FABAD

JOURNAL of PHARMACEUTICAL SCIENCES

ISSN 1300-4182
e-ISSN: 2651-4648
www.fabad.org.tr

Volume: 48 • Issue: 2 • August 2023

An Official Journal of The Society of Pharmaceutical Sciences of Ankara (FABAD)

The Society of Pharmaceutical Sciences of Ankara (FABAD)
FABAD Journal of Pharmaceutical Sciences
Volume: 48 Issue: 2 August 2023

The FABAD Journal of Pharmaceutical Sciences is published three times a year by the
Society of Pharmaceutical Sciences of Ankara (FABAD)

The FABAD Journal of Pharmaceutical Sciences is indexed in Chemical Abstracts,
Analytical Abstracts, International Pharmaceutical Abstracts, Excerpta Medica (EMBASE), Scopus and TR Index
All manuscripts and editorial correspondences should be sent via e-mail to Nesrin GÖKHAN KELEKÇİ
(Editor-in-Chief): fabadankara@gmail.com

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FABAD J. Pharm. Sci., 48, 2, 183-200, 2023
Doi: 10.55262/fabadeczacilik.1177914
RESEARCH ARTICLE

The Effect of *Troxerutin* Intraperitoneal Consumption on the Symptoms of Morphine Withdrawal Syndrome in Male Mice

Nasrin Hosseinzad MANIE[*], Ramin Ghasemi SHAYAN[**o]

The Effect of Troxerutin Intraperitoneal Consumption on the Symptoms of Morphine Withdrawal Syndrome in Male Mice

SUMMARY

*Chronic morphine use is associated with increased oxidative stress and inflammatory factors. Troxerutin is a natural bioflavonoid containing antioxidant effects that could relieve morphine withdrawal syndrome. The aim is to experiment with the effects of troxerutin on morphine dependence in mice. Troxerutin was prepared in three doses (50, 100, and 200 mg/kg) via a normal saline solution. The experiment was performed in five groups of 7 mice. One group received eight days of increasing doses (10, 20, 30, 40, 50, and 60 mg/kg) of morphine subcutaneously with normal saline (10 ml/kg) intraperitoneal, one group received only normal saline, and the other three groups received three different doses of troxerutin solved in normal saline with morphine. On the ninth day, withdrawal symptoms were recorded after the naloxone injection and blood samples were examined. Consequently, the total withdrawal score in 50 mg/kg was p<0.001***, and in the 100 mg/kg Troxerutin-morphine group was p<0.01**, significantly lower than the morphine-saline group. Antioxidant tests showed a significant increase in the level of Total Antioxidant Capacity (TAC) (p<0.001***) and a decrease in the level of Malondialdehyde (MDA) of serum (p<0.001***) in all three doses of troxerutin. In the locomotion test, no significant motility dysfunction or paralysis was observed in mice after using troxerutin. (All P>0.05). Briefly, Troxerutin reduces the symptoms of morphine withdrawal syndrome. The results of antioxidant tests declared that troxerutin possibly due to its antioxidant properties, increases the level of TAC and decreases the level of MDA in the serum of mice.*

Key Words: *Morphine, troxerutin, withdrawal syndrome, dependence, MDA, TAC*

Erkek Farelerde Trokserutinin İntraperitoneal Uygulamasının Morfin Yoksunluk Sendromu Belirtileri Üzerine Etkisi

ÖZ

*Kronik morfin kullanımı artmış oksidatif stres ve inflamatuar faktörler ile ilişkilidir. Trokserutin doğal bir biyoflavonoiddir ve morfin yoksunluk sendromunu hafifletebilecek antioksidan etkilere sahiptir. Amaç, trokserutinin farelerde morfin bağımlılığı üzerindeki etkilerini incelemektir. Trokserutin, normal salin solüsyonu ile üç doz (50, 100 ve 200 mg/kg) halinde hazırlanmıştır. Deney, 7'şer fareden oluşan beş grupta gerçekleştirildi. Bir grup sekiz gün artan dozlarda (10, 20, 30, 40, 50 ve 60 mg/kg) subkütan morfin ve intraperitoneal normal salin (10 ml/kg) aldı. Bir grup sadece normal salin aldı. Diğer üç grup morfin ile birlikte normal salin içinde çözülmüş üç farklı doz trokserutin aldı. Dokuzuncu gün nalokson enjeksiyonu sonrası yoksunluk belirtileri kaydedildi ve kan örnekleri incelendi. Sonuç olarak, toplam yoksunluk skoru 50 mg/kg'da p<0,001*** ve 100 mg/kg Trokserutin-morfin grubunda p<0,01** hesaplandı, morfin-salin grubuna göre anlamlı derecede düşük bulundu. Antioksidan testleri, her üç trokserutin dozunda da Toplam Antioksidan Kapasitesi (TAC) seviyesinde önemli bir artış (p<0.001***) ve serum Malondialdehit (MDA) seviyesinde bir düşüş (p<0.001***) gösterdi. Hareket testinde trokserutin kullanıldıktan sonra farelerde belirgin bir motilite disfonksiyonu veya felç gözlenmedi. (Hepsi P>0.05). Kısaca, trokserutin, morfin yoksunluk sendromu belirtilerini azalttı. Antioksidan testlerinin sonuçları, trokserutinin muhtemelen antioksidan özelliklerinden dolayı fare serumundaki TAC seviyesini artırdığını ve MDA seviyesini düşürdüğünü ortaya koymuştur.*

Anahtar Kelimeler: *Morfin, trokserutin, yoksunluk sendromu, bağımlılık, MDA, TAC*

Received: 21.09.2022
Revised: 17.02.2023
Accepted: 27.02.2023

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INTRODUCTION

Pain is an unpleasant sensation that informs the person about the existence of injuries to body tissues and the occurrence of possible dangers, which is a natural defense mechanism. Pain is, in most cases, self-limiting. But if this condition persists and is not relieved, the pain itself is a disorder (Cappendijk, de Vries, & Dzoljic, 1993; Hosseinzadeh & Jahanian, 2010; Hosseinzadeh & Nourbakhsh, 2003; Vela, Ruiz-Gayo, & Fuentes, 1995).

