately, a hierarchical relationship exists among the concepts of AI, ML, and DL: AI represents the broadest umbrella term, encompassing the general field of intelligent systems, while ML constitutes a subfield of AI that focuses on data-driven learning. Within ML, deep learning emerges as a specialized approach that leverages multi-layered neural network architectures to enable advanced pattern recognition and representation learning.

ML approaches are generally categorized into three main types: supervised learning, unsupervised learning, and semi-supervised learning. Each of these paradigms offers specialized techniques tailored to distinct data structures and learning objectives (Janiesch et al., 2021).

Supervised Learning

Supervised learning is based on training a model using labeled datasets. In this approach, each input example is presented to the algorithm along with its corresponding correct output label. The objective of the model is to learn the patterns within these input-output pairs and make accurate predictions on new, unseen data with similar structure.

The training process typically involves two main phases. In the first phase, the model is trained on a labeled training dataset. In the second phase, the model's generalization ability is evaluated using a test dataset composed of previously unseen data. During this process, the model's predictions are compared to the true outputs, and the discrepancy is quantified using a loss function. Optimization techniques are then employed to minimize this error (Mahesh, 2020; Nasteski, 2017).

Supervised learning methods primarily address two types of problems: classification and regression. Classification tasks aim to assign input data to predefined categories. For instance, classifying lung nodules as benign or malignant based on imaging data exemplifies a classification problem. In contrast, regression tasks focus on predicting continuous numerical variables. An example of this is estimating the op-

timal drug dosage for patients based on demographic and clinical data using regression-based models (Ryan et al., 2023).

This approach is widely applied across both traditional (shallow) machine learning algorithms and deep learning architectures. Traditional methods include algorithms such as Decision Trees, Naive Bayes, and Support Vector Machines, while deep learning models such as Convolutional Neural Networks (CNNs) and Recurrent Neural Networks (RNNs) are also trained within the supervised learning paradigm.

Unsupervised Learning

Unsupervised learning is a subfield of machine learning that operates on unlabeled data. In this approach, the algorithm is trained without any predefined labels or known output values associated with the input data. The primary goal of the model is to uncover similarities, patterns, or structural relationships within the data and to organize the input in a meaningful way.

Unlike supervised learning, unsupervised learning does not involve a "correct answer." The algorithms freely analyze the internal structure of the data and attempt to identify hidden patterns or relationships. This process is typically associated with tasks such as clustering and dimensionality reduction. Clustering aims to group data samples with similar characteristics, while dimensionality reduction techniques seek to reduce the number of variables in high-dimensional data while preserving critical information, thereby enabling more concise and interpretable data representations.

Because unsupervised learning attempts to reveal inherent structures in data without external guidance, the learning process is generally more ambiguous and complex than in supervised approaches. Nevertheless, it offers significant advantages, particularly in large and complex datasets, by enabling automatic grouping of data or the discovery of previously unrecognized patterns (Greene, Cunningham, & Mayer, 2008; Mahesh, 2020).

This approach is implemented across both shallow ML algorithms and deep learning architectures. For instance, K-means clustering and Principal Component Analysis (PCA) are widely used conventional methods, while Autoencoders and Generative Adversarial Networks (GANs) represent deep learning models commonly employed in unsupervised learning frameworks.

Semi-Supervised Learning

Situated between supervised and unsupervised learning, *semi-supervised learning* seeks to improve model performance by leveraging both labeled and unlabeled data. Particularly in tasks such as classification and clustering, the incorporation of unlabeled data can help the model make more robust generalizations. For example, in classification problems, unlabeled instances complementing a limited number of labeled examples may enable more accurate definition of decision boundaries. Similarly, in clustering applications, prior knowledge indicating that certain data points belong to the same group can facilitate the formation of more coherent clusters.

Semi-supervised learning becomes especially valuable when labeled data is scarce or difficult and costly to obtain—scenarios commonly encountered in domains such as computer-aided diagnosis or drug discovery. In these contexts, insights derived from large volumes of unlabeled data can enhance model accuracy and lead to better overall performance compared to models trained solely on labeled datasets (Van Engelen & Hoos, 2020).

Within the realm of deep learning, semi-supervised learning is implemented through models trained on partially labeled datasets. In certain instances, models such as GANs are also employed under this category. Additionally, RNN architectures like Gated Recurrent Units (GRUs) and Long Short-Term Memory (LSTM) networks are occasionally used within semi-supervised learning paradigms. One of the key advantages of this approach lies in its ability to significantly reduce the reliance on large amounts of labeled data (Alzubaidi et al., 2021).

Data Resources

In machine learning-based drug discovery studies, the availability of high-quality and structured data resources is of critical importance.

PubChem is the world's largest open-access chemical information database, providing comprehensive data on chemical compounds and their biological activities. It includes chemical structures, physicochemical properties, toxicity data, and 2D/3D structural information, as well as protein interaction data derived from biochemical assays. The data are organized into three main categories: *Substance* (raw chemical records), *Compound* (unique structures), and *BioAssay* (results of biological experiments).

ChEMBL is a freely accessible database specifically developed for drug discovery purposes, containing biologically active molecules. Established in 2002 by EMBL-EBI, it compiles compounds and their associated bioassay results from medicinal chemistry literature, approved drugs, and clinical trial data. Its latest version includes information on over 1.9 million compounds, more than 10,000 drugs, and upwards of 12,000 target proteins.

DrugBank is one of the most widely used and reliable open-access drug reference databases. Launched in 2006, it offers extensive bioinformatics and cheminformatics data on drugs and their corresponding target proteins. Drug-target interaction data in DrugBank are curated from scientific publications, textbooks, and other major databases.

Protein Data Bank (PDB) serves as the primary source for three-dimensional structural information on proteins and protein-ligand complexes. As one of the earliest open-access biological data repositories, it provides 3D structures of proteins, nucleic acids, and their complexes, mostly derived through X-ray crystallography and, to a lesser extent, nuclear magnetic resonance (NMR) spectroscopy.

UniProt is among the most comprehensive protein-centric databases, based on protein sequences obtained from genome sequencing projects. It pro-

vides detailed functional annotations of proteins, and its *Swiss-Prot* subset contains manually curated information on more than 560,000 verified protein entries.

BRENDA, is an extensive enzyme database established in 1987. It contains data on approximately 84,000 enzymes and nearly 205,000 enzyme–ligand relationships. These records are manually curated from over 140,000 scientific publications. The ligands include substrates, products, activators, inhibitors, and cofactors. Users have full access to the entire dataset.

BindingDB, is an open-access database that focuses on binding affinities between drug-like small molecules and protein targets. Its content is derived from scientific articles and patents and is integrated with other databases such as ChEMBL and PubChem.

PDBbind, is a specialized database that aggregates experimentally determined binding affinity data for biomolecular complexes from the PDB. Introduced in 2004, it aims to bridge the gap between protein structural information and binding energetics. The dataset is based on complex structures available in the PDB and their associated binding affinity data reported in the scientific literature (Niazi et al., 2024).

Data Representations

The successful application of machine learning and deep learning algorithms in drug discovery critically depends on the ability to represent both drug candidates and target proteins in numerically interpretable formats. These representations are regarded as a fundamental step in computational modeling, as they allow for the encoding of the structural, chemical, and biological properties of molecules and proteins. In general, such representations are categorized into three main types: string-based, fingerprint-based, and graph-based (2D/3D) formats.

One of the most common approaches for ligand representation is the Simplified Molecular Input Line Entry System (SMILES), which expresses a compound as a sequence of characters. This method linearizes the molecular graph to generate a textual representation. However, due to its sequential nature, SMILES

often fails to capture spatial relationships within the molecule, potentially leading to the loss of critical structural information.

To overcome these limitations, fingerprint-based representations are employed. These representations encode molecules as binary vectors based on the presence or absence of specific substructures-assigning “1” to indicate presence and “0” for absence. Among the most widely used are Extended Connectivity Fingerprints (ECFP) or Morgan fingerprints. These representations have shown strong performance in molecular similarity analysis and property prediction tasks.

In 2D graph-based molecular representations, atoms are treated as nodes and bonds as edges. Each node is described by a feature vector capturing its chemical or structural characteristics, while an adjacency matrix encodes the connectivity between atoms. Some representations are further enriched with edge features, offering a more detailed view of the molecular structure. This format aligns seamlessly with Graph Neural Network (GNN)-based models, enabling more accurate analysis of molecular topology and interactions.

3D graph-based representations consider the spatial coordinates of atoms in a molecule and incorporate stereochemical information encoded through x, y, and z coordinates. These representations are especially effective in quantum-level property prediction tasks and in identifying binding pockets.

Similar strategies are employed for protein representations. Protein sequences are typically transformed into binary vectors using one-hot encoding. However, such sequence-based representations do not inherently capture structural information. To address this, contact maps -a form of 2D graphical representation- are commonly used. These maps encode pairwise relationships between amino acid residues in matrix format, allowing for the integration of both sequence and structural information.

Finally, 3D graph-based protein representations are constructed using structures obtained from data-

bases such as PDB and AlphaFold. In these graphs, nodes may represent either amino acid residues or atoms, and edges denote the physical or chemical interactions between them. These 3D representations are particularly valuable in advanced tasks such as identifying protein–ligand interaction sites, as they enhance model accuracy and generalizability (Wu et al., 2024a).

Traditional (Shallow) Learning (SL)

Traditional machine learning, also referred to as shallow learning (SL), encompasses the majority of machine learning models proposed prior to 2006. Shallow learning aims to capture patterns in data using relatively simple and direct approaches. These methods typically involve a limited number of parameters and shallow model architectures, focusing on identifying explicit relationships within the data without learning complex representations. Due to their simplicity, shallow learning algorithms offer faster training processes and higher interpretability. However, when it comes to modeling multi-layered or complex structural relationships, their performance is often inferior to that of deep learning techniques (Xu, Zhou, Sekula, & Ding, 2021).

Within the framework of shallow learning, a wide range of algorithms has been developed to address different types of problems. This section provides a detailed overview of core models including Decision Trees, Naive Bayes, Support Vector Machines (SVM), Ensemble Learning methods, PCA, and K-means clustering.

Decision Tree

Decision trees are a machine learning method primarily used for classification tasks. This technique is based on representing decisions and their possible consequences in a tree-like structure, enabling systematic modeling of the decision-making process. The construction of a decision tree begins with the entire dataset and involves recursively splitting the data into two subsets based on the value of a specific feature. This splitting continues at each step until the resulting subsets contain instances belonging to a single class.

To determine the decision nodes, the concept of

entropy is often employed. For each feature, the entropy of the resulting subsets is calculated, and the feature-value pair that results in the lowest total entropy is selected as the decision node. Accordingly, each node in the decision tree represents a feature and a threshold value associated with that feature.

During classification, the data instance is evaluated starting from the root node, and at each decision node, it is compared with the corresponding feature and directed to the appropriate branch. This process continues until a leaf node is reached. The leaf node indicates the predicted class of the instance, thereby completing the classification process.

In a decision tree, each node represents a condition or decision, while branches denote the possible outcomes of those decisions. In other words, nodes reflect the attributes used for classification, and the branches correspond to the possible values of those attributes (Mahesh, 2020; Nasteski, 2017).

Naive Bayes

Naive Bayes is a simple yet effective classification technique that falls under the category of supervised learning methods and is fundamentally based on probability theory. The algorithm aims to estimate the conditional probability of each class $\mathcal{Y}$ given an object $\mathcal{X}$, expressed as $P(\mathcal{Y} | \mathcal{X})$. Through conditional probability computations, Naive Bayes is widely utilized in classification tasks and decision support systems.

Thanks to its straightforward computational requirements, the Naive Bayes algorithm is capable of delivering fast results and exhibits reliable performance even on small datasets, with low variance. Moreover, it allows for incremental learning by seamlessly incorporating new data into the system, enabling continuous model updates. The algorithm is also notably robust in the presence of errors and missing values within the dataset.

The flexible and robust nature of the Naive Bayes algorithm has facilitated its widespread and effective application across various domains (Mahesh, 2020; Nasteski, 2017; Webb, Keogh, & Miikkulainen, 2010).

Support Vector Machines (SVM)

SVMs are a widely used and effective technique in the field of machine learning, primarily employed for classification and regression tasks within the supervised learning paradigm. SVM aims to represent each input in a high-dimensional feature space and to determine the optimal hyperplane that separates the data samples. This hyperplane is constructed to maximize the margin between classes, thereby minimizing classification error.

The SVM algorithm is not limited to linearly separable problems; it can also be applied to nonlinear classification tasks through the use of a technique known as the kernel trick. This approach maps the original data into a higher-dimensional feature space, where a linear separation becomes possible.

SVMs are particularly effective in domains characterized by complex data structures and have shown strong performance on high-dimensional datasets, especially where the number of features exceeds the number of samples. However, without proper regularization, SVM models are prone to overfitting, particularly in noisy or sparse data environments.

On the downside, SVM is often considered a “black-box” algorithm, meaning that the underlying decision-making process can be difficult to interpret. Specifically, the rationale behind the selection and optimization of the separating hyperplane is not readily transparent, which limits the explainability of model outputs (Jiang, Gradus, & Rosellini, 2020; Mahesh, 2020).

Ensemble Learning (EL)

EL is a technique in supervised machine learning that combines multiple models to produce a collective decision. In this approach, each model functions as a base learner, trained on labeled data to generate predictions for new, unlabeled instances. EL may incorporate various machine learning algorithms, including decision trees, artificial neural networks, or linear regression. The central rationale behind this method is that the individual errors made by different models can offset each other, thereby improving overall pre-

dictive accuracy. As a result, ensemble methods often achieve higher performance compared to any single model acting alone.

This approach reflects the principle of “wisdom of the crowd” in machine learning. A classic demonstration of this idea was provided by British philosopher and statistician Sir Francis Galton. In a contest held at a livestock fair, Galton asked participants to estimate the weight of an ox. Although none of the individual guesses were perfectly accurate, the average of all the predictions closely approximated the actual weight. This experiment highlighted the potential of aggregated predictions to yield more accurate results than individual estimates. Inspired by this concept, ensemble models aim to combine the outputs of multiple learners to generate more reliable and accurate predictions.

Moreover, ensemble models are particularly efficient when the base learners have low computational costs. This enables both more accurate and faster predictions to be achieved.

Ensemble learning methods are generally categorized into two main frameworks: dependent and independent.

In the dependent framework, the output of each model influences the training of subsequent models. Here, misclassified instances in previous iterations are given greater importance during the training of new models. A well-known example of this approach is the AdaBoost algorithm.

In contrast, the independent framework trains each model separately, without influence from others. The outputs are then aggregated using techniques such as majority voting. In this framework, a bootstrapping technique is often employed to generate subsets of the data—where some samples may be used multiple times and others not at all. The Random Forest algorithm, which applies the bagging method to decision trees, is a representative example of an independent ensemble learning strategy (Arifa, Aditsania, & Kurniawan, 2022).