Opioid drugs like morphine are derivatives of natural, semi-synthetic, and synthetic alkaloids that act on the body's major opioid receptors mu (μ), delta (δ), and kappa (κ). They express their analgesic effect and regulate the sensation of pain by acting on the N-methyl-D-aspartate (NMDA) and substance P receptors to relieve the unpleasant feeling of pain (Maldonado, Negus, & Koob, 1992).

Morphine is an alkaloid obtained from the poppy plant scientifically named *Papaver somniferum* and acts on endogenous opioid receptors (Etemadzadeh, 1993). Morphine is frequently used in surgeries and procedures and also in severe cases of several sicknesses when the patient is not responding to any painkiller. Then, the last phase of soothing pain comes with the use of morphine or other opioid drugs of this family like pethidine or oxycodone to reduce the disagreeable pain feeling. Nevertheless, the use of morphine must not be chronic since it will cause other unrepairable complications.

So far, several mechanisms have been proposed for the development of morphine dependence and the development of withdrawal symptoms (Etemadzadeh, 1993; Maldonado et al., 1992; Maldonado et al., 1997). Among the mechanisms involved in the phenomenon of dependence, an increase in inflammatory cytokines, and an increase in the amount of nitric oxide (NO) in the central amygdala, are important determined factors (Hosseinzadeh & Jahanian, 2010).

Yet, there are multiple probable mechanisms due to the references which are discussed below in the process of morphine dependence leading to withdrawal syndrome. Still, these various mechanisms should be investigated more in detail to recognize the precise operative lane of causing dependence to minimize its issues.

When there is acute use of morphine, the level of Cyclic-adenosine-mono phosphate (cAMP) decreases, while chronic use of morphine would increase the level of cAMP by negative feedback through the activation of the adenylate cyclase enzyme (Masood, Schäfer, Naseem, Weyland, & Meiser, 2020). As a result, electrical discharge of neurons occurs and morphine dependence happens. Moreover, morphine would increase the activity of the glutamatergic system; thus the obstacle of controlling stress and anxiety would disappear and this leads to dependence. Besides that, chronic use of morphine would cause down-regulation of the receptors. Finally, morphine could cause oxidative stress. There are multiple mechanisms for creating oxidative stress. One of them might be the increase of the NO synthase (NOS) hydroxyl synthesis enzyme. As a result, there will be a great climb in the level of NO (a free radical agent) which could cost oxidative stress. Furthermore, chronic morphine use would directly increase the level of superoxide (a free radical agent) in the kidney. Another mechanism would be the increase in lipid peroxidation. Oxidative stress occurs when there is an imbalance between the reactive species and the inhibitory system of these toxins. Free radicals cause neuropathic pain and behavioral disorders. Oxidative stress also disrupts cellular communication which on severe occasions, could cause necrosis.

On the other hand, chronic morphine use has been shown to increase pro-inflammatory mediators such as interleukins and TNF-α. These mediators increase the activity of cyclooxygenases, which is one of the possibilities of morphine dependence (Yamaguchi et al., 2001; Quimby & Luong, 2007).

Anyhow, *troxerutin* (tri-hydroxyl-ethyl rutin) is a natural plant bioflavonoid that prevents damage to the tissues (Maurya, Salvi, & Krishnan Nair, 2004; Sui, Zang, & Bai, 2019). *Troxerutin* inhibits the painful oxidative pathways and inflammatory cytokines and plays an important role in relieving the symptoms of morphine-induced withdrawal syndrome (Zhang et al., 2009). *Troxerutin* is originally obtained from the herbaceous plant *Sophora japonica* of the *Fabaceae* family, which is native to China and Japan and is widely distributed in Asia, Oceania, and the Pacific Islands (Adam, Pentz, Siegers, Strubelt, & Tegtmeier, 2005).

Antioxidant, anti-tumor, sedative and soothing, antimicrobial, analgesic, and anti-inflammatory properties have been reported in it (Maurya et al., 2004; Adam et al., 2005; Zhang et al., 2009; Farajdokht et al., 2017). *Troxerutin* has been used in studies to treat chronic venous insufficiency by improving capillary function, which prevents abnormal capillary leakage and capillary vascular insufficiency (Turton, Kent, & Kester, 1998; Adam et al., 2005; Zhang et al., 2009; Farajdokht et al., 2017). It is also very effective in relieving the nervous system of mice by relieving oxidative stress caused by D-galactose and improving the activity of Phosphoinositide 3-kinase / Akt (Turton et al., 1998; Maurya et al., 2004; Adam et al., 2005; Farajdokht et al., 2017).

Therefore, considering all these influences of *troxerutin*, in the present study, the effect of chronic *troxerutin* use on morphine dependence in male mice was investigated. The main option of *troxerutin* in this thesis is considered its antioxidant properties though it could regulate the oxidative stress system and maintain health, which might include dependence.

Troxerutin in the following experiment is not extracted from the plants; instead, it is in a ready and commercial capsule form. Subsequently, the changes in the levels of total antioxidant capacity (TAC) and malondialdehyde (MDA) in the serum of male mice after morphine administration have been experimented with. According to the design hypotheses, chronic use of *troxerutin* with morphine was expected to reduce the symptoms of morphine withdrawal syndrome. Furthermore, it was expected to increase the level of TAC and decrease the level of MDA in the serum of animals.

MATERIAL AND METHOD

In this design, the mice were kept in groups of 7 in transparent polypropylene cages with steel rods. First of all, disposable latex gloves and masks had to be used. To induce dependence syndrome, 10 mg/ml of morphine sulfate ampoule (Iran Pharmaceutical Company) was used based on the weight of each mouse. Naloxone ampoules (0.4 mg/ml) were injected into mice based on their weight before calculating withdrawal symptoms. For a 25 g mouse, we needed 0.125 mg of naloxone. As naloxone ampules were 0.4 mg/ml, 0.125 mg of naloxone gave us 0.3125 ml which had to be injected into a 25 g mouse. It could be arranged for each mouse owning to its precise weight. (Meybodi, Zarch, Zarrindast, & Djahanguiri, 2005; Wiebelhaus, Walentiny, & Beardsley, 2016; Lewter et al., 2022). *Troxerutin* 300 mg capsules commercially produced by Actavis Company were used. To dissolve the powder inside the capsule, as a solvent and the carrier of *troxerutin*, a 0.9% sodium chloride solution for injection was used. Then the capsule (300 mg) of *troxerutin* was opened and the powder inside was weighed accurately with electric scales (Guo, Wang, Bi, & Sun, 2005). Of course, the entire powder included not only *troxerutin* but also the other ingredients (filler, lubricant, binder, disintegrants, and…) of the capsule. However, to reach the dosages (50, 100, and 200 mg/kg) of *troxerutin*, we had to use proportionality ratios to calculate how much we have to extract from that amount of powder dissolved in saline. So it was estimated based on the weight of each mouse (Guo et al., 2005). So the entire powder was dissolved in normal saline and for each dose of *troxerutin* for each mouse, it was calculated how much of that mixture (powder-saline) should be extracted. Meanwhile,

for the morphine amount calculation, the ampules of morphine were 10 mg/ml. The adjusted increasing doses of morphine (20, 30, 40, 50, and 60) were calculated as 20 mg of morphine in 1000 gr would bring 0.6 mg of morphine for a 30 g mouse. Subsequently, 10 mg of morphine in 1 ml would cause 0.06 ml of morphine. Other dosages could be estimated based on the weight of each mouse (Alaei, Esmaeili, Nasimi, & Pourshanazari, 2005; Hassan et al., 2020). Electric scales were used to weigh the mice every day before the procedures since most of the animals had lost weight by taking chronic morphine. We used a stopwatch to increase the accuracy of the process of making signs and counting the animal's movements throughout the test. Also, after each injection, the contents of the morphine ampoule and the dissolved *troxerutin* powder were stored in the refrigerator. An insulin syringe was used to inject morphine and *troxerutin* into the animals and a 5-cc syringe was used to draw the contents of the morphine ampoule and normal saline solution. A cylindrical and glass enclosure was used to place the animals and prevent them from escaping to count and carefully examine the symptoms. Then blood samples were taken from the apex of the hearts of the animals and poured into bracket tubes using 2-cc syringes, respectively. The serum and blood cells were separated by centrifugation and the serums were stored in a freezer at -70 ° C for 24-48 hours and finally tested in a special laboratory with special kits and the results of TAC and MDA were reported (Oskuye et al., 2019; Ahmadi, Mohammadinejad, Roomiani, Afshar, & Ashrafizadeh, 2021).

Grouping

In this study, 5 groups of 7 male adult albino mice weighing 25-30 grams mice were used according to the mentioned conditions:

First Group (the Control Group (Morphine + Saline))

In a group of 7 mice, 10 ml/kg saline was first injected IP every 12 hours for 8 days. Morphine was injected SC every 12 hours for half an hour after saline injection every day. On the first day (20 mg/kg), second and third day (30 mg/kg), fourth and fifth day (40 mg/kg), sixth and seventh day (50 mg/kg), and on the eighth and ninth day (60 mg/kg) of morphine injection was performed. On the ninth day, morphine was injected only in the morning. On the ninth day, 1 hour after the morning dose of morphine, mice had been injected with a dose of naloxone (5 mg/kg) IP and showed signs of withdrawal syndrome (number of jumping, standing on feet, body grooming, genital grooming, teeth chattering, wet-dog-like shakes and abdomen writhing), which are essential parameters for assessing dependence. For 30 minutes, the number of symptoms was counted and recorded separately via filming for accuracy. The results of this group were considered as the basic responses of the animals and their comparison with other groups.

Second Group (The Control Group (Saline + Saline)

In a group of 7 male mice to evaluate the appropriateness of the dependence method, just like the previous group, but instead of morphine, normal saline was injected incrementally every 12 hours for 9 days at a dose of 10 ml/kg SC. Normal saline 10 ml/kg was also injected IP every 12 hours. On the ninth day, 1 hour after the morning dose of saline, a dose of naloxone (5 mg/kg) was injected IP, and withdrawal symptoms were recorded for 30 minutes. The results of this group indicate the behavior of the animal in this study. It should be noted that saline is considered the carrier of troxerutin.

Third, Fourth, and Fifth Groups (Groups Receiving Three Different Doses of Troxerutin with Morphine (Troxerutin Doses: 50, 100, and 200 mg/kg)

The respective groups consisted of three groups of 7 male mice that received increasing doses of morphine every 12 hours for 9 days. Troxerutin commercial capsules (Actavis Company) were opened and the powder was weighed and dissolved in the carrier of troxerutin (normal saline which was completely solu-

ble) (Elangovan & Pari, 2013; Kaeidi et al., 2020). The mice had only morphine and naloxone injections on the ninth day, and subsequently, withdrawal symptoms were recorded for half an hour.

Investigation of the Effect of *Troxerutin* on Animal Motility

A locomotion test was performed in morphine + saline groups and the groups received three doses of *troxerutin* with morphine. On the ninth day, before the morphine injection, a locomotor activity test was performed by the open-field method. A box measuring 40 × 40 cm was designed, which was divided into 25 smaller squares of the same size of 8 × 8 cm with a marker. To determine whether *troxerutin* would cause paralysis or other movement dysfunctions and abnormalities, the mice were consecutively placed in the middle of the screen of the box, and immediately after the stopwatch was activated, by checking the time, it was determined that the mice would cut the lines several times in 20 minutes. Each time the animal was crossing the lines, it was a point. At the end of the experiment, the total times it had cut the lines was estimated.

Registration of Withdrawal Symptoms

To evaluate the withdrawal symptoms after naloxone administration, the animals were placed individually under a clear glass cylindrical chamber, and withdrawal symptoms included: number of jumping, standing on feet, genital grooming, abdomen writhing, body grooming, teeth chattering, and wet dog shakes were recorded and evaluated by the camera for 30 minutes. Naloxone had to be injected at 5 mg/kg.