Principal Component Analysis (PCA)

PCA is a widely used technique for reducing the dimensionality of large datasets. By projecting data onto a lower-dimensional space, PCA facilitates faster and more efficient analysis. The method aims to represent numerous variables using smaller groups while preserving the essential information in the dataset and minimizing noise, thus revealing the most meaningful underlying structure.

Also known as the Karhunen–Loève transform, PCA is utilized in various applications such as dimensionality reduction, data compression, and visualization. Extended versions of this method, including probabilistic PCA and kernel PCA, have also been developed. These approaches contribute to highlighting key features and uncovering hidden structural relationships, particularly in large and complex datasets.

The computational process of PCA is grounded in linear algebra and can be efficiently executed by computers. By isolating only the most informative components, PCA enhances the performance and speed of subsequent machine learning algorithms. Additionally, it helps mitigate the challenges associated with high-dimensional data and reduces the risk of overfitting in regression-based models (Kurita, 2019; Naeem, Ali, Anam, & Ahmed, 2023).

K-Means

The K-Means algorithm is one of the most widely used techniques within unsupervised learning. Its primary objective is to partition a dataset into K predefined groups, or clusters, based on data similarity. The algorithm operates by assigning data points to clusters in such a way that the distance between the data points and the centroids of their respective clusters is minimized.

Initially, K centroids are randomly selected. Each data point is then assigned to the nearest centroid based on a distance metric, typically Euclidean distance. Subsequently, the centroids are updated by calculating the mean position of all data points assigned to each cluster. These steps are iteratively repeated un-

til the cluster assignments stabilize and the centroids no longer change significantly.

K-Means is particularly effective for large-scale and numerical datasets due to its computational efficiency and simplicity. However, since the initial selection of centroids can significantly influence the final clustering outcome, it is often recommended to perform multiple runs with different initializations to enhance robustness and accuracy (Mahesh, 2020; Naeem et al., 2023).

DEEP LEARNING (DL)

DL, a more advanced subfield of machine learning, aims to capture complex patterns in data through multilayered ANNs. These networks are inspired by the information processing mechanisms of the biological nervous system and are composed of interconnected artificial neurons designed to process inputs and generate outputs. Each neuron performs basic mathematical operations by weighting and summing the incoming signals, which are then transformed into outputs through activation functions. This layered organization of numerous neurons lays the foundation for learning more intricate representations and decision-making processes (Montesinos López, Montesinos López, & Crossa, 2022; Singh & Banerjee, 2019).

Artificial neurons, inspired by their biological counterparts, operate by aggregating incoming signals, applying weights to them, and transforming the results into outputs through activation functions once a predefined threshold is reached. In ANNs, the input layer is responsible for receiving data from the external environment, hidden layers process this data through nonlinear transformations, and the output layer generates the final prediction or decision. The connections between neurons are mediated by adjustable weights that govern the flow of information and are optimized during the learning process. In certain neural architectures, feedback connections where the output of a layer is fed back into a previous layer enable more dynamic and interactive learning mechanisms.

Deep learning overcomes the limited representational capacity of traditional neural networks, which are often constrained to a single hidden layer, by introducing multiple hidden layers to learn more complex and hierarchical patterns. These architectures, referred to as DNNs, are capable of mapping intricate relationships between inputs and outputs, particularly when trained on large-scale datasets. As the number of hidden layers increases, so does the model's learning capacity, enabling it to extract progressively higher-level abstractions from the data (Montesinos López et al., 2022).

The topology of an artificial neural network defines the overall structure of the network and the pattern of connections among neurons. Different types of connections such as inter-layer, intra-layer, and self-connections play a crucial role in determining the network's information processing capacity. The weights assigned to each connection govern the strength and direction of information flow, serving as fundamental parameters during the learning process (Abiodun et al., 2018).

Today, deep learning has become an indispensable approach in domains that rely heavily on large-scale datasets, particularly for tasks such as pattern recognition, feature extraction, data-driven prediction, and complex decision-making. While previous sections have explored how AI-based techniques are integrated into the drug discovery pipeline -from target identification to lead compound optimization- this section introduces the core principles of deep learning and outlines selected neural network architectures that have demonstrated particular relevance in pharmaceutical research.

Feedforward Networks (FFNNs)

FFNNs represent the most fundamental architecture of artificial neural networks. In these networks, information flows in a single direction—from the input layer, through the hidden layers, to the output layer. Connections exist only between successive layers; there are no intra-layer or skip connections. This

unidirectional flow of information contributes to the relatively simple and interpretable structure of feedforward neural networks, facilitating their analytical tractability (Abiodun et al., 2018).

The architecture of feedforward neural networks consists of three fundamental components: the input layer, hidden layers, and the output layer. The input layer serves as the initial interface where external data enters the network. Each input neuron represents an independent variable in the model and directly influences the output depending on the training conditions. Data are received at the input layer, passed through one or more hidden layers via feedforward propagation, and finally reach the output layer.

The output layer transforms the processed data into the final output of the network. The number of neurons in this layer is determined by the specific task the network is designed to perform. For instance, in classification tasks, one output neuron may be assigned to each category, whereas in tasks like noise reduction, the input and output layers might need to contain the same number of neurons.

Hidden layers, located between the input and output layers, enhance the network's learning capacity. These layers, which are not directly connected to the external environment, extract pattern-based features from the input data and contribute significantly to the generation of the final output. The number of neurons in the hidden layers plays a critical role in determining model performance. If too few neurons are used, the network may fail to learn complex patterns, resulting in underfitting. Conversely, an excessive number of neurons may increase the risk of overfitting and unnecessarily prolong the training process, thereby raising computational costs.

Therefore, when designing a feedforward neural network, both the number of layers and the number of neurons per layer must be carefully determined. These parameters directly affect the model's learning capacity and should be optimized based on the nature of the problem and the structure of the data (Ben-Bright et al., 2017).

In training feedforward neural networks, trial-and-error-based strategies are commonly employed to determine the optimal architecture. One such strategy is the Forward Selection Method, which begins by constructing a model with a small number of hidden neurons and gradually increases the number of neurons based on training and test performance. Typically, the process starts with two hidden neurons, and additional neurons are incrementally added as performance improves.

In contrast, the Backward Selection Method starts with a model containing a relatively large number of hidden neurons and gradually reduces the number of neurons based on performance evaluations. Neurons are removed one by one until a noticeable decline in performance is observed. Both approaches aim to balance model capacity and optimize the learning process.

In addition to these strategies, pruning techniques are also employed to reduce unnecessary complexity and accelerate the training process. Pruning involves analyzing the connection weights within the network and eliminating neurons associated with connections whose weights are close to zero. This approach not only reduces computational cost but also facilitates the development of a more parsimonious and generalizable model (Ben-Bright et al., 2017).

Convolutional Neural Networks (CNNs)

CNNs are among the core deep learning approaches, distinguished by their multilayered archi-

tectures and high accuracy, particularly in visual data processing tasks.

The architecture of CNNs is generally composed of three main components: convolutional layers, pooling layers, and fully connected layers.

Convolutional layers process input data and intermediate feature maps using a set of kernels to generate new feature maps.

Following the convolutional layers, pooling layers are applied to reduce the dimensionality of the feature maps and to decrease the number of model parameters.

Finally, fully connected layers transform the two-dimensional feature maps into one-dimensional vectors and are primarily responsible for tasks such as classification. These layers often account for the majority of a CNN's total parameters, which significantly increases computational cost.

The training of a CNN model typically consists of two main phases: forward propagation and backpropagation. During forward propagation, the input data are processed using the current weights and biases, and the predicted output is compared with the true labels to compute the loss. Subsequently, in the backpropagation phase, gradients are calculated using the chain rule, and the model parameters are updated accordingly. After a sufficient number of iterations, the learning process is completed. The workflow of the general CNN architecture is shown in Figure 11 (Guo et al., 2016).

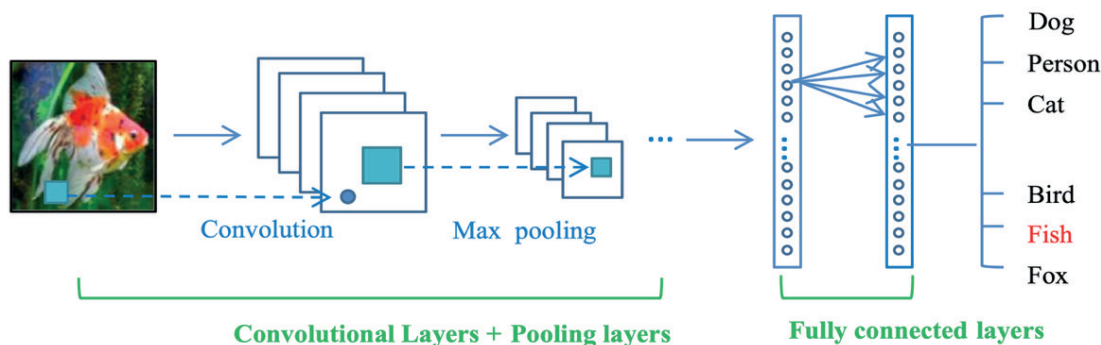


Figure 11. Schematic workflow of a general CNN.

The large number of parameters involved in deep neural networks often leads to undesirable issues such as overfitting. To address this challenge, various regularization strategies have been developed. One such strategy is the Dropout technique, which randomly deactivates a subset of feature detectors during each training iteration to prevent the model from overfitting to the training data. This technique enhances the model's generalization capability and reduces the risk of overfitting. Dropout is also considered a form of ensemble learning, as it effectively trains multiple sub-models simultaneously.

In CNNs, which are widely used in visual object recognition tasks, data augmentation techniques are frequently employed to expand the training dataset. These techniques enhance data diversity without additional labeling costs, thereby supporting better generalization. AlexNet stands as an early and notable example of leveraging data augmentation to improve performance.

Another effective strategy is pre-training, which involves initializing a network with previously learned parameters instead of random weights. This approach accelerates the learning process and improves generalization performance. For instance, models such as AlexNet, trained on the ImageNet dataset, are often fine-tuned for new tasks and datasets, allowing for faster adaptation and improved results.

Another strategy, fine-tuning, is a crucial step for adapting models to specific tasks and datasets. For instance, models such as AlexNet, trained on the ImageNet dataset, are often fine-tuned for new tasks and datasets, enabling faster adaptation and improved performance. During this process, class labels are typically required to compute loss functions for model optimization. However, in certain scenarios, these labels may be unavailable. To address this limitation, similarity learning methods have been proposed, allowing the model to adapt without the need for explicit class annotations.

All of these regularization strategies can be applied individually or in combination to further enhance the

overall performance and robustness of CNN models (Guo et al., 2016).

CNNs Models

Among the prominent CNN architectures, AlexNet stands out as one of the earliest deep learning models that brought significant attention to the field. Comprising five convolutional layers and three fully connected layers -eight layers in total- AlexNet processes fixed-size input images through successive convolution and pooling operations, ultimately producing the final output via fully connected layers. Trained on the ImageNet dataset, AlexNet integrated regularization techniques such as data augmentation and Dropout, and its victory at the 2012 ImageNet Large Scale Visual Recognition Challenge (ILSVRC) marked a turning point for deep learning research and applications (Guo et al., 2016) .

Aiming to increase network depth by reducing the size of convolutional kernels, the Visual Geometry Group (VGG) model focuses on building deeper and more structured networks using small convolutional filters. VGG achieved second place in the 2014 ILSVRC competition with a Top-5 error rate of 7.3%, demonstrating the positive correlation between network depth and performance. In particular, VGG-16 has become widely used in image processing tasks due to its simple architecture and strong compatibility with transfer learning.

The winner of the same competition, GoogLeNet, introduced the Inception module, which allows multiple convolution operations with different kernel sizes to be executed simultaneously. Despite its depth, GoogLeNet effectively reduced the number of parameters and offered a more computationally efficient architecture. By incorporating sparse connections and global average pooling, the model significantly lowered computational costs while maintaining a lightweight design.

Following the success of GoogLeNet, the Inception architecture was developed with the goal of increasing network depth while maintaining computational effi-

ciency. Evolving from Inception V1 to V2 and V3, this architecture optimizes multi-scale processing capabilities, enabling deep learning models to be effectively applied to larger and more complex datasets.

The Residual Network (ResNet) model was developed to address the degradation problem caused by increasing the number of layers in deep neural networks. ResNet adopts the residual learning approach, which facilitates the training of very deep networks by incorporating sequential residual blocks. This architecture enables the model to learn identity mappings more effectively, thereby improving gradient flow and reducing the risk of performance degradation as the network depth increases. For instance, a ResNet model with 152 layers possesses a deeper architecture compared to shallower networks like VGG, while achieving lower error rates and higher accuracy (Zhao et al., 2024).

Graph Neural Networks (GNNs)

Graph structures consist of nodes, which represent entities, and edges, which denote the relationships or connections between these nodes. Such structures play a crucial role in modeling complex relational systems, including social networks, citation networks, and molecular structures. GNN-based approaches have found significant applications in pharmaceutical research, particularly in modeling molecular structures and in the discovery of novel drug candidates.

GNNs have been developed to process graph-structured data characterized by irregular and dynamic topologies, drawing inspiration from traditional CNNs. While CNNs excel at capturing spatial dependencies in grid-like data and RNNs are effective at learning sequential correlations, GNNs demonstrate superior performance on graph-based data by modeling complex dependencies through nodes and edges (Khemani, Patil, Kotecha, & Tanwar, 2024).

A graph is a structure composed of nodes interconnected by edges. Edges represent the connections between nodes. Graphs are employed in relevant domains to model and analyze the relationships between objects or entities.

GNNs are neural networks that operate on data structures consisting of nodes and edges. The fundamental components of a GNN include nodes, edges, layers, activation functions, pooling, aggregation, and other common neural network components.

A node is a point or vertex in a graph that can be connected to other nodes. In GNNs, each node is associated with a feature vector that contains the properties of the corresponding entity and primarily represents the node's attributes. Node classification is a powerful technique in classification problems.

Nodes play a critical role in enabling the network to learn from the graph data structure and its connections. During GNN training, information between nodes is propagated via the edges that connect them. This process allows the network to learn from the relationships among nodes and to make predictions for previously unseen nodes in the graph.

Edges are the connections between nodes in a graph. Edge features can provide significant information regarding the relationships between nodes. This facilitates understanding the types of connections shared by nodes in one-to-one, one-to-many, or many-to-many relationship contexts. Edge prediction plays an important role in link prediction, node classification, and graph-level classification.

Edge features can be represented as a vector and are typically used together with node features, enabling the network to learn from both the attributes of individual nodes and the relationships between nodes.

GNN layers are the fundamental components that enable the network to learn from the connections within the graph data structure. Each layer aggregates information from the feature vectors of neighboring nodes and combines it with the current node's feature vector to generate new representations.