Calculate the Total Score of Withdrawal Syndrome (TWS)

The total score of withdrawal syndrome symptoms was calculated to summarize the symptoms and obtain an index for the set of recorded symptoms and determine the severity of withdrawal syndrome based on previous studies and a modified system of other researchers. For each index, it was divided by the standard value (Table 1).

These numbers were then summed for each mouse and averaged for each group. The sum of these symptoms was reported as the total score of withdrawal symptoms. To eliminate the differences in the animals' responses to the different withdrawal symptoms, using this relationship helps us to have a general indicator to show the effect of the drug on the onset and severity of the symptoms.(Gordon, 2004; Habibi Asl, Ahmadi, Hasanzadeh, & Charkhpour, 2007; Lammers, Kruk, Meelis, & Van der Poel, 1988; Parvizpour et al., 2013)

Table 1. Table of values given to different symptoms of Withdrawal Syndrome

Behavior (Symptom of morphine withdrawal syndrome)	**Weight factor (Value given to the desired mark)**
Jumping	4
Wet dog shake	5
Abdomen writhing	5
Head shake	5
Handshake	5
Genital rooming	5
Body grooming	10
Facial grooming	10
Teeth chattering	10
Swallowing	10
Standing on feet	20

Blood Sampling Method

At the end of the morphine and troxerutin injection courses in all groups of mice, each mouse was anesthetized with ketamine-midazolam; ketamine at a dose of 100 mg/kg and midazolam at a dose of 5 mg/kg were used. Then 1 ml of blood was taken from their heart apex. The sample was stored in a test tube without the presence of an anticoagulant at room temperature for 20 minutes until blood clotted. The clotted sample was then centrifuged at 3000 rpm for 7 minutes and after separating the blood serum, it was poured into a 1 ml microtube and stored in a -70 ° C freezer for the above tests.

Malondialdehyde (MDA)

The basis of serum MDA measurement method is based on a reaction with thiobarbituric acid (TBA), extraction with normal butane, absorption measurement by spectrophotometric method, and comparison of absorption with a standard curve.

Preparation of Solutions

1% orthophosphoric acid: This solution was prepared in a 250 ml balloon by dissolving 85% phosphoric acid and bringing it to volume using deionized water. Thiobarbituric acid 0.67%: 1.675 g of thiobarbituric acid ($C_2H_4N_2O_2S$) was collected in a 250 ml balloon with deionized water and used freshly prepared.

Test Method

500 µl of serum was dissolved in 3 ml of 1% phosphoric acid. After vortexing, 1 ml of 0.675% thiobarbituric acid was added to the test tube and after complete vortex, placed inside a boiling marijuana pan for 45 minutes. After that, the tubes were cooled under cold water and were added 3 ml of normal butanol was, and vortexed for 1 to 2 minutes. Then for 10 minutes, at 3000 rpm (round-per-minute), they were centrifuged and after separating the organic phase (supernatant), light absorption was measured at 532 nm against normal butane blank and the results were determined after transferring to the standard curve and were reported as serum MDA concentration of the samples.

Total Antioxidant Capacity (TAC)

It is a way to measure the antioxidant capacity of all the antioxidants in a biological sample.

Concentration of Reagents

The concentrations of reagents used in the present study are listed below (Table 2):

Table 2. The concentration of reagents in the TAC test

80mmol/l	Phosphate Buffered Saline pH7.4	Buffer
6.1μmol/l	Metmyoglobin	Chromogen
6.1μmol/l	2,2'-and-bis (3-ethyl benzothiazoline-6-sulphonic acid)	
250 μmol/l	Hydrogen Peroxide (in Stabilised form)	Substrate
Lot specific	6-Hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid	Standard

Solution Preparation

Chromogen: 1 vial of chromogen was mixed with 10 ml of buffer.

Substrate: 1 ml of Substrate was diluted with 1.5 ml of buffer.

Standard: 1 vial of the standard was diluted with 1 ml of deionized distilled water.

Test Method

First, MDA was measured by spectrophotometer using thiobarbituric acid (TBA) method and then the TAC test was performed. This method was performed by antioxidant capacity measuring kits, which is a simple, repeatable, and standard method. These kits work by the colorimetric method and are based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidant compounds of the sample, which are paired with suitable thermogenesis and produced a colored product.

In the cuvette sample: 20 µl of the sample was mixed with 1 ml of chromogen.

In the cuvette Blank: 20 µl of DDH2O was mixed

with 1 ml of chromogen.

In the standard cuvette: 20 µl of the standard was mixed with 1 ml of chromogen.

At a wavelength of 600 nm in the Alcyon 300 at 37 ° C and in front of the air, the initial light absorption of the cuvettes was measured (A1).

Then 200 µl of substrate was added to each cuvette and after three minutes the light was absorbed again. (A2)

Calculations: (formula1)

A2 - A1 = ΔA of sample/standard/blank

Factor = $conc\ of\ standard/\ (\Delta A\ blank-\ \Delta A\ standard)$

Mmol/l = factor × $(\Delta A\ Blank\ -\ \Delta A\ Sample)$

Statistical Analysis

Statistical analysis using Sigma plot version 12.2 software was done. The results of recording analgesic effects were expressed as (Mean ± S.E.M). One Way ANOVA test was applied to emphasize just one factor (*troxerutin* effects) and the Tukey post-test was used to compare the results of more than two groups more accurately in male mice. The minimum difference between groups with p <0.05 was considered statistically significant. P <0.05 was considered the level of statistical significance. So that p <0.05 *, p<0.01 ** and p<0.001 *** were reported.

Ethical Approval Information

Under the approval of the Faculty of Pharmacy Thesis Research Council of the Iran Pharmacist Association and the Regional Ethics Committee of Pazhoohan in the Tabriz University of Medical Science, this thesis has been approved on the date of 2020.08.31 with the ethical code of (IR. TBZMED. VCR.REC.1399.167) and with the tracking code of 65384.