In GNNs, activation functions are employed to introduce non-linearities to the outputs of each layer, allowing the network to learn complex patterns in the data. The choice of activation function in a GNN de-

depends on the nature of the input data and the specific application. Different activation functions can have varying effects on the training process and the performance of the network.

Pooling and aggregation are used in GNNs to reduce the dimensionality of the feature space and to enable the network to handle graphs of varying sizes. Pooling refers to the process of consolidating information from multiple nodes or subgraphs into a sin-

gle representation. Aggregation, on the other hand, involves combining information from neighboring nodes into a single representation for each node. Aggregation is often used in conjunction with pooling to create more compact representations of the graph data. By pooling and aggregating information from multiple nodes and subgraphs, the network can learn more effectively and produce scalable representations of graph data.

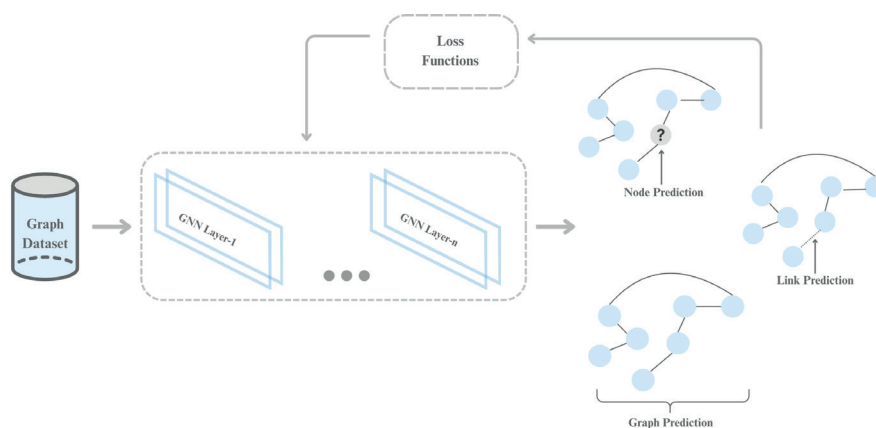


Figure 12. Schematic workflow of a general GNN.

Figure 12 illustrates the workflow of a GNN schematically. The graph dataset is input to the network and passed through GNN layers that incorporate the fundamental components described above. The loss function is applied as in other deep learning systems, and the network is trained until a predefined error threshold or number of iterations is reached. The task is typically a classification at the node, edge, or graph level. Finally, the trained model is evaluated by making predictions on the test data (Sharma, Singh, & Ratna, 2024).

Node-level tasks focus on determining the identity or function of each node within a graph. They are typically used when working with unlabeled data; for instance, predicting whether a specific individual is a smoker.

Edge-level tasks (link prediction) concentrate on analyzing the relationships between pairs of nodes in a graph. An example of such a task is assessing the likelihood or compatibility of a connection between two en-

tities. On platforms such as Netflix, an edge-level task may involve predicting the next video to recommend based on a user's viewing history and preferences.

Graph-level tasks aim to predict an overall property or behavior that encompasses the entire graph. For example, in the evaluation of a newly synthesized chemical compound, a graph-level task may seek to determine whether the molecule has the potential to serve as an effective drug (Khemani et al., 2024).

GNN Models

GNNs have been developed in various architectures based on their application domains and can be broadly categorized into four main types: RecGNNs, ConvGNNs, Graph Autoencoders (GAEs), and Spatio-Temporal Graph Neural Networks (STGNNs) (Park, Yi, & Ji, 2020).

RecGNNs are among the pioneering architectures of GNNs and aim to learn node representations through an iterative information exchange mecha-

nism. This approach refines the concept of message passing and serves as the foundation for the development of ConvGNNs.

ConvGNNs generalize traditional convolution operations from grid-structured data to graph-structured data and generate high-level node representations by stacking multiple graph convolution layers. ConvGNNs are widely used in a variety of tasks, including node classification and graph classification.

GAEs are based on an unsupervised learning approach and aim to encode nodes or entire graphs into a latent vector space from which the original graph structure can be reconstructed. They are effectively used in applications such as network embedding and graph generation.

STGNNs aim to learn latent patterns from dynamic data by simultaneously modeling spatial and temporal dependencies within graph-structured information. These models are widely applied in time-sensitive tasks, particularly in traffic speed prediction and human activity recognition (Wu et al., 2021).

Recurrent Neural Networks (RNNs)

RNNs are deep learning models specifically designed to process sequential data. Unlike traditional feedforward neural networks (FFNs), RNNs can re-

tain information from previous inputs in their memory and utilize this information when processing new data. This capability enables the network to effectively learn temporal dependencies and sequential patterns (Lipton, Berkowitz, & Elkan, 2015; Park, Yi, & Ji, 2020; Yang et al., 2019a).

RNNs are an extension of FFNs; however, unlike FFNs, RNNs incorporate recurrent connections between hidden units, thereby providing the model with temporal context information. This structure allows RNNs to effectively process sequential dependencies in time-series data. The network is designed with a hidden state mechanism that retains information from previous inputs, and its core architecture consists of an input layer, a hidden layer, and an output layer. At each time step, the hidden units receive information both from the current data point and from the hidden state of the previous step; consequently, the output is computed using both the current and accumulated past information, facilitating a cyclic flow of information within the network. This recurrent mechanism enables the learning of long-term dependencies (Lipton et al., 2015; Mienye, Swart, & Obaido, 2024). Figure 13 presents a structural comparison between the general FFN and RNN architectures.

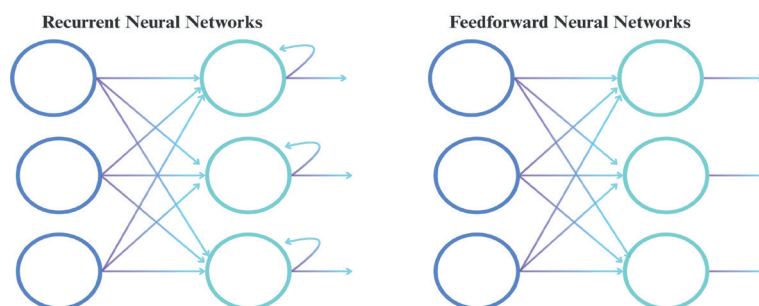


Figure 13. Structural Comparison of general FFNs and RNNs.

Training RNNs is a complex process, particularly due to the challenges encountered when learning long-term dependencies. During training, the Backpropagation Through Time (BPTT) algorithm is applied (Lipton et al., 2015; Park et al., 2020; Yang et al., 2019a).

During backpropagation, the propagation of errors through time often leads to the vanishing gradient and exploding gradient problems. The exploding gradient problem arises when the gradient norms grow excessively during training, causing unstable learning.

To address this issue, the Truncated Backpropagation Through Time (TBPTT) method has been proposed, which limits the span of error propagation, enabling more stable and balanced learning.

On the other hand, the vanishing gradient problem occurs when gradients exponentially decay over time, hindering the model's ability to learn long-term dependencies (Pascanu, Mikolov, & Bengio, 2013). To overcome this limitation, the Long Short-Term Memory (LSTM) network has been proposed, enabling effective learning of long-term dependencies.

Although structurally similar to conventional RNNs, LSTMs employ memory cells instead of hidden units, allowing them to learn long-term dependencies. Each memory cell maintains its own internal state, which helps prevent information loss over time. LSTMs utilize input and output gates to control when information is written to or read from memory and employ a constant error carousel mechanism to mitigate vanishing or exploding gradients. Thanks to these capabilities, LSTMs are widely applied in fields such as natural language processing, speech recognition, and handwriting recognition (Lipton et al., 2015).

As an alternative to LSTMs, the Gated Recurrent Unit (GRU) architecture has been proposed, offering comparable performance while featuring a simpler structure and fewer parameters, thereby accelerating the training process (Mienye et al., 2024).

Another successful RNN architecture is the Bidirectional Recurrent Neural Network (BRNN). BRNNs can learn both past and future context by processing input sequences in both forward and backward directions. The BRNN architecture consists of two separate hidden layers: one processes the inputs in the forward temporal direction, while the other operates in reverse. This bidirectional processing allows the model to make predictions at each time step based on both previous and subsequent inputs. However, the dependence on future information may limit the applicability of BRNNs in online learning or real-time data processing scenarios. Nevertheless, for fixed-length

sequences, this bidirectional structure offers significant performance advantages.

LSTM and BRNN architectures possess complementary characteristics, and their combination—Bidirectional LSTM (BLSTM)—has demonstrated superior performance in sequential data tasks such as phoneme classification and handwriting recognition (Lipton et al., 2015).

Autoencoders (AEs)

Autoencoders (AEs) serve as fundamental building blocks that enable the hierarchical structuring of deep neural network models. These networks are designed to learn and reconstruct input data. By organizing, compressing, and extracting high-level features from the data, they facilitate unsupervised learning and the discovery of nonlinear features. In unsupervised learning, the primary objective is to transform raw data into more meaningful and information-rich representations.

AEs are feedforward neural networks that transmit information unidirectionally and are particularly prominent in tasks such as feature learning and dimensionality reduction. Consequently, they are widely applicable across different data types and domains.

The most basic autoencoder, which does not incorporate additional complexity or architectural modifications, is referred to as the “vanilla autoencoder.” A basic autoencoder typically consists of an input layer (encoder), one or more hidden layers (latent space), and an output layer (decoder). This structure compresses the input data into intermediate representations and subsequently reconstructs the original input from these representations (Berahmand, Nasiri, & Karimi, 2024). Figure 14 provides a visual depiction of the architecture of a general autoencoder neural network.

Conventional autoencoders typically employ a single-layer encoder. However, this limitation can impede the learning of deep and abstract features. To address this issue, deeper network architectures and layer-wise learning approaches have been developed. In this context, Stacked Autoencoders (SAEs) are con-

structured by hierarchically stacking multiple basic autoencoders layer by layer.

SAEs adopt the principle of layer-wise learning, wherein each autoencoder compresses the data into a lower-dimensional representation to extract high-

er-level features. The final output is obtained from the combined outputs of all individual autoencoders. This approach enables the learning of deeper and more abstract representations and allows for effective modeling of complex data structures.

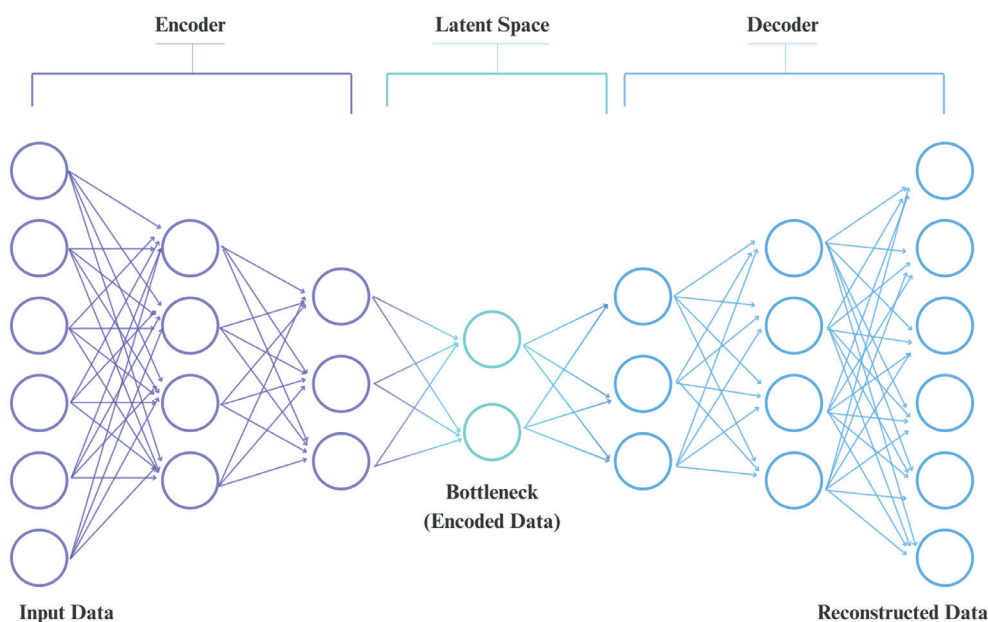


Figure 14. Schematic depiction of the architecture of a general AE.

The training of autoencoders involves hyperparameter and architectural design choices that directly affect model performance. These decisions are typically optimized through experimental evaluation and validation procedures to achieve task-specific performance objectives.

The depth of the network and its capacity to capture complex data patterns are primarily determined by the number of hidden layers. Increasing the number of layers can enhance representational capacity but also introduces optimization challenges and raises the risk of overfitting.

The bottleneck structure refers to the latent layer that compresses the most essential features of the input data. The dimensionality of this layer governs the trade-off between information retention and compression. An excessively small bottleneck may lead to significant information loss.

Activation functions enhance the network's ability to model nonlinear relationships, thereby improving its representational power. Functions such as Sigmoid, Tanh, ReLU, and SELU are commonly employed in autoencoder architectures.

Optimization algorithms update the weights and biases to minimize the loss function. Techniques such as Stochastic Gradient Descent (SGD), Adam, and Adagrad are selected based on dataset size and model complexity.

The learning rate determines the step size of weight updates; excessively high or low values can adversely affect the training process.

The batch size refers to the number of samples used in each optimization step. Smaller batches provide faster but noisier updates, whereas larger batches offer more stable gradients at the cost of higher memory usage. The optimal batch size should be chosen

according to the scale of the dataset and the available computational resources (Berahmand et al., 2024).

AE Models

Autoencoders can be adapted for different data types and tasks through architectural modifications and objective function adjustments, offering a broad range of applications.

Regularized Autoencoders (RAEs) aim to represent data in a compressed latent space while applying regularization constraints to ensure that the learned representations exhibit meaningful and structured characteristics. These regularization techniques promote the learning of sparse representations, manifold structures, or orthogonal features, enhancing interpretability and robustness.

Robust Autoencoders are designed to handle noisy or corrupted inputs and improve model resilience against common real-world data issues such as outliers and missing values. Robust AE models are commonly classified into three main categories: Denoising Autoencoders (DAE), Marginalized Denoising Autoencoders (mDAE), and $L_{2,1}$ -norm Autoencoders, each offering varying degrees of robustness and reconstruction fidelity.

GAEs go beyond traditional data compression by learning the underlying probability distribution of the data, thereby enabling the generation of new samples resembling the training data. Among these, Variational Autoencoders (VAEs) have attracted significant attention in unsupervised learning due to their ability to represent and compress complex data distributions. In pharmaceutical research, VAEs have been effectively utilized in the discovery and design of novel drug molecules, with a notable example being the pioneering work conducted by Gómez-Bombarelli et al. (2018) (Gangwal et al., 2024).

Convolutional Autoencoders (CAEs) employ convolutional layers in both encoder and decoder modules instead of fully connected layers, allowing them to capture spatial dependencies within the data. CAEs have demonstrated high performance in image-relat-

ed tasks, including image denoising, inpainting, segmentation, and super-resolution.

Recurrent Autoencoders (RAEs) are tailored for sequential data (e.g., time series) and incorporate architectures such as Long Short-Term Memory (LSTM) or Gated Recurrent Units (GRU) in their encoder and decoder modules. This allows them to effectively preserve and learn from temporal dependencies within the sequence data.

GAEs aim to enhance the efficiency of graph analysis by transforming graph-structured data into lower-dimensional embeddings. In this architecture, the encoder compresses the input graph into a vector representation, and the decoder reconstructs the original graph structure from this latent space. When combined with models like Graph Convolutional Networks (GCNs), GAEs can effectively capture node attributes and topological relationships, offering significant advantages in graph-based learning tasks (Berahmand et al., 2024).

Generative Adversarial Networks (GAN)

Developed in 2014, GANs represent a significant milestone in generative artificial intelligence (GAI)-based modeling. These models are capable of learning the fundamental distribution of real-world data and generating various types of synthetic data, such as images, videos, and even molecular structures. By employing an adversarial training strategy, GANs enable the creation of new compounds with desired properties. In particular, GAN-based approaches have been effectively utilized in drug discovery to expand the chemical space and identify novel chemical structures (Gangwal et al., 2024).

GANs consist of two adversarial neural networks: a generator (G) and a discriminator (D). Typically constructed using convolutional and/or fully connected layers, these two models are trained in opposition to one another, continuously improving in response to each other's performance (Creswell et al., 2018).

In the GAN architecture, the generator network produces synthetic data samples and presents them

to the discriminator. The generator aims to map from a latent variable space to the data space, generating samples that closely resemble the true data distribu-

tion. Conversely, the discriminator is trained to determine whether a given input originates from the real dataset or has been generated by the generator.

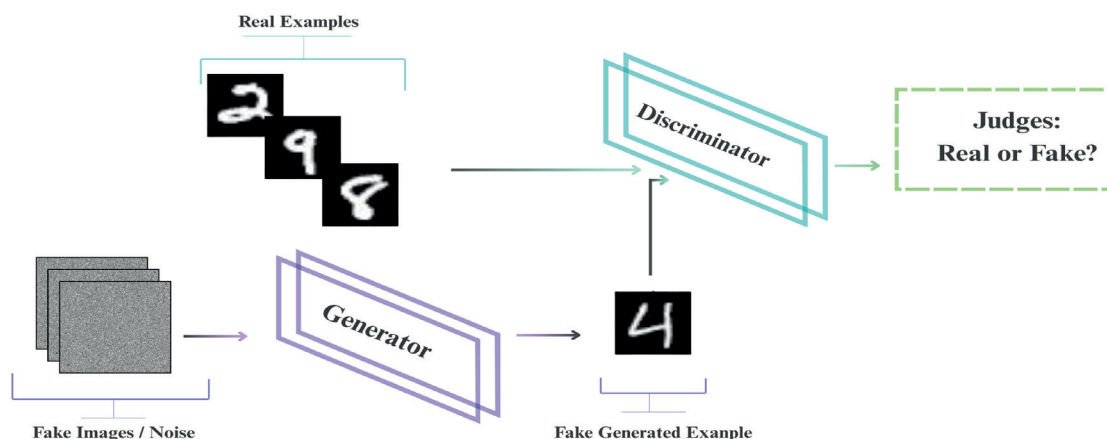


Figure 15. Schematic workflow of a general GAN.

The discriminator is optimized to assign high probabilities to real data and low probabilities to synthetic (fake) data. Meanwhile, the generator seeks to deceive the discriminator into classifying its outputs as real, thereby maximizing the likelihood that its generated data will be perceived as genuine. This adversarial process is grounded in zero-sum game theory: while the generator minimizes its loss the discriminator simultaneously strives to maximize its classification accuracy. Figure 15 illustrates the schematic workflow of the GAN architecture.

The ultimate objective of GANs is to establish a Nash equilibrium between the generator and discriminator networks. Theoretically, this equilibrium is reached when the distribution of data produced by the generator aligns with the true data distribution. At this point, the generator has effectively learned the real data distribution and can produce novel, highly realistic samples that are indistinguishable from genuine inputs (Gui, Sun, Wen, Tao, & Ye, 2021; Oussidi & Elhassouny, 2018). However, achieving this equilibrium is challenging, as real and generated data occupy only limited regions within the same space.

When the discriminator rapidly attains high accuracy, the gradients propagated to the generator

approach zero, causing the learning process to stall. Additionally, the alternating parameter updates of the two networks can introduce instability during training; under such conditions, the generator tends to produce highly similar outputs for different inputs—a phenomenon known as mode collapse.

One of the first major improvements addressing these issues was introduced through the Deep Convolutional GAN (DCGAN) architecture. The reduction of fully connected layers and the use of batch normalization enhanced the stability and efficiency of training deeper networks. Moreover, employing leaky ReLU activation functions in the intermediate layers of the discriminator yielded superior performance compared to conventional activations.

Subsequently, several heuristic methods were proposed to further stabilize GAN training. Among these, feature matching allows the generator to learn by imitating the intermediate activations of the discriminator rather than solely attempting to deceive it. The mini-batch discrimination technique introduces an additional input feature to the discriminator, preventing the generator from producing identical or overly similar outputs. Additionally, label smoothing sets the target label for real samples to 0.9 instead of

1.0, thereby softening the discriminator's decision boundary, reducing overconfidence, and providing the generator with more balanced gradients.

To mitigate the vanishing gradient problem—a key challenge—f-GAN was proposed by generalizing the loss function used in classical GANs. This approach provides greater flexibility to the training process.

Subsequently, the Wasserstein GAN (WGAN) model was developed to address the vanishing gradient problem inherent in classical GANs. WGAN provides the generator with more meaningful and stable gradients, thereby enhancing the overall stability of the training process. In this model, the discriminator—referred to as the “critic”—does not perform probabilistic classification; instead, it measures the distance between the real and generated data distributions.

In general, GANs are highly powerful models capable of generating new data from random noise. However, challenges such as vanishing gradients, convergence difficulties, and mode collapse make the training process highly complex. Successful training of GANs depends on maintaining a balanced adversarial dynamic between the generator and the discriminator. Consequently, through various developed approaches over time, the training strategies of GANs have become more stable, balanced, and efficient (Creswell et al., 2018).

GAN Models

The original GAN (Vanilla GAN) architecture paved the way for the emergence of various structural and methodological variants. One of the major challenges in GAN training is the vanishing gradient problem, which can severely impede model convergence. To address this issue, the Wasserstein GAN (W-GAN) model was developed, measuring the difference between real and generated data distributions using the Wasserstein distance and enforcing a Lipschitz continuity constraint on the discriminator; this results in a more stable training process.

Nevertheless, W-GAN may still experience subop-

timal sample generation or convergence issues under certain conditions. To overcome these limitations, the Loss-Sensitive GAN (LS-GAN) model was proposed, aiming to achieve a more balanced learning process by controlling the discriminative capacity of the discriminator. Both approaches preserve the core structure of GANs while employing different strategies to enhance training stability (Sengar, Hasan, Kumar, & Carroll, 2024).

Since the Vanilla GAN architecture does not have direct access to label information during data generation, its ability to perform controlled data synthesis is limited. To address this, the Conditional GAN (CGAN) was developed. CGAN integrates auxiliary information, such as class labels, into both the generator and discriminator, enabling data generation conditioned on specific attributes (Navidan, Dehghan-tanha, & Parizi, 2021).

Extending the CGAN approach, InfoGAN was developed to strengthen the relationship between observed data and latent variables. This model maximizes the generator's loss while minimizing the discriminator's loss, thereby increasing the mutual dependency between generated data and latent codes. Consequently, it enhances the interpretability of the generative process and provides a more structured and disentangled generation mechanism (Sengar et al., 2024; Wang, She, & Ward, 2021).

Designed for semi-supervised learning, the Auxiliary Classifier GAN (AC-GAN) allows the generated samples to be classified according to both real/fake status and class labels, enabling more targeted and label-informed data generation (Navidan et al., 2021).

A significant architectural advancement in GANs was achieved with the Deep Convolutional GAN (DCGAN), which employs deconvolution operations in the generator to better model spatial patterns and utilizes convolutional layers for more realistic data generation.

The Laplacian Pyramid Adversarial Network (LAPGAN) aims to produce high-resolution outputs

from low-resolution inputs. Using a multi-scale learning approach based on Laplacian pyramids, it generates sharper and more detailed images through progressive resolution enhancement (Wang et al., 2021).

While classical GANs focus solely on generating data from latent variables, the Bidirectional GAN (BiGAN) introduces a mechanism to map real data into the latent space, providing a unified framework for both data generation and representation learning.

Adversarial Autoencoders combine traditional autoencoder architectures with the adversarial learning framework to learn more structured and semantically rich latent representations. Building on this concept, the Adversarial Variational Bayes (AVB) model was developed, enhancing the Variational Autoencoder (VAE) framework with adversarial learning to obtain more expressive data representations (Creswell et al., 2018).

REAL-WORLD APPLICATIONS

In recent years, the integration of AI technologies into drug discovery processes has garnered significant attention due to their potential to reduce development costs and accelerate timelines. AI-based approaches are increasingly being adopted, particularly with the aim of identifying novel therapeutic candidates more efficiently and at lower cost.

AI-Assisted Molecular Discovery Approaches in Scientific Literature

In a study conducted by Chen et al. in 2020, an artificial intelligence-based approach was developed to identify potential dual inhibitors targeting fibroblast growth factor receptor 4 (FGFR4) and epidermal growth factor receptor (EGFR). In this work, four different machine learning models including SVM and Random Forest (RF) were trained to predict the biological activities of both targets. The IC_{50} values of 843 compounds for FGFR4 and 5,088 compounds for EGFR were collected from BindingDB to support the modeling process.

The results demonstrated that the SVM model achieved the highest prediction accuracy for both targets. This model was subsequently applied to predict the biological activities of a set of in house compounds previously synthesized and archived by the research group. To evaluate the accuracy of the predictions, the kinase inhibition potentials of selected compounds were experimentally tested. Among these, compound 1 exhibited significant inhibitory activity against both FGFR4 ($IC_{50} = 86.2$ nM) and EGFR ($IC_{50} = 83.9$ nM) kinases.

In conclusion, this study demonstrates the feasibility of *in silico* modeling for predicting dual-target inhibition against FGFR4 and EGFR kinases, offering a valuable methodological framework for assessing the biological activity of compounds in early-stage drug discovery. Compound 1, selected based on predictive modeling, showed potent inhibitory activity against both targets and emerged as a promising candidate for further investigation. Compound 1 is illustrated in Figure 16 (Chen et al., 2020).

In the previously discussed study, classical machine learning algorithms were prominent; however, the following example demonstrates the modeling capacity of these technologies in drug discovery through a deep learning-based approach enriched with graph neural network architectures.

Zhi, Zhao, Lee, & Chen (2021) developed an AI-assisted multilayered approach for the discovery of novel dihydroorotate dehydrogenase (DHODH) inhibitors that may be effective in the treatment of small-cell lung cancer (SCLC). In this study, both GNN architectures and traditional machine learning algorithms namely, RF and SVR were utilized to evaluate the biological activity of candidate compounds through various modeling techniques. The workflow employed in this process is presented below in Figure 17.

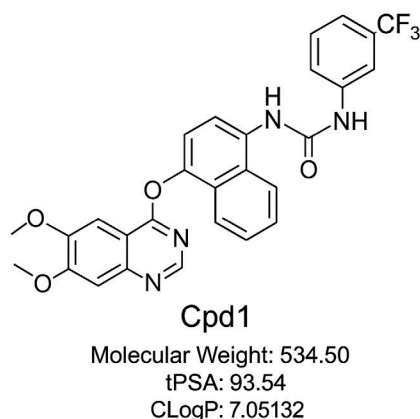


Figure 16. Structure of Compound 1 identified by Chen et al. As a dual FGFR4/EGRF inhibitor.

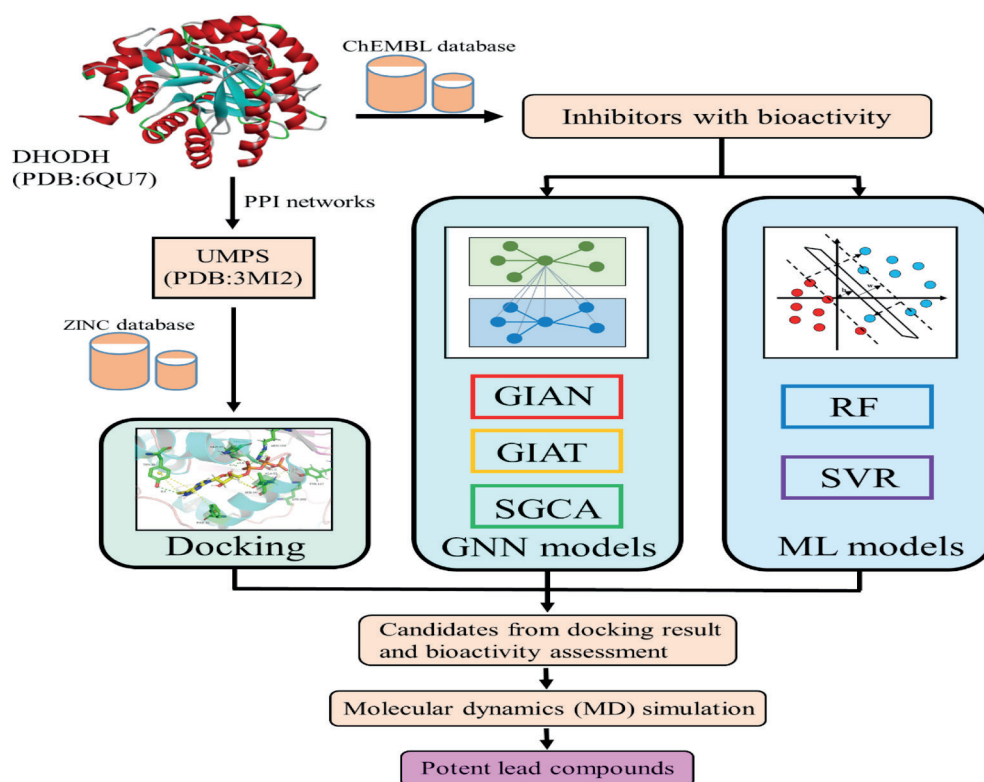


Figure 17. Workflow diagram of the study conducted for the discovery of DHODH inhibitors.

The modeling process was based on two primary data sources: data on known inhibitors with biological activity were retrieved from the ChEMBL database, while potential novel compound candidates were obtained from the ZINC database. The retrieved structures were subjected to molecular docking analysis against DHODH (PDB: 6QU7) and the related pro-

tein UMPS (PDB: 3MI2). Binding scores and interaction profiles were evaluated to select lead compounds.