RESULTS AND DISCUSSION

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Jumping) in Male Mice

According to the results of this study, intraperitoneal injection of *troxerutin* significantly increased the number of jumping following naloxone administration in the group receiving 200 mg/kg *troxerutin* with morphine, with p <0.01 ** compared with the morphine-saline control group. (Figure 1)

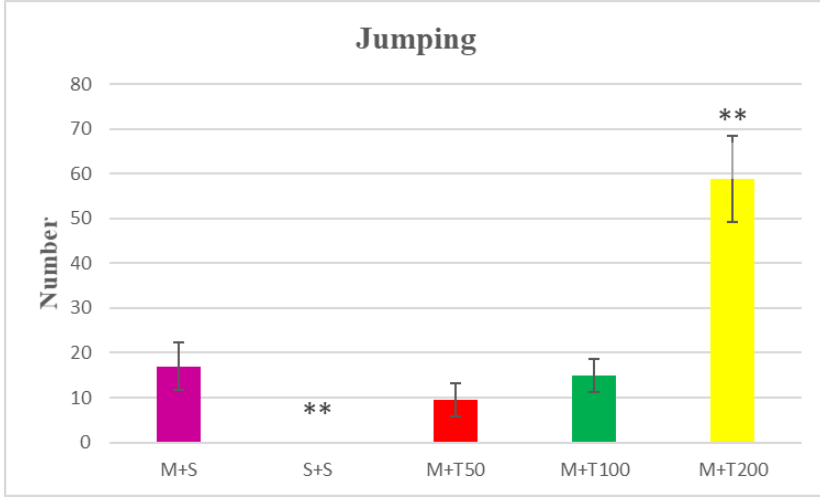

Figure 1. The effect of different doses of *troxerutin* on the number of jumping movements in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.01**) compared to the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Times Standing on Feet) in Male Mice

Based on the results of this study and statistical comparison, because the responses obtained from the groups were abnormal, the data obtained from this symptom were not used to calculate the total withdrawal score.

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Genital Grooming) in Male Mice

According to the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline in significantly reduced the number of genital grooming. (Figure 2)

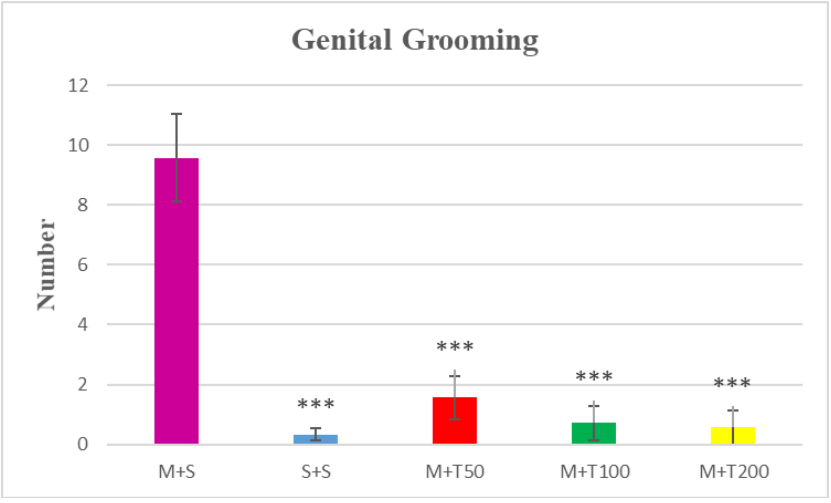

Figure 2. The effect of different doses of *troxerutin* on the number of genital grooming movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Body Grooming) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving 50 mg/kg and 100 mg/kg of *troxerutin* with morphine, with p <0.001 ***, and in the group receiving 200 mg/kg of *troxerutin* with morphine with p <0.01 ** compared to the control group, morphine-saline recipients significantly reduced the number of body grooming (Figure 3).

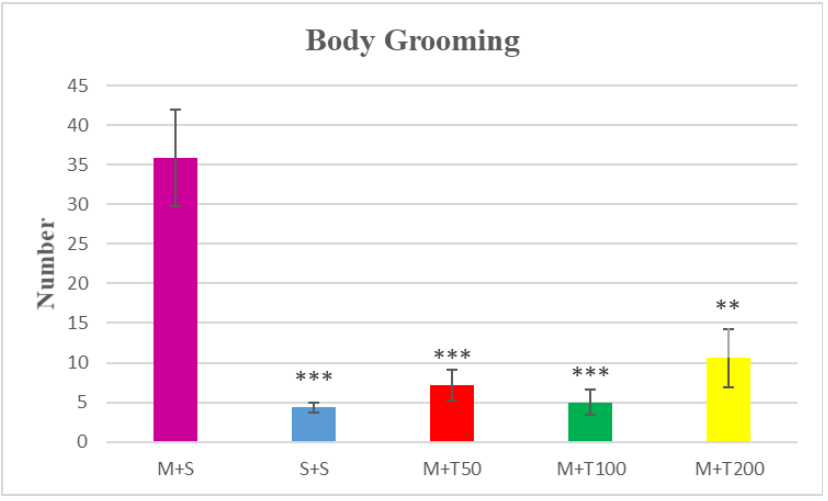

Figure 3. The effect of different doses of *troxerutin* on the number of body grooming movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as ($p < 0.001^{***}$) and ($p < 0.01^{**}$) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Teeth Chattering) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving 50 mg/kg and 200 mg/kg *troxerutin* with morphine, with $p < 0.001$ *** compared with the control group receiving morphine-saline significantly reduced the number of teeth chattering. Also, no significant difference ($p < 0.05$) was observed in the group receiving 100 mg/kg *troxerutin (Figure 4).*

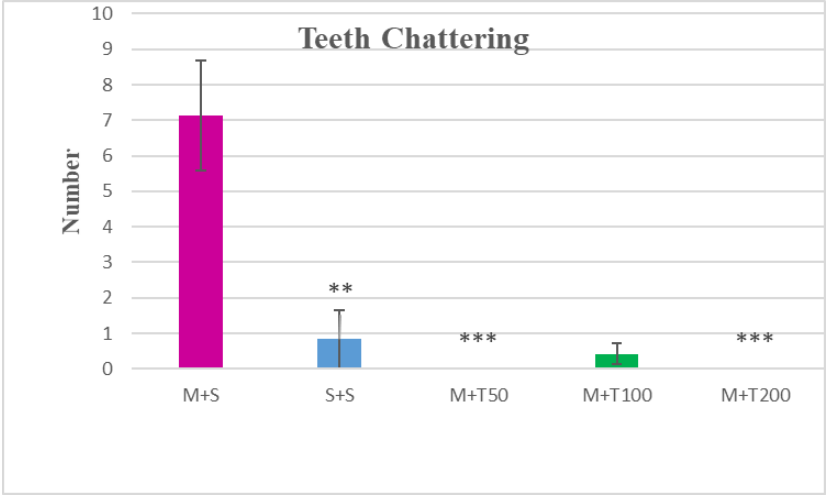

Figure 4. The effect of different doses of *troxerutin* on the number of teeth chattering movements in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as ($p < 0.001^{***}$) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Wet Dog Shakes) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline significantly reduced the number wet dog shake movements (Figure 5).

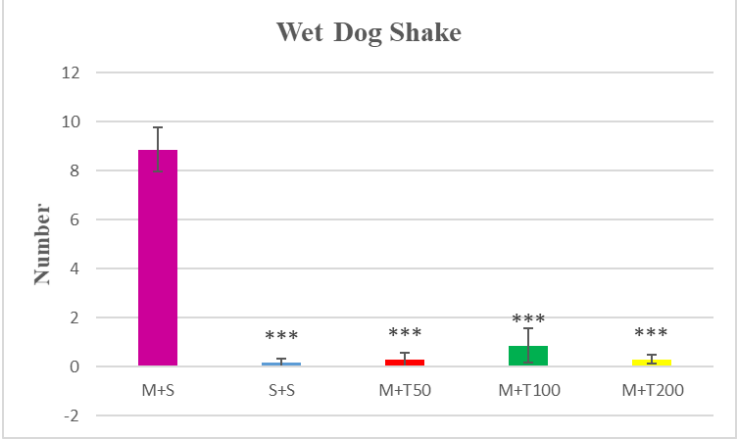

Figure 5. The effect of different doses of *troxerutin* on the number of wet dog shake movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Abdominal Writhing on the Ground) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the 50 and 200 mg/kg *troxerutin* dose groups with morphine, with p <0.01 **, significantly decreased the number of abdominal writhing compared to the morphine-saline control group. Also, no significant difference (P <0.05) was observed in the group receiving a 100 mg/kg dose (Figure 6).

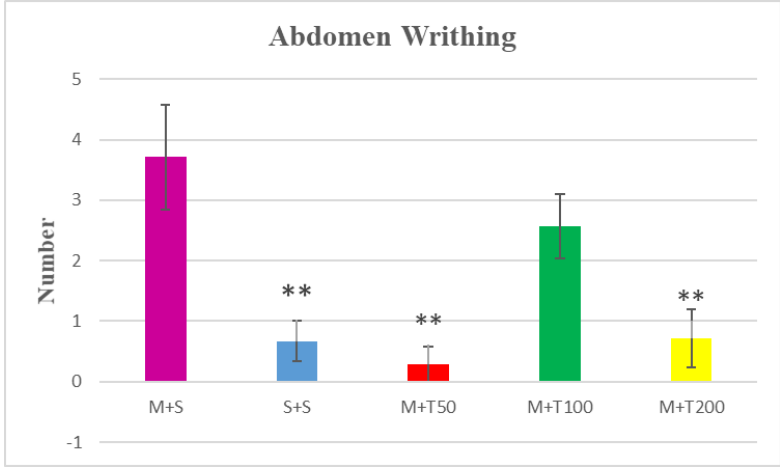

Figure 6. The effect of different doses of *troxerutin* on the number of abdominal writhing movements on the ground in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.01**) compared to the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on the Total Symptoms of a Withdrawal Syndrome in Male Mice Based on Total Withdrawal Score (TWS)

Based on this behavioral study, intraperitoneal administration of *troxerutin* with morphine at a dose of 50 mg/kg with *troxerutin* with morphine at p <0.001 *** and at a dose of 100 mg/kg with morphine at p <0.01 ** compared with the morphine-saline control group, it significantly reduced the symptoms of withdrawal syndrome (Figure 7).

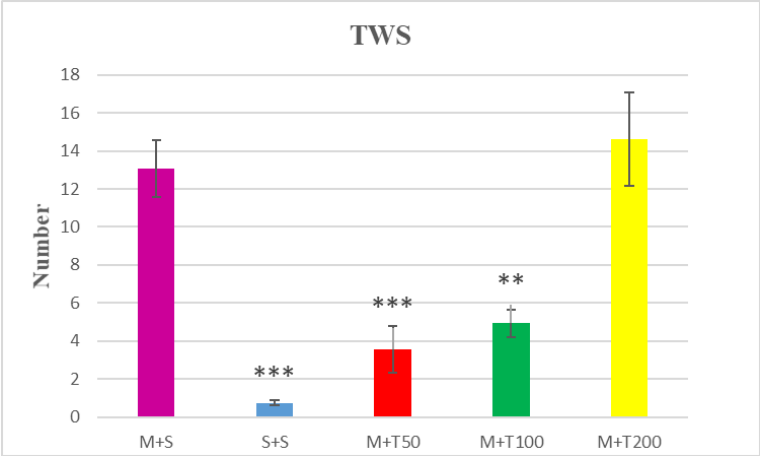

Figure 7. The effect of different doses of *troxerutin* on total withdrawal score in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) and (p <0.01**) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Serum TAC Levels in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline significantly increased the TAC levels of serum (Figure 8).

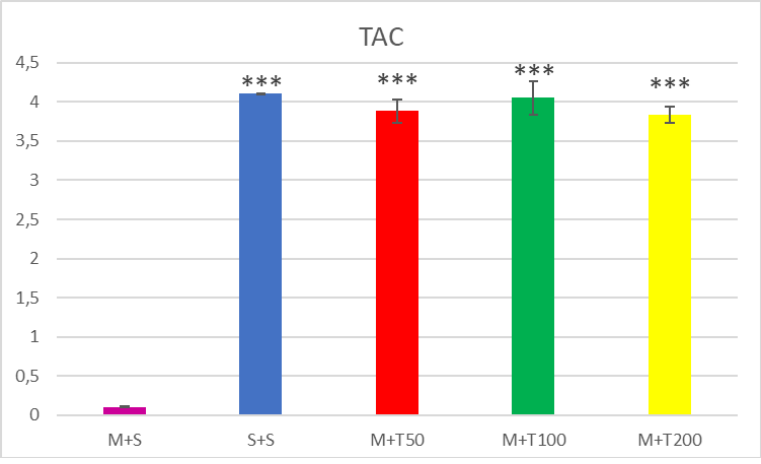

Figure 8. The effect of different doses of *troxerutin* on serum TAC levels in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M= Morphine, S= Saline, T= Troxerutin

The Effect of Different Doses of *Troxerutin* on Serum MDA Levels in Male Mice

Based on this behavioral study, intraperitoneal administration of *troxerutin* with morphine at a dose of 50, 100, and 200 mg/kg with *troxerutin* with morphine at p <0.001 *** compared with the morphine-saline control group, significantly decreased the MDA levels of serum (Figure 9).

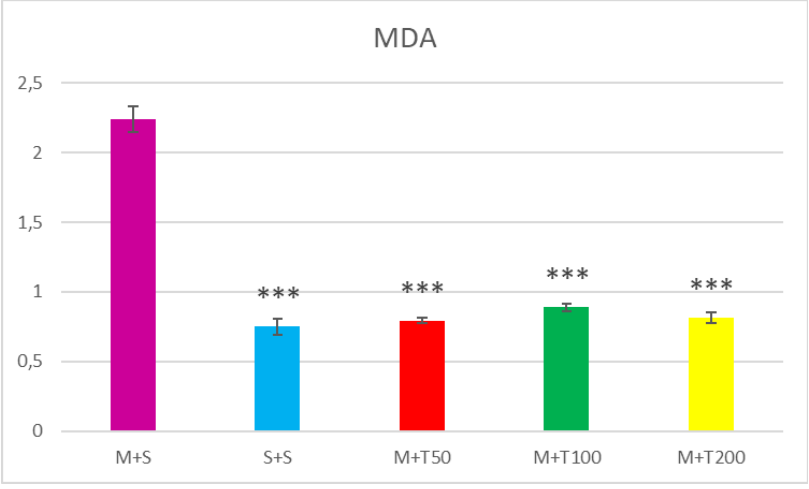

Figure 9. The effect of different doses of *troxerutin* on serum MDA levels in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M= Morphine, S= Saline, T= Troxerutin

The Effect of *Troxerutin* on Locomotor Factors in Locomotor Activity Testing

The results of the locomotion test show that there is no statistically significant difference between the groups receiving morphine with doses of 50, 100, and 200 mg/kg *troxerutin* with the control group receiving morphine-saline in estimating the number of entering the squares in mice. Therefore, it can be said that the effect of *troxerutin* on relieving the symptoms of withdrawal syndrome has nothing to do with its effect on the points related to the motion activities of the animals through the brain (Figure 10).

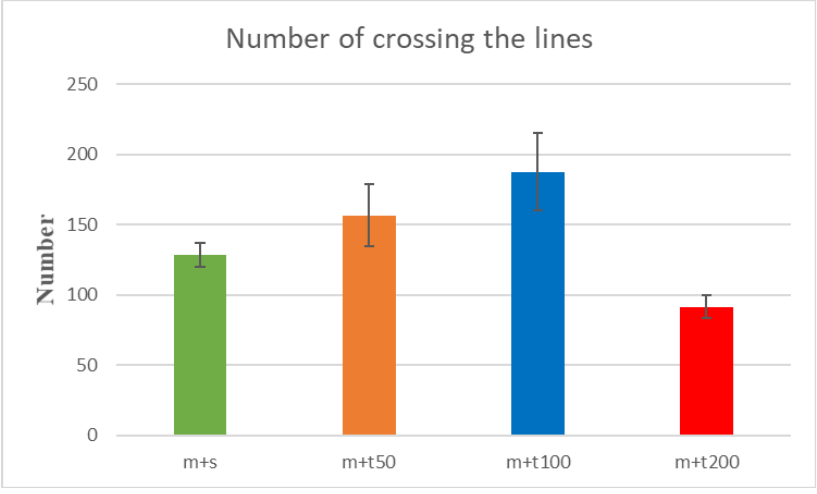

Figure 10. The effect of *troxerutin* on locomotor activity test locomotor factors in male mice compared to the morphine + saline control group. The results are expressed as mean ± SEM (n = 7).

M = Morphine, S=Saline, T=Troxerutin

The results of the present study showed that the 9-day administration of increasing doses of morphine causes dependence and subsequent administration of naloxone (5 mg/kg), causes withdrawal symptoms in mice. Also, intraperitoneal administration of *troxerutin* with morphine significantly reduces the overall symptoms of morphine withdrawal syndrome. The justification for *troxerutin* dosage selection was since dosages of 100, 200, and 300 mg/kg were practically tested previously to reach an efficient amount of the medication, so we declined the dosage for one step to do an experiment and probably reduce possible side effects of the drug to observe whether *troxerutin* would cause the same medicinal effect (Zamanian, Hajizadeh, Esmaeili Nadimi, Shamsizadeh, & Allahtavakoli, 2017;Raja, Saranya, & Prabhu, 2019; Gao et al., 2020).