Subsequently, the selected compounds were analyzed using three different GNN architectures, as well as RF and SVR models. The results demonstrated that graph neural networks achieved higher accuracy in predicting biological activity compared to traditional

methods. The top performing compounds were then subjected to MD simulations, in which the binding stability and interaction dynamics of the ligand–protein complexes were thoroughly assessed.

As a result of these analyses, three compounds ZINC8577218, ZINC95618747, and ZINC4261765 were identified as exhibiting high binding stability

and potential inhibitory effects on the DHODH target. These findings highlight the potential of deep learning-assisted modeling as a powerful tool for identifying effective compounds in early-stage drug discovery (Zhi et al., 2021). The structural representations of these three lead candidate compounds are presented in Figure 18.

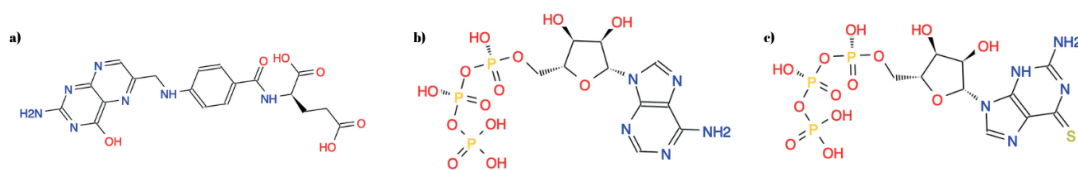


Figure 18. 2D structural representations of the compounds identified as potential DHODH inhibitors: a) ZINC8577218, b) ZINC4261765, and c) ZINC95618747.

Applications of AI-Based Molecule Discovery in The Pharmaceutical Industry

In January 2020, the British pharmaceutical company Exscientia announced the initiation of Phase I clinical trials for DSP-1181, a compound developed for the treatment of obsessive-compulsive disorder (OCD). DSP-1181 was designed using an artificial intelligence platform that screens chemical libraries to identify the most promising candidates, making it the first AI-designed drug candidate to enter human clinical trials. Exscientia developed this molecule in collaboration with Japan-based Sumitomo Dainippon Pharma and completed the process from initial screening to preclinical testing in just 12 months. In contrast, this stage typically takes an average of 4–6 years in the pharmaceutical industry, and only about 1 in 1000 screened molecules generally advance to clinical phases (Burki, 2020).

Similarly, Insilico Medicine has made notable progress in AI-driven drug discovery. In February 2022, the company initiated a Phase I clinical trial for ISM001-055, a small-molecule inhibitor developed for the treatment of idiopathic pulmonary fibrosis (IPF). The discovery process of ISM001-055 was entirely AI-driven, from target selection via the PandaOmics™ platform to molecule design using the Chemistry42™

engine. The company emphasized that the total time from target identification to Phase I trial initiation was less than 30 months (Insilico Medicine, 2022).

In January 2022, within the framework of a collaboration between Insilico Medicine and Fosun Pharma, ISM004-1057D was developed as a modulator of the QPCTL enzyme, which plays a regulatory role in the CD47–SIRPα axis—an important signaling pathway in immuno-oncology. This molecule has been advanced to the preclinical stage as a potential first-in-class small-molecule inhibitor targeting this pathway (Kirkpatrick, 2022).

For the treatment of anemia associated with chronic kidney disease, a PHD1/2 inhibitor was discovered using Insilico Medicine's Pharma.AI platform. Leveraging structural information of target proteins, a structure-based virtual screening approach was implemented through the Generative Chemistry application to design candidate molecules targeting the PHD1/2 enzymes. These candidates were optimized over several iterations in terms of physicochemical properties as well as *in vitro* and *in vivo* ADME profiles, and subsequently evaluated in preclinical studies. In June 2023, an Investigational New Drug (IND) application was submitted in China, and Phase I clinical trials are currently ongoing (Insilico Medicine, 2025).

In the field of rare diseases, HLX-1502, developed by Healx, stands out as a promising candidate. Discovered through Healx's AI-driven platform, HLX-1502 has been granted Fast Track, Orphan Drug, and Rare Pediatric Disease designations by the U.S. Food and Drug Administration (FDA). The compound completed its Phase I clinical trials in 2024 and is currently being evaluated in Phase II as of 2025 (Healx, 2024).

Exscientia and Recursion are two additional key players attracting attention for their work in AI-driven drug discovery. These companies aim to accelerate the drug development process and deliver novel therapeutic options for rare and serious diseases through their technology-based platforms.

Within this context, Exscientia's CDK7 inhibitor REC-617, intended for the treatment of advanced solid tumors, is expected to have Phase I monotherapy safety and pharmacokinetic/pharmacodynamic data disclosed by the end of 2024. Additionally, updated Phase I dose-escalation data for the RBM39-targeting compound REC-1245 are anticipated in the first half of 2026. Initial patient dosing for the MALT1 inhibitor REC-3565 and the LSD1 inhibitor REC-4539 is projected to occur in Q1 and the first half of 2025, respectively. For REC-4881, a MEK1/2 inhibitor being developed for familial adenomatous polyposis, Phase 1b/2 safety and efficacy data are expected in the first half of 2025. As for REC-2282, an HDAC inhibitor targeting neurofibromatosis type 2, results from the PFS6 (6-month progression-free survival) analysis are scheduled to be released in 2025.

Furthermore, the development candidate for REV-1025, an ENPP1 inhibitor for the treatment of hypophosphatasia, is expected to be identified in Q4 of 2024. An update on the Phase II progress of REC-3964, developed to prevent recurrent *Clostridium difficile* infections, is planned for Q1 2026. Finally, IND-enabling studies are ongoing for REC-4209, an investigational compound targeting idiopathic pulmonary fibrosis, whose molecular target has not yet been disclosed (Exscientia, 2024).

In the field of antibiotic discovery, a study published in *Cell* in February 2020 reported the identification of a novel antibiotic using a deep learning-based model. The researchers trained a neural network on a dataset comprising 2,335 compounds to identify molecules capable of inhibiting *E. coli* growth. Subsequently, the model was applied to a library of 6,111 molecules. Based on the model's predictions, the compound coded as SU3327 was identified for its antibacterial activity and renamed "Halicin"—a reference to the artificial intelligence system HAL 9000 from Stanley Kubrick's film 2001: A Space Odyssey. Halicin demonstrated efficacy not only against *E. coli* but also against multidrug-resistant pathogens such as *Clostridium difficile* and *Acinetobacter baumannii*. However, this compound has not yet progressed to clinical trials (Burki, 2020).

Overall, AI-driven drug discovery studies have demonstrated significant success in accelerating development processes and identifying novel molecular entities. AI technologies, particularly in the stages of target identification, molecule design, and prioritization, have overcome limitations inherent in traditional approaches and introduced a new paradigm. Real-world applications confirm that AI serves not only as a theoretical promise in drug discovery but also as a transformative tool in practice.

ARTIFICIAL INTELLIGENCE-ENABLED DRUG DEVELOPMENT & EVALUATION PROCESSES

Preclinical Stages

In preclinical stages, evaluating drug exposure in humans and optimizing the pharmacokinetic (PK) profile represent key objectives of drug discovery and development efforts (Healx, 2024). Since the late 1990s, it has become increasingly evident that inadequate pharmacokinetic properties of candidate molecules are a leading cause of clinical failures, prompting significant paradigm shifts within the pharmaceutical industry (Exscientia, 2024). Even today, poor ADME characteristics of small-molecule drug candidates

remain one of the primary reasons for development setbacks. However, recent years have witnessed considerable advancements in addressing these issues (Obrezanova, 2023).

During the drug discovery process, prior to first-in-human (FIH) studies, dose estimation and exposure assessment are typically carried out using *in vivo* animal models and *in vitro* systems derived from human sources. Physiologically Based Pharmacokinetic (PBPK) models simulate the time-dependent ADME behavior of drugs through mathematical formulations and contribute significantly to predicting biopharmaceutical profiles in humans, especially in advanced development stages. However, due to their high cost and limited scalability, PBPK models often fall short in enabling the rapid screening of large numbers of candidate compounds (Obrezanova, 2023).

One of the main challenges in PBPK modeling lies in the lack of compound-specific pharmacokinetic parameters for newly developed drugs. The absence of experimentally validated parameter data for most novel molecules limits the predictive accuracy of these models. To overcome this gap, several researchers have proposed integrated systems that combine simplified PBPK frameworks with ML algorithms to predict various PK parameters. This emerging approach is regarded as a multi-step process for the characterization and optimization of key ADME properties (Chou & Lin, 2023).

In the initial phase, databases containing *in vivo* time-concentration profiles and pharmacokinetic parameters are either constructed or derived from existing resources. Within this framework, the structural and physicochemical properties of selected drugs, along with *in vitro* ADME experimental data, must also be compiled.

In the second phase, ML/AI-based computational models are developed using these datasets, and the models are then employed to predict various ADME parameters—such as the tissue-plasma partition coefficient (K_p), clearance (Cl), and unbound fraction

(fu)—based on the structural and physicochemical attributes of the compounds. The predicted values are subsequently integrated into a general PBPK model.

In the third phase, time-concentration profiles in plasma and tissues are simulated using the integrated model, allowing for the estimation of key PK parameters such as the area under the curve (AUC) and maximum concentration (C_{max}). The resulting simulation data can either enhance existing databases or contribute to the development of new datasets for future modeling cycles (Chou & Lin, 2023).

In pharmacokinetic simulations specifically developed for animal models, a broad spectrum of methodologies has been employed—ranging from traditional ML techniques to advanced approaches such as deep learning. For example, Obrezanova and colleagues utilized a dataset comprising over 3,000 compounds to model time-concentration curves for nine different PK parameters following both intravenous and oral administration in rats. In their study, high-accuracy curve predictions were achieved for intravenous applications; however, only limited success was obtained for oral routes (Obrezanova, 2023).

There are certain structural challenges associated with modeling human pharmacokinetics using ML and AI techniques. One major limitation is the relatively small amount of available data for human PK modeling compared to preclinical animal data. While datasets for humans are typically limited to around 1,400 compounds, animal-based datasets encompass 5,000 to 60,000 compounds. Furthermore, due to the inherent constraints of clinical trials—such as high costs, long durations, and ethical or regulatory barriers—the expansion of human PK data occurs at a much slower pace.

In addition, compounds entering clinical trials are often selected based on “desirable” pharmacokinetic profiles, such as low clearance and high bioavailability. As a result, ML training datasets tend to underrepresent compounds with “suboptimal” properties, such as low bioavailability or high clearance. This under-

representation hinders the models' ability to learn the full spectrum of pharmacokinetic behavior and limits their generalizability.

Moreover, human data involve substantially greater complexity compared to controlled animal studies, due to inter-individual variability, disease states, dietary factors, and variations in measurement techniques. Consequently, a rigorous data curation process is essential when working with human-derived datasets.

In this context, Miljkovic et al. utilized a curated dataset of 1,000 clinical compounds to predict 12 different intravenous and oral human PK parameters. They employed random forest algorithms along with 2D chemical descriptors and selected *in vivo* rat PK parameters. The models developed for predicting oral C_{max}, AUC, and volume of distribution (V_d) demonstrated sufficient accuracy to be considered usable during the design stage and were validated using clinical data provided by AstraZeneca (Obrezanova, 2023).

Accurate and rapid prediction of pharmacokinetic parameters in the preclinical phase is a critical requirement for enhancing the efficiency of drug

discovery processes. In this regard, *in silico* ADMET modeling, which enables pharmacokinetic predictions based solely on chemical structures and fundamental molecular properties, has emerged as a valuable scientific tool. These approaches improve the effectiveness of project teams in designing and selecting molecules with favorable ADMET profiles and aim to reduce the number of compounds that need to be synthesized and experimentally tested by directing resources toward more promising candidates (Chou & Lin, 2023).

In recent years, the growing availability of experimental ADMET data, combined with advances in artificial intelligence algorithms, has led to the development of numerous AI-based tools for predicting ADMET properties. These tools significantly contribute to the efficient evaluation of candidate molecules in drug discovery and offer researchers substantial advantages in terms of both time and cost. Among these tools, five publicly available ADMET prediction platforms developed in the early 2020s have undergone extensive benchmarking and stand out as particularly notable. A summary of key information regarding these models is presented in Table 1 (Tran, Tayara, & Chong, 2023).

Table 1. AI-based ADMET prediction models and key features

Model	Latest Version	Number of Prediction	ML Algorithm	Website / Source
ADMETlab 3.0	3.0 (2024)	119	GNN	https://admetlab3.scbdd.com/
FP-ADMET	1.0 (2021)	≈50	RF	gitlab.com/vishsoft/fpadmet
AdmetSAR 3.0	3.0 (2023)	107	RF, SVM, k-NN	https://lmmd.ecust.edu.cn/admetSar3/index.php
Interpretable-ADMET	1.0 (2022)	59	GCNN, GAT	cadd.pharmacy.nankai.edu.cn/interpretableadmet
HelixADMET	1.0 (2022)	52	RF, GNN	paddlehelix.baidu.com/app/drug/admet/train

AI-assisted Pharmaceutical Formulation Development

Pharmaceutical formulation development is a multi-stage process aimed at designing safe, effective, and patient-compliant products by combining active pharmaceutical ingredients (APIs) with appropriate excipients (Afrin & Gupta, 2025). Traditional formu-

lation strategies predominantly rely on experimental trial-and-error approaches and often utilize statistical tools such as Design of Experiments (DoE) or Quality by Design (QbD) throughout the process. However, the multidimensional nature of formulation parameters frequently leads to prolonged timelines, high costs, and intensive labor requirements (Bao et al., 2023).

For instance, approximately 40% of newly developed compounds face solubility/bioavailability issues during early-stage screening. A large proportion of next-generation molecules exhibit poor aqueous solubility, which reduces bioavailability and increases the risk of formulation failure (Bhalani, Nutan, Kumar, & Singh Chandel, 2022). These challenges can result in reduced therapeutic efficacy or compromised product stability, further complicating the development process. Several reports emphasize that conventional formulation practices are labor-intensive, time-consuming, and costly due to their empirical basis (Afrin & Gupta, 2025). Consequently, there is a growing demand for more efficient, faster, and predictive methods in pharmaceutical formulation development.

In this context, AI, ML, and DL techniques have initiated a significant transformation within pharmaceutical technology in recent years. With a computational pharmaceuticals approach, drug formulations are being designed, optimized, and evaluated using big data and simulation techniques, enabling the extraction of meaningful relationships from experimental datasets. ML models allow for the prediction of drug–excipient interactions and compatibilities, while DL networks can forecast critical parameters such as drug release profiles. As a result, formulation design has moved beyond sole reliance on laboratory experimentation, evolving into a more systematic, rapid, and high-accuracy process (Dong, Wu, Xu, & Ouyang, 2023; Yang et al., 2019b).

The integration of AI into pharmaceutical formulation development has led to substantial paradigm shifts compared to conventional approaches. Primarily, AI-powered methods reduce both the number of experimental trials and overall development costs and timelines (Yang et al., 2019b). Leveraging historical datasets, predictive models can estimate critical properties such as solubility, bioavailability, and release kinetics in advance, thereby enabling more accurate formulation decisions. Furthermore, AI-based systems can uncover structure–property relationships that may be overlooked by traditional techniques,

thereby facilitating the identification of optimal formulation combinations and the development of innovative strategies (Challener, 2024).

Recent projects and platforms have demonstrated significant progress in AI-assisted pharmaceutical formulation development. For instance, the FormulationAI platform provides pre-trained AI models capable of predicting formulation properties across a variety of systems, from cyclodextrin complexes to liposomal delivery systems. Users can input information about active pharmaceutical ingredients and excipients to obtain predictions for multiple formulation characteristics (Dong et al., 2023).

Similarly, the DE-INTERACT project has developed an AI-based system capable of predicting drug–excipient compatibility with 98% accuracy by utilizing molecular descriptors and model ensemble techniques. Platforms like Intrepid Labs, which operate as “self-driving” laboratories, combine AI and robotics to automate experimental optimization workflows, significantly shortening formulation development timelines (Intrepid Labs, 2024). Additionally, the AI-driven autonomous lab established through the collaboration between Atinary Technologies and Takeda has markedly improved formulation efficiency (Atinary Technologies, 2023) (Hang et al., 2024).

Collectively, these advances illustrate how the integration of AI, ML, and DL technologies is transforming pharmaceutical formulation development into a more rapid, efficient, and intelligent process. As these technologies become more widely adopted, it is expected that the timelines for drug discovery and development will be significantly shortened, while simultaneously reducing research and development costs.

AI Applications in Clinical Trials

The development and commercialization of a new drug typically span a lengthy period of 10 to 15 years and require high costs ranging between approximately 1.5 to 2.0 billion USD. Nearly half of this time and

financial investment is consumed during drug discovery, optimization, and preclinical research stages, while the remaining 50% is allocated to clinical research processes. The high failure rate observed during clinical trials stands out as one of the major obstacles in drug development. Only one-third of compounds that reach Phase II proceed to Phase III, and more than one-third of those that reach Phase III fail during the approval process. Considering that Phase III studies alone account for nearly 60% of total R&D costs, a failed clinical trial can result in a financial loss ranging from 0.8 to 1.4 billion USD, representing a significant portion of R&D investment.

At this point, AI technologies emerge as promising tools to make clinical research processes more efficient, faster, and effective. ML and DL techniques analyze patterns within large and diverse data sources—such as electronic health records (EHR), medical literature, and clinical databases—to optimize patient-trial matching processes and enable a more reliable assessment of clinical endpoints (Harrer, Shah, Antony, & Hu, 2019).

In 2024, the Research Affairs Committee of the American College of Clinical Pharmacy (ACCP) conducted a comprehensive review of the role of AI in clinical pharmacy research and scientific publishing. Their evaluation highlighted how AI technologies can be effectively utilized at various stages of scientific research—including research question development, study design, data analysis, and results reporting (Chan et al., 2025).

Patient recruitment is one of the major challenges encountered in clinical trials. In Phase I clinical studies, approximately 80% of trials experience delays in patient enrollment. Traditional recruitment methods require healthcare professionals to manually review large volumes of medical records, a process that is time-consuming, costly, and prone to human error. Artificial intelligence offers significant advantages in this regard by leveraging electronic health records, social media platforms, and other digital data sources to

rapidly identify potential participants who meet specific study criteria. For instance, Deep 6 AI technology is capable of screening millions of patient records to swiftly detect eligible candidates for clinical trials (Wu et al., 2024b).

Wout Brusselaers, founder and CEO of Deep 6 AI, has emphasized that their system is not limited to traditional insurance records or structured EHR data. Instead, it harnesses the power of AI to mine deeper and less structured data sources, enabling the identification of target patient populations with high precision and speed.

Traditional data collection and evaluation methods often suffer from limitations such as low efficiency, limited capacity, susceptibility to error, and a lack of real-time monitoring. Artificial intelligence offers promising solutions to these challenges. ML techniques can manage and analyze large-scale clinical datasets swiftly and effectively, thereby contributing to the early identification of overlooked issues and hidden risks. Moreover, the analysis of real-time health data collected from wearable devices enables continuous and dynamic monitoring of participants' health status, providing researchers with accurate and up-to-date insights.

Artificial intelligence also plays a critical role in the clinical trial design phase. AI-powered predictive analytics can analyze historical clinical trial data to forecast potential outcomes, supporting researchers in making more informed decisions regarding optimal dosage selection, appropriate patient cohort identification, and the early detection of possible adverse events (Wu et al., 2024b).

In addition, AI holds great potential for enhancing operational efficiency and patient management in clinical research. AI applications in study design and patient stratification processes contribute to improved trial performance and support the development of targeted therapeutic strategies. Machine learning algorithms can analyze prior clinical data, electronic health records, and genomic information to facilitate

the creation of more efficient study protocols and accurate identification of suitable patient populations.

One study reported a 25% reduction in protocol amendments and a 15% increase in patient enroll-

ment rates through the application of ML techniques. These tangible improvements in clinical trial efficiency are summarized in Table 2 (Huang, Yang, Wen, Xia, & Yuan, 2024).

Table 2. The impact of artificial intelligence on clinical trial efficiency

Criterion	Traditional Approach	AI-Assisted Approach	Improvement Rate
Protocol Amendments	4.2 per study	3.15 per study	25% reduction
Patient Enrollment Rate	60%	69%	15% increase
Study Completion Time	3.5 years	2.8 years	20% reduction
Cost Reduction	-	-	18% reduction

Integration of artificial intelligence into the healthcare and pharmaceutical sectors: ethical and regulatory perspectives

The integration of AI technologies into the healthcare and pharmaceutical sectors has sparked a range of ethical debates. One of the key concerns pertains to the potential impact of these technologies on the labor market; for instance, it has been suggested that AI-driven systems may reduce employment opportunities by automating tasks traditionally carried out by healthcare professionals. In addition, questions surrounding the ownership of discoveries generated by machine learning algorithms—and whether such findings can be patented—represent unresolved ethical and legal challenges.

The degree to which physicians should rely on health profiles generated by AI systems, the extent to which these systems should influence diagnostic processes, and the level of trust patients place in such recommendations are all matters of growing concern. The absence of assurance regarding the impartiality and completeness of the data used to train these algorithms undermines trust in AI applications. Furthermore, there is a significant risk that existing social inequalities may be exacerbated by biased models trained on non-representative datasets. In particular, insufficient representation of disadvantaged patient groups in training data may lead to discriminatory practices, thereby violating the principle of equity in healthcare delivery.

The prediction of an individual’s predisposition to certain diseases based on pharmacogenomic data poses a significant ethical dilemma. While such predictive information can facilitate preventive medical interventions and offer potential health benefits, it may also exert adverse effects on an individual’s psychological well-being and emotional stability. Moreover, if health insurance providers were to gain access to such data, they might begin to demand future risk reports from individuals, potentially leading to new ethical and privacy-related concerns.

In response to these and similar ethical challenges, various international platforms have been developed to foster global discourse on the ethical implications of artificial intelligence. One notable initiative is the *Moral Machine*, which provides a simulation environment to explore ethical dilemmas encountered by autonomous vehicles. With input from over 40 million participants, the platform has allowed researchers to examine cross-cultural variations in moral decision-making. Findings have suggested that certain ethical principles may be shared across cultures, indicating the presence of some universal moral values.

Milena Pribić, a representative of IBM, has emphasized that insufficient prioritization of AI ethics by institutions may result in adverse long-term consequences. Therefore, it is imperative that AI systems be designed and implemented in alignment with fundamental ethical principles. In clinical practice, protocols such as CONSORT-AI and SPIRIT-AI have been

introduced to enhance transparency and accountability by ensuring that AI-driven interventions are reported in a comprehensive and standardized manner within the context of clinical trials (Arabi, 2021).

In a study conducted by Karimian, Petelos, and Evers in 2021 (Karimian, Petelos, & Evers, 2022), the ethical challenges potentially arising from the integration of artificial intelligence into the healthcare sector were systematically examined. The review was based on the Ethics Guidelines for Trustworthy AI, published by the European Commission, which outlines seven fundamental ethical principles: human agency and oversight, technical robustness and safety, privacy and data governance, transparency, diversity and non-discrimination, societal and environmental well-being, and accountability.

Among the most frequently discussed ethical issues in the reviewed literature were fairness, the preservation of human autonomy, explainability, and patient privacy. Notably, the principle of non-maleficence—"do no harm"—was found to be under-represented in the existing academic discourse. Furthermore, the study highlighted a substantial gap in the development of practical evaluation frameworks aimed at assessing the compliance of AI-based systems with these ethical principles. Only a limited number of publications offered actionable solutions for protecting patient privacy in healthcare settings, and empirical evidence supporting the operationalization of other ethical principles was reported to be scarce (Karimian et al., 2022).

In conclusion, the development and implementation of artificial intelligence technologies within ethical boundaries are of paramount importance to maintain a balance between technological progress and human values. AI solutions that operate in collaboration with human expertise and complement ethical gaps such as intuition and emotion have the potential to enhance societal benefit when properly managed. However, the continuous ethical evaluation and responsible governance of these technologies are essen-

tial for building a more equitable and trustworthy AI ecosystem (Arabi, 2021).

Pharmacogenomics, Bioinformatics, and Data Privacy

Pharmacogenomics (PGx), a cornerstone of personalized medicine, aims to maximize drug efficacy and minimize adverse drug reactions by integrating individuals' genetic characteristics into therapeutic decision-making processes.

The integration of AI with pharmacogenomic data introduces an innovative approach to precision medicine and facilitates the development of individualized treatment strategies. For instance, models utilizing ML algorithms can predict drug responses based on patients' genetic profiles, thereby enabling safer treatment options and optimized dosage regimens. In domains such as oncology—where therapeutic options may be limited—these models have contributed to the identification of novel genetic biomarkers associated with drug response through the analysis of transcriptomic data, thereby advancing targeted drug discovery processes.

However, the ethical, legal, and social implications associated with technologies developed using PGx data are receiving increasing attention. Among these concerns, genetic data privacy remains one of the most pressing issues. Such data not only reveal personal information but also encode familial biological relationships and ancestral origins. This inherent sensitivity renders full anonymization challenging and increases the risk of re-identification through cross-referencing even when data are presumed to be anonymized. Consequently, the use of genetic data for training AI models necessitates the highest standards of data security and a strong commitment to maintaining patient trust.

To address these concerns, the development of ethical guidelines for the use of AI in healthcare plays a pivotal role. For example, Char (2020) proposed a four-phase framework for assessing ethical challenges in AI-based healthcare applications. This framework

includes: (i) development and deployment of AI tools, (ii) establishment of regulatory and oversight mechanisms, (iii) ethical evaluation of AI-driven decisions, and (iv) consideration of broader ethical concerns. Such a comprehensive approach promotes the responsible and transparent implementation of AI systems in healthcare settings. Additionally, techniques such as federated learning—categorized under decentralized data processing strategies—help reduce the need for inter-institutional data sharing, thereby contributing to the protection of patient privacy. These methods allow AI models to be trained on decentralized datasets while preserving the confidentiality of sensitive health information.

In conclusion, the development of AI-based pharmacogenomic models must be grounded in ethical principles, ensuring the careful handling of genetic data and the protection of patient confidentiality.

Employing fair and representative datasets that reflect genetic diversity and actively addressing algorithmic biases are essential for building trustworthy and ethically sound AI systems. Such an approach not only enhances clinical efficacy but also reinforces public confidence in AI-driven healthcare technologies (Haga, 2024).

RESULTS AND DISCUSSION

This study has comprehensively demonstrated how AI technologies can be integrated across all stages of the drug-discovery process—from target identification and validation to lead optimization, from synthesis planning to ADME/Tox prediction and preclinical assessment. Beyond structure- and ligand-based virtual screening, the capacity of state-of-the-art models—particularly graph neural networks—to capture complex molecular relationships delivers clear gains in candidate selection, target fit, and optimization speed. In addition to these modeling advances, we outline data-representation choices, algorithmic architectures, and training strategies. Synthesis planning and synthetic-accessibility prediction enhance resource efficiency and sustainability,

while early computational ADME/Tox filters enable a more controlled and predictable triage of candidates with the potential to advance to the clinic. Industrial and academic case studies surveyed under Real-World Applications show that AI is not merely a theoretical promise but a transformative tool with practical impact. Nevertheless, realizing AI's full potential requires continuous improvements in data volume and quality, explainability, reproducibility, and governance (data security, ethics, standards). AI technologies are emerging not only as supportive tools in pharmaceutical research but as strategic components at the center of innovative drug-discovery paradigms. To this end, building large and balanced datasets; strengthening labeling and curation; defining performance metrics transparently and comparably; making model decision logic auditable; and corroborating results through independent validation are critical. Regulatory compliance and transparent reporting should be supported by end-to-end traceability, versioning, and audit trails.

At the preclinical stage, we recommend systematically aligning *in silico* predictions with *in vitro/in vivo* readouts and regularly monitoring out-of-model errors (dataset shift, bias). From a sustainability perspective, adopting low-energy, low-resource workflows; prioritizing synthesis routes consistent with green-chemistry principles; and establishing automation and active-learning cycles that improve the cost-time balance are valuable.

In summary, the assessments presented here indicate that AI is a complementary accelerator in pharmaceutical R&D; when robust data governance, explainability, reproducibility, and regulatory compliance are ensured, it reduces risk while increasing accuracy and efficiency. To further strengthen AI integration into pharmaceutical R&D, we recommend enhancing interdisciplinary collaboration, prioritizing explainable-AI approaches, and restructuring health-specific ethical and regulatory standards accordingly.

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AUTHOR CONTRIBUTION STATEMENT

Conception (NGK), Literature research and writing (DN), Reviewing the text (NGK, DN), Supervision (NGK).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Pharmacotherapy in Interstitial Cystitis / Painful Bladder Syndrome

Merve DENİZALTI*, Nezahat Tuğba DURLU-KANDİLCİ**

Pharmacotherapy in Interstitial Cystitis / Painful Bladder Syndrome

İnterstisyel Sistit / Ağrılı Mesane Sendromunda Farmakoterapi

SUMMARY

Interstitial cystitis / Painful bladder syndrome is a chronic disease characterized by suprapubic pain associated with bladder filling, accompanied by an overactive detrusor and inflammation of the bladder. Although the etiology and pathophysiology of this disease are not unlightened yet, some pathophysiological theories such as the glycosaminoglycan theory, altered permeability, neural regulation, mast cell, and neuroendocrine theories have been suggested. Different treatment methods have been developed based on these theories. The developed pharmacotherapies can be classified as conservative treatment, oral treatment, intravesical treatment, and current treatment methods. Agents used in oral treatment include pentosan polysulfate sodium, tricyclic antidepressants, histamine receptor antagonists, immunosuppressants, and AQX-1125 (Rosiptor). Agents also used in intravesical therapy include dimethyl sulfoxide, lidocaine, heparin, pentosan polysulfate sodium, chondroitin sulfate, hyaluronic acid, and Bacillus Calmette-Guerin. The use of botulinum toxin A (BTX-A) has also been approved. Finally, current treatment methods include phosphodiesterase-5 inhibitors, monoclonal antibodies, cannabinoids, and liposomes use. The goal of most treatments is to control symptoms.