Nonetheless, morphine is one of the oldest opioid drugs commonly used to relieve moderate to severe pain worldwide. It is widely used after surgery and in cancer patients. Due to its frequent consumption, side effects such as dizziness, nausea, constipation, and also physical dependence are seen in consumers (Hutchinson et al., 2008). Chronic use of morphine can lead to dependence and addiction, but the mechanisms involved are not fully understood. To understand the accurate mechanisms, it is necessary to first review the mechanisms of dependence on morphine. One of these biochemical mechanisms is the role of NMDA excitatory amino acid receptors. Glutamate is the messenger of primary stimulation in most central nervous system receptors. Almost all nerve cells are activated and depolarized with glutamate and its receptors (Wang & Wang, 2006).

In several studies, the use of NMDA receptor antagonists has been shown to prevent morphine tolerance. Another effect of stimulation of NMDA receptors by excitatory amino acids is to increase the production of NO. Some articles have also shown the involvement of the nitric oxide synthase pathway as an inhibitor of tolerance and dependence (Salehpour, Habibi Asl, Charkhpur, & Mahmoudi, 1398). In long-term use of opioids, it is seen that due to nervous adaptations, a series of behaviors such as craving, restlessness, insomnia, and high blood pressure are observed in person, and over time these behaviors become part of the mood. The use of NMDA receptor antagonists has significantly reduced many of these behaviors which are caused by addictive substances such as opioid compounds (Asl, Hassanzadeh, Khezri, & Mohammadi, 2008).

In the present study, the use of three doses of *troxerutin* (50, 100, and 200 mg/kg), reduced the withdrawal symptoms consisting of body grooming, genital grooming, and wet dog shake movements. Also, a decrease in the number of jumping movements was observed at 50 and 100 mg/kg doses of *troxerutin* compared to the control group. Additionally, a decrease in the number of teeth chattering and the abdomen writhing at a dose of 50 and 200 mg/kg *troxerutin* was observed compared to the control group. According to the results of this study, between different doses of *troxerutin* (50, 100, and 200 mg/kg) with morphine, *troxerutin* 50 mg/kg with $p < 0.001$ ***, and *troxerutin* 100 mg/kg with $p < 0.01$ **, were efficient to significantly reduce the symptoms compared to the control group. Anyhow, *troxerutin* in 200 mg/kg seemed to increase the jumping symptoms in animals significantly showing an enormous gap. The possible explanation could be illustrated this way by increasing the dose of *troxerutin*, over-stimulation of receptors in the neurologic system could have occurred; precisely whether the dosage of drugs increases illogically, it could cause toxicity. The same may have happened in this experiment, either. Perhaps the involvement of the limbic system could be another factor of occurring a serious difference (Jun Lu et al., 2012; Sharma, 2014). Nevertheless, further investigations are required.

To sum up, *troxerutin* at 50 and 100 mg/kg doses were more effective in reducing morphine dependence. Also, chronic usage of *troxerutin* significantly

increased the level of TAC and decreased the level of MDA in the serum of the mice with $p < 0.001^{***}$. Even though the dose of 200 mg/kg was effective in some symptoms, the total score was estimated and the gap in jumping symptoms prevented the efficacy of 200 mg/kg so far. Briefly, *troxerutin* has many biological properties. It can significantly reduce damage to various tissues such as the brain, liver, and kidney, and is a potential candidate for the prevention and treatment of cerebral palsy (Bruppacher, Rieckemann, Naser-Hijazi, & Wüstenberg, 1998; Zhang et al., 2015).

In the present study, the results show that *troxerutin* is a free radical scavenger, affecting the enzymes which are determined through the oxidative stress pathways, or maybe the reduction of lipid and lipoprotein peroxidation; and also involving the receptors directly by their antioxidant effects. In conclusion, it could reduce the symptoms of morphine withdrawal syndrome in mice. In recent years, *troxerutin*, especially in combination with *coumarin*, has been used in the treatment of chronic venous diseases and varicose veins (Babri et al., 2014; Farajdokht et al., 2017; Gohel & Davies, 2009; Panat, Maurya, Ghaskadbi, & Sandur, 2016). Researches show that even with a single dose of morphine, the production of Reactive Oxygen Species (ROS) increases and the level of regenerative glutathione (GSH) in brain cells decreases (Ma et al., 2019). Increased levels of glutathione-sulfide (GSSG), decreased levels of intracellular GSH, the deduction in the activity of enzymes like catalase (CAT), Superoxide Dismutase (SOD), and glutathione peroxidase enzyme (GPx) as well as overproduction of cerebral glutamate, has been reported consequently (Jun Lu et al., 2010; Zamanian et al., 2021). By increasing the activity of antioxidant enzymes containing catalase, superoxide dismutase, and glutathione peroxidase, repairing GSH deficiency, and reducing MDA and ROS levels, it can reduce morphine dependence signs. Studies have also shown that *troxerutin* not only can reduce ROS and MDA levels but also can increase the activity of GPx. By inhibiting the activation of the inflammatory pathway like c-Jun N-Terminal Protein Kinase 1/Nuclear factor- κB Kinase/Nuclear factor- κB in the hippocampus, *troxerutin* reduces the inflammatory factors of α-TNF, Interleukin (IL)-1β, and IL-6. (Saranya et al., 2020) Furthermore, it reduces the activity of NOS and the production of NO; which could be the reason why there is a noticeable increase in the level of TAC and a decrease in the level of oxidative stress-related factors like MDA inside the serum of tested animals (J. Lu et al., 2013; Medeiros et al., 2007; Najafi, Noroozi, Javadi, & Badalzadeh, 2018).

Briefly, according to the results of this study, it can be said that chronic administration of *troxerutin* in some doses can reduce the symptoms of morphine dependence in mice. Symptoms of morphine withdrawal syndrome were significantly reduced when *troxerutin* was administered at a dose of 50 mg/kg ($p < 0.001^{***}$) and 100 mg/kg ($p < 0.01^{**}$). Likewise, the level of TAC in the serum of the mice was significantly increased and the level of MDA was significantly decreased at all doses (50, 100, and 200 mg/kg) with ($p < 0.001^{***}$). Yet, the particular mechanism of action requires extra study, but it can be said that *troxerutin*, possibly by performing its effect on the immune system and inhibiting the production of inflammatory cytokines, inhibits inflammation of microglia, and also with its antioxidant effects, it inhibits oxidative stress and causes the decline