Keywords: Interstitial cystitis / Painful bladder syndrome, inflammation, conservative treatment, oral treatment, intravesical treatment

ÖZ

İnterstisyel sistit/ Ağrılı mesane sendromu, mesanenin dolu olmasına bağlı olarak suprapubik ağrı, aşırı aktif detrusor ve mesanenin inflamasyonu ile karakterize kronik bir hastalıktır. Bu hastalığın etiyojisi ve patofizyolojisi kesin olarak bilinmemekle birlikte glikozaminoglikan teorisi, değişen geçirgenlik, nöronal düzenleme, mast hücre ve nöroendokrin teorisi gibi bir çok patofizyolojik teori ileri sürülmüştür. Bu teoriler dikkate alınarak farklı tedavi yöntemleri geliştirilmiştir. Geliştirilen farmakoterapiler konservatif tedavi, oral tedavi, intravezikal tedavi ve yeni nesil tedavi yöntemleri olarak sınıflandırılabilir. Oral tedavide kullanılan ajanlar pentosan polisülfat sodyum, trisiklik antidepresanlar, histamin reseptör antagonistleri, immünosupresanlar ve AQX-1125 (Rosiptor); intravezikal tedavide kullanılan ajanlar ise dimetil sülfoksit, lidokain, heparin ve pentosan polisülfat sodyum, kondroitin sülfat ve hyaluronik asit, Bacillus Calmette-Guerindir. Botulinum toksin A (BTX-A) kullanımını da onay almıştır. Son olarak yeni nesil tedavi yöntemleri fosfodiesteraz-5 inhibitörleri, monoklonal antikorlar, kanabinoidler ve lipozom kullanımı gibi yöntemleri içermektedir. Tedavilerin birçoğunun hedefi semptomların kontrolünü sağlamaktır.

Anahtar Kelimeler: İnterstisyel sistit / Ağrılı mesane sendromu, inflamasyon, konservatif tedavi, oral tedavi, intravezikal tedavi.

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* ORCID: 0000-0001-5363-606X, Hacettepe University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Türkiye

** ORCID: 0000-0001-7236-2041, Hacettepe University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Türkiye

INTRODUCTION

INTERSTITIAL CYSTITIS / PAINFUL BLADDER SYNDROME (IC/PBS) PATHOPHYSIOLOGY

Interstitial cystitis / Painful bladder syndrome (IC/PBS) is a chronic inflammatory bladder syndrome characterized by a sudden urge to urinate together with painful micturition, increased urinary frequency, and bladder overreactivity. These symptoms of urinary urgency, urinary bladder pain, and irritative symptoms last more than 6 months (Zhao et al., 2016). Chronic inflammation observed in the bladder may directly affect its function.

Although the pathophysiological mechanisms underlying IC/PBS are not fully understood, several hypotheses have been proposed, including glycosaminoglycan (GAG) layer deficiency with increased epithelial permeability, microbial infection theory, mast cell activation, neurogenic or neuro-urothelial interactions, and autoimmune mechanisms (Patnaik et al., 2017). Among these, the most widely accepted hypothesis proposes that an initial defect or injury of the bladder mucosal barrier subsequently induces chronic inflammation. The urothelium exhibits a unique structural organization characterized by polysaccharides such as chondroitin sulfate, hyaluronic acid, and glycoproteins (Lewis, 2000). This configuration establishes a non-adhesive, highly impermeable barrier that protects the urothelium against bacterial adherence as well as urinary irritants. Structural disruption or biochemical deficiency within this barrier impairs its integrity, allowing urinary solutes to penetrate the suburothelial space of the bladder wall. The suburothelial compartments and the bladder wall (Parsons, 2011). Histopathological analyses of bladder biopsies from patients with IC/PBS have demonstrated aberrant expression of uroplakins, chondroitin sulfate, and tight junction proteins (Slobodov et al., 2004). Consequently, urinary constituents such as potassium ions may traverse the defective urothelium, inducing depolarization of muscle and neuronal

cells, activating inflammatory signaling cascades, and provoking mast cell degranulation, thereby contributing to the pathogenesis of IC/PBS-associated lower urinary tract symptoms

Chronic inflammation has a role in the pathogenesis of IC/PBS. Biopsies from IC/PBS patients have demonstrated mast cell infiltration, leukocyte accumulation of inflammatory cells within the suburothelial layers and bladder wall, accompanied by increased vascularity and bladder wall thickening. The mechanisms underlying infection-induced pain are not fully understood, experimental studies have shown that stimulation with defective mutants of *E. coli* lacking in O-antigen biosynthesis may induce allodynia that persists long after the infection has been treated (Rudick et al., 2012). Therefore, IC/PBS may represent a sterile/chronic inflammatory condition triggered by a prior episode of microbial cystitis. The exact cause of IC/PBS remains unclear; however, it is widely accepted that multiple factors contribute to its pathophysiology.

As of yet, there are no other ways to study the effects of cystitis on the bladder comprehensively, including the changes in bladder structure and function, but to use animal models. Because the exact cause of IC/PBS in patients remains elusive, it is not possible to precisely reproduce the condition under experimental conditions. However, animal models have proven useful in investigating the underlying mechanisms of bladder inflammation and for evaluating potential therapeutic strategies to alleviate clinical symptoms. Rodents are the most widely used species used to study IC/PBS, and many models for inducing cystitis in rodents have been developed over the years, such as neurogenic cystitis caused by heat, cold, bacterial and viral infection, and antigen exposure. Intravesical instillation of irritants such as lipopolysaccharide (LPS), acids (hydrochloric acid and acetic acid), turpentine, mustard oil, croton oil, and acrolein is commonly used to induce experimental cystitis in rodents (Birder & Andersson, 2018). Cyclo-

phosphamide-induced IC/PBS is a well-established model in rodents that mimics some of the symptoms of this condition observed in humans, such as pain, inflammation, and increased urinary frequency and urgency. In experimental IC/PBS models induced by cyclophosphamide, contractile responses of detrusor smooth muscle to different agonists such as carbachol, trypsin, or sphingosine-1-phosphate are altered. Moreover, aging has been reported to change the detrusor function in these models (Anjum et al., 2017; Denizalti et al., 2018; Denizalti et al., 2022; Denizalti et al., 2023; Durlu-Kandilci et al., 2015; Masago et al., 2009). However, the exact cause of IC/PBS and how to treat bladder dysfunction in patients has not yet been identified.

PHARMACOLOGICAL TREATMENT APPROACHES

According to the American Urological Association's updated 2022 guideline, treatments for IC/PBS are categorized into four main groups: behavioral and non-pharmacological interventions, oral pharmacotherapy, intravesical therapies, and major surgical procedures. When necessary, concurrent and multimodal therapeutic approaches are recommended. The multimodal treatment strategy for IC/PBS should be based on the potential pathophysiological mechanisms of the disease. Given the multifactorial etiology of IC/PBS, achieving a satisfactory clinical response may require combining multiple therapeutic modalities targeting the underlying pathological processes. Multimodal treatment strategies grounded in the underlying pathophysiological mechanisms and clinical characteristics of IC/PBS may lead to more successful therapeutic outcomes. Therefore, it is emphasized that clinical management of IC/PBS should be individualized according to each patient's unique clinical profile (Clemens et al., 2022; Jhang et al., 2025).

Pharmacotherapy in IC/PBS is diverse, reflecting the multiple pathophysiological mechanisms underlying the disease. Mechanisms of bladder dysfunction include chronic bacterial infection, disruption of the

glycosaminoglycan (GAG) layer, activation of mast cells, autoimmune-mediated mechanisms, and autonomic nervous system dysfunction. Research exists on treatment options targeting each of these mechanisms, but there is still no definitive cure (Chen & Kuo, 2020). First-line treatment includes conservative, non-pharmacological approaches such as behavioral modifications, pelvic floor exercises, controlled fluid intake, and bladder training. Second-line treatment includes oral medication such as pentosan polysulfate, tricyclic antidepressants, histamine, and leukotriene receptor inhibitors, immunosuppressants, and AQX-1125 (Rosiptor), whereas third-line therapy involves intravesical administration (Garzon et al., 2020).

Conservative treatment (non-pharmacological management)

First-line treatment includes conservative management such as behavioral and dietary modifications, heat or cold therapy, pelvic floor exercises, and psychological stress reduction. Dietary restrictions, such as limiting the intake of coffee, tea, alcohol, chocolate, and spicy foods, have been shown to alleviate IC/PBS symptoms. In addition, proper regulation of diet and fluid intake helps reduce constipation and normalizes urinary frequency (Bosch & Bosch, 2014; Friedlander et al., 2012). Pelvic floor exercises (such as knee-chest position, legs-spreading, or squatting techniques) and bladder training are also known to alleviate IC/PBS symptoms. Furthermore, personal care and stress reduction strategies contribute to overall symptom improvement and quality of life (Hanley et al., 2009; Weiss, 2001).

Oral treatment

Oral pharmacotherapy is a second-line treatment that should be administered in combination with conservative treatment methods. If conservative treatment does not correct the symptoms alone, oral pharmacological treatment such as pentosan polysulfate sodium (PPS), amitriptyline, hydroxyzine, cimetidine and cyclosporine A is administered as a treatment (Table 1.).

- **Pentosan polysulfate sodium (PPS)**

Pentosan polysulfate sodium (PPS) is the only oral pharmacotherapy agent approved by the US Food and Drug Administration (FDA) for the treatment of IC/PBS. The disorder is thought to be associated with disruption of the GAG layer covering the bladder urothelium. PPS, a synthetic sulfated polysaccharide, acts by restoring this damaged GAG layer and thereby reducing urothelial permeability. It is administered orally at a dose of 300 mg/day for 8 months. It is no longer widely recommended due to side effects such as diarrhea, vomiting, rectal bleeding, and ophthalmic effects (Garzon et al., 2020).

- **Tricyclic antidepressants**

Amitriptyline is a tricyclic antidepressant commonly used in the management of IC/PBS. It acts by blocking the reuptake of serotonin and norepinephrine. Although not specifically approved for IC/PBS, it is widely prescribed for neuropathic pain. Its anticholinergic side effects include blurred vision, dry mouth, constipation, weight gain, and sedation. Other oral agents may be used in combination with amitriptyline to enhance therapeutic outcomes (Garzon et al., 2020).

- **Histamine receptor antagonists**

It has been shown that mediators released from mast cells during hypersensitivity reactions can cause urinary symptoms. Histamine receptor antagonists have been reported to reduce mast cell activation in patients with IC/PBS. Cimetidine, an H2 receptor antagonist, provides significant improvement in su-

prapubic pain and nocturia (Dasgupta et al., 2001; Thilagarajah et al., 2001). Hydroxyzine, an H1 antagonist with anticholinergic activity, has been shown to have no significant difference from placebo, but it is effective in combination with PPS (Fall et al., 2008).

- **Immunosuppressants**

Significant elevations in proinflammatory chemokines and cytokines have been demonstrated in the urine and bladder tissue of patients with IC/PBS (Crane et al., 2019). Cyclosporine A (CyA), a calcineurin inhibitor that suppresses T cell activation, has been reported to be effective in patients refractory to single-agent or oral combination therapies, resulting in reduced pain, increased maximum bladder capacity, and improved voiding volume. However, despite its promising long-term therapeutic benefits, CyA is associated with adverse effects such as hypertension, nephrotoxicity, hyperglycemia, dyslipidemia, and neurotoxicity, as well as an increased risk of infection (Crescenze et al., 2017; Sairanen et al., 2004; Sairanen et al., 2005; Wang & Zhang, 2016).

- **AQX-1125 (Rosiptor)**

AQX-1125, an activator of SH2-containing inositol-5'-phosphatase 1 (SHIP1), has recently been investigated as a novel oral therapeutic agent for the treatment of IC/PBS. In women with moderate-to-severe IC/PBS, a significant reduction in pain and urinary symptoms was reported following 6 weeks of oral AQX-1125 treatment (Nickel et al., 2016; Nickel et al., 2019).

Table 1. Alternative oral pharmacological agents for the management of IC/PBS

Agent	Proposed Mechanism of Action	Evidence/Remarks	Key References
Pentosan polysulfate sodium (PPS)	Replenishment of the glycosaminoglycan (GAG) layer; epithelial barrier protection; mild anti-inflammatory effects	Most extensively studied oral agent; modest efficacy; long-term use may be limited by pigmentary maculopathy risk.	Clemens et al., 2022; Jhang et al., 2025
Amitriptyline	Tricyclic antidepressant with analgesic and neuromodulatory properties; antimuscarinic effects	Demonstrated symptom improvement in clinical trials; sedation and anticholinergic side effects are limiting factors.	Cacciatore et al., 2024; Hanno et al., 2011
Hydroxyzine	H1 receptor blockade; mast-cell modulation and anti-inflammatory effects	May be particularly beneficial in patients with mast cell-related pathophysiology	Clemens et al., 2022; Hanno et al., 2011
Cimetidine	H2 receptor blockade; modulation of mast cell activation and histamine pathways	Limited evidence; small-scale studies show partial symptom improvement	Cacciatore et al., 2024
Cyclosporine A	Calcineurin inhibition; suppression of T-cell-mediated inflammation	Shown effective in refractory cases with Hunner lesions; requires close monitoring for nephrotoxicity and immunosuppression	Jhang et al., 2025

Intravesical therapy

Intravesical therapy is a third-line treatment option. It involves direct instillation of therapeutic agents into the bladder via a catheter. It is often combined with low-pressure, short-term hydrodistension. It provides easy and safe access to the bladder for prolonged drug exposure. The advantages of intravesical therapy include prolonged contact time between the drug and the urothelium, reduced systemic side effects, urothelial repair, and higher drug concentrations in the bladder wall compared to systemic administration. Other pathologies must be ruled out before these treatments can be administered, and they are recommended when less invasive treatments have failed. In general, maintenance therapy is generally required in patients who show improvement in symptoms (Garzon et al., 2020). The most commonly used intravesical agents are dimethyl sulfoxide (DMSO), lidocaine, heparin, pentosan polysulfate sodium, chondroitin sulfate, hyaluronic acid, and Bacillus Calmette-Guerin (BCG) (Table 2.).

- Dimethyl sulfoxide (DMSO) and lidocaine

DMSO is the only intravesical therapeutic agent approved by the FDA for the management of IC/PBS, and is commonly administered in combination with other agents. It is considered the most widely used intravesical intervention. Although its precise mechanism of action has not been fully elucidated, DMSO is thought to act by attenuating inflammation, relaxing bladder smooth muscle, and modulating of mast cell-mediated inflammatory activity. The standard instillation procedure consists of administering 50 mL of a 50% DMSO solution into the bladder via a catheter. Although no universally accepted treatment protocol exists, the regimen is commonly applied once weekly for a six-week period (Garzon et al., 2020; Hanno et al., 2011). Lidocaine is a local anesthetic with anti-inflammatory properties that provides symptomatic improvement both alone and in combination with other intravesical agents. Intravesical lidocaine is recommended by clinical guidelines for short-term relief of acute symptom exacerbations (Nickel et al., 2009).

- Heparin and pentosan polysulfate sodium (PPS)

The pathogenesis of IC/PBS is thought to be related to abnormalities in the bladder urothelium caused by disruption of the GAG layer. Heparin is a polysaccharide, one of the components of the GAG layer covering the urothelium. It is thought that intravesical instillation of exogenous GAG components, either as monotherapy or in combination with other drugs, replenishes this disrupted GAG layer. Heparin has an anti-inflammatory effect and is known to induce urothelial growth, smooth muscle, and fibroblast proliferation. Improvement in symptoms has been reported in 56-73% of patients after intravesical heparin therapy (3 months). Clinical studies indicate that heparin is more effective when used in combination with other agents rather than when used as monotherapy (Garzon et al., 2020; Giusto et al., 2018).

PPS is a heparin analog used as an intravesical drug to restore the GAG layer, in addition to its oral use. The combination of both oral and intravesical administration has been shown to provide greater improvement (Garzon et al., 2020).

- Chondroitin sulfate and hyaluronic acid (HA)

Both chondroitin sulfate and hyaluronic acid are components of the GAG layer covering the urothelium, and intravesical administration restores the damaged protective barrier. The combined use of these agents rather than as monotherapy is recommended (Cox et al., 2016; Garzon et al., 2020; Thakkinstian & Nickel, 2013).

Hyaluronic acid is a glycoprotein that is an important component of the GAG layer. It alleviates the inflammatory process by both inhibiting leukocyte aggregation and blocking ICAM-1 receptors by binding to lymphocytes and endothelial cells. It has also been reported to suppress mast cell degranulation in IC/PBS. Similarly, chondroitin sulfate has been identified as a key component of the GAG layer of the bladder. When used to replenish the GAG layer, it is thought to be more beneficial than HA (Ha & Xu, 2017).

Due to the multifactorial etiology of IC/PBS, combined intravesical therapy is preferred to enhance the therapeutic efficacy. iAluRil, containing 1.6% hyaluronic acid and 2% chondroitin sulfate, is commonly used in formulations for this purpose. This mixture is administered intravesically once weekly for 8 weeks. No significant adverse effects have been reported associated with iAluRil combination therapy (Ha & Xu, 2017).

- **Bacillus Calmette-Guerin (BCG)**

IC/PBS is thought to represent an autoimmune condition based on its histopathological features and the presence of autoantibodies in similar to those in other autoimmune diseases. IC/PBS patients have been shown to exhibit five times higher interleukin-6 production levels. The exact mechanism of BCG therapy remains unclear, and considering BCG's side effect profile and low therapeutic benefit, its use in IC/PBS treatment is limited (Ha & Xu, 2017).

Botulinum toxin A (BTX-A)

Botulinum toxin, produced by *Clostridium botulinum*, is recognized as the most potent neurotox-

in. However, when administered in controlled doses, it can be effective in several clinical conditions. The American Urological Association (AUA) recommends this treatment for refractory overactive bladder. The FDA approved botulinum toxin for the treatment of neurogenic detrusor overactivity and refractory overactive bladder in 2011 (Spinu et al., 2020).

Botulinum toxin A (BTX-A) is a neurotoxic protein that can be administered intravesically into the detrusor smooth muscle. It is suggested that it inhibits the release of the neurotransmitter acetylcholine from cholinergic afferent nerve terminals in the suburothelial layer of the bladder, thereby providing antinociceptive and anti-inflammatory activity (Chen & Kuo, 2020; Chuang et al., 2004). Despite its therapeutic efficacy, it also has adverse effects such as urinary tract infections, viral conditions, and increased residual volume after urination (Spinu et al., 2020). However, intravesical BTX-A injection is still considered a safe and effective treatment option even after repeated treatments due to the limited duration of effect (9-10 months) (Lee & Kuo, 2015; Pinto et al., 2013; Shie et al., 2012).

Table 2. Experimental and investigational intravesical agents for IC/PBS

Agent	Proposed Mechanism of Action	Evidence/Remarks	Key References
Hyaluronic acid	GAG layer replenishment; enhancement of epithelial barrier and mucosal repair	Multiple studies and meta-analyses show symptom reduction; heterogeneous protocols.	EAU Guidelines, 2025; Plotti et al., 2024
Chondroitin sulfate	Restoration of the urothelial GAG barrier	Often combined with HA; partial symptomatic benefit in clinical studies	Plotti et al., 2024
Heparin	Anti-inflammatory and barrier-protective effects	Mixed results; occasionally used in combination protocols	Hanno et al., 2011
Lidocaine	Local anesthetic; blockade of sensory afferent signaling	Provides short-term relief; recommended for symptom flares in EAU guidance	EAU Guidelines, 2025
Botulinum toxin A	Inhibition of acetylcholine release; sensory and motor neuromodulation	Randomized trials show improvement in pain and bladder capacity; repeated injections may be required	Cacciatore et al., 2024; Jhang et al., 2025
Resiniferatoxin (RTX)	TRPV1 receptor desensitization and afferent nerve modulation	Results from randomized studies are inconsistent; it remains investigational	Cacciatore et al., 2024
Experimental anti-inflammatory formulations (e.g., NF-κB inhibitors)	Modulation of inflammatory signaling pathways; reduction of neurotrophin expression	Preclinical and early clinical evidence only; promising preliminary outcomes	Jhang et al., 2025

EMERGING/NOVEL THERAPIES

IC/PBS is a complex disease, and its exact etiology is not yet fully understood. More research is being conducted to discover the underlying full pathology, and new treatment methods are being developed. Several emerging therapies have shown promising results in small randomized trials (Colemeadow et al., 2020).

Improved intravesical drug delivery systems

New methods aimed at enhancing intravesical drug delivery, such as liposomes, the lidocaine-releasing intravesical system (LiRIS), and nanoparticles, appear promising in early trials (Colemeadow et al., 2020).

Liposomes and liposomal drug delivery are thought to be a promising new treatment for lower urinary tract dysfunction. Liposomes are drug carriers composed of phospholipids and sphingomyelins. They can adhere to the urothelium membrane surface and, after intravesical administration, support endocytosis by facilitating the entry of drugs, toxins, and oligonucleotides into the epithelium. When administered, they form a film layer that acts as a mechanical barrier in the bladder urothelium. They have been found to reduce inflammation and irritation in IC/PBS bladders and have been reported to renew the GAG layer by 50%. Although liposomes are ineffective when compared to a placebo, they have been considered as a carrier for botulinum toxin A in refractory IC/PBS patients as an alternative to injection (Garzon et al., 2020). Due to the large size of botulinum toxin A (150 kDa), it cannot access the afferent nerves immediately below without injection. The transport of the toxin from the urothelium to the deeper layers of the bladder is limited. Therefore, it has been combined with liposomes to enable the transport of Botulinum A to deeper layers (Chuang et al., 2009).

The goal is to transport Botulinum A from the urothelium via liposomes and to protect Botulinum A encapsulated within the liposomes from being broken down by proteases in urine without altering its efficacy (Chuang et al., 2009). Improvement was found to

be 54% in the lipo-Botulinum A group and 32% in the placebo group, with no urinary retention or other side effects observed in either group (Chuang et al., 2014).

Tacrolimus is a potent lipophilic immunosuppressive drug. It exerts its effect by inhibiting IL-2-dependent T cell activation. However, systemic tacrolimus administration causes side effects such as nephrotoxicity and hypertension. The low solubility of tacrolimus in water also limits its intravesical distribution for treatment. Studies have shown that when liposomes are used for tacrolimus delivery, serum levels are low, while tacrolimus levels accumulated in the bladder are almost the same (Nirmal et al., 2013; Rajaganapathy et al., 2015).

Another slow-release drug delivery system, the lidocaine-releasing intravesical system (LiRIS), aims to provide continuous release of lidocaine over 2 weeks. A single-arm pilot study reported promising short-term efficacy in pain and urinary symptoms with a 64% success rate (Colemeadow et al., 2020).

Nanoparticles are widely used in the treatment of bladder diseases due to their controllable release kinetics and long-term drug retention advantages. They enable drugs to penetrate the bladder wall without damaging the urothelium structure (Sharif et al., 2019). Regulating mucoadhesion is essential to increase drug penetration. The combination of chitosan and halosite nanotubes has been shown to improve mucoadhesion and control drug release (Barthelmes et al., 2011). Trimethoprim has also been incorporated into chitosan-thioglycolic acid nanoparticles to improve the therapeutic outcome of IC/PBS. The efficacy of trimethoprim-loaded chitosan-thioglycolic acid nanoparticles was found to be 14 times higher. Nanoparticle technology has yielded successful results for improved therapeutic effects in IC/PBS (Sharif et al., 2019).

Silodosin

Stress plays a very important role in IC/PBS. Patients who experienced traumatic events in their early lives exhibit a distinct symptomatic phenotype char-

acterized by intense pain and high urinary frequency (Charrua et al., 2015a; Chiu et al., 2017). Symptoms worsen during stressful periods in IC/PBS patients. Pain triggered or exacerbated by stress is thought to be caused by increased norepinephrine, especially in IC/PBS patients. Studies have shown that stress increases the excitability of bladder nociceptors through the activation of alpha1-adrenergic receptors. Silodosin is a high-affinity alpha1-adrenergic receptor antagonist. It has been reported that a group of patients resistant to oral, systemic, and intravesical treatments respond well to silodosin (Charrua et al., 2015b).

Anti-tumor necrosis factor-alpha (TNF- α) agents

Adalimumab is a biological agent known as an anti-tumor necrosis factor-alpha (TNF- α) agent or TNF- α blocking agent, which is a proinflammatory cytokine. Adalimumab is a recombinant monoclonal antibody that acts by neutralizing human TNF- α . Although a 53% success rate was recorded in a study with Adalimumab, a monoclonal antibody against the proinflammatory cytokine TNF- α , there was no significant difference compared to placebo due to the high placebo response in this study (Bosch, 2014).

Certolizumab pegol is a new anti-TNF- α agent used in the treatment of autoimmune diseases. Certolizumab pegol is thought to have potential advantages over other TNF- α antagonists in the treatment of IC/PBS. In a study with certolizumab pegol, there was a significant reduction in IC/PBS symptoms at week 18 (Di et al., 2021).

Anti-nerve growth factor (NGF) agents

Nerve growth factor (NGF) is one of the key factors contributing to the survival of sensory and sympathetic neurons during growth and is thought to be a peripheral mediator of various inflammatory painful conditions. It has been reported to be elevated in the urine and bladder tissue of IC/PBS patients. NGF increases bladder hyperactivity by sensitizing peripheral and central nerve endings (Hefti et al., 2006; Schnegelsberg et al., 2010). Recently, tanezumab, an anti-NGF agent, exerts its effect by blocking the in-

teraction of NGF receptors with tropomyosin-related kinase A (high-affinity receptor) and p75 (low-affinity receptor). A study reported improvements in pain scores after 6 weeks of treatment (Abdiche et al., 2008; Brown et al., 2012; Evans et al., 2011).

Cannabinoids

Cannabinoids are known to have analgesic and anti-inflammatory properties and have been studied in various chronic pain conditions. In mouse models, activation of the cannabinoid 2 receptor has been shown to reduce the severity of chronic cystitis (Krenn et al., 2003).

Phosphodiesterase-5 inhibitors

Phosphodiesterase-5 (PDE5) inhibitors are thought to inhibit smooth muscle contraction and prevent mast cell degranulation by inhibiting potassium release. In a study of 48 women given low-dose PDE5 inhibitor sildenafil, improvements in urodynamic bladder capacity were observed after 3 months of treatment, and no serious side effects were seen (Chen et al., 2014).

CONCLUSION

IC/PBS is a multifactorial and a complex syndrome, together with pelvic pain, frequent urination, and urgency symptoms, the etiology of which is not yet fully understood, and for which there is no definitive treatment yet. After patients are correctly diagnosed, personalizing treatment with a multifaceted, individualized approach is key to managing the disease effectively. Conservative treatment, including patient education, behavioral modification, dietary advice, stress reduction and, physical therapy, is a fundamental initial management strategy for all patients. If conservative treatment is ineffective, oral treatments such as amitriptyline, pentosan polysulfate sodium, cimetidine and, hydroxyzine are initiated. Among oral treatments, amitriptyline may yield the best response. However, its side effects are not tolerated by the majority of patients. Although there is insufficient evidence for hydroxyzine and cimetidine, they may be included

in treatment due to their low incidence of side effects. The new SH2-containing inositol-5'-phosphatase 1 (SHIP1) activator AQX-1125 initially showed promising results, but trials concluded that it is a safe but ineffective therapeutic agent. Intravesical drug therapy is tried if less invasive treatments fail. Although DMSO shows a high recovery rate, symptoms return within 8 weeks in more than 35% of patients. The combined use of heparin and lidocaine has been reported to yield significantly effective results. Pentosan polysulfate sodium provides greater improvement when administered orally and intravesically at the same time, but it has many side effects. Studies support the combined intravesical administration of chondroitin and hyaluronic acid. Botulinum toxin A is tried if previous treatment steps fail and the patient is willing to risk catheterization, but viral infections observed after this therapy restrict its use. Oral cyclosporine is effective even in patients who have failed multiple oral treatments. However, it generally causes significant side effects, requiring intensive monitoring, and therefore cannot be widely used.

The use of liposomes and lidocaine-releasing intravesical system (LiRIS), newly developed drug delivery systems for IC/PBS, appears promising in early trials. Tacrolimus delivered via liposomes has been shown to have the same effect without systemic side effects. When the newly researched drug, alpha1-adrenoceptor antagonist silodosin, was administered, significant improvement was seen in 65% of patients in the experimental group. When sildenafil, a phosphodiesterase-5 inhibitor, was given to patients, improvements in bladder capacity were seen, and no serious side effects were observed. Adalimumab, one of the monoclonal antibodies, showed no significant difference compared to placebo. In studies with certolizumab pegol, superior benefit was demonstrated compared to placebo. In a study conducted for tanezumab, improvements in pain scores were recorded. The goal of most treatments used in clinics today is unfortunately, to control symptoms only; therefore, further experimental studies are needed to develop new effective drugs.

AUTHOR CONTRIBUTION STATEMENT

Literature research and writing (MD), Reviewing the text (NTDK).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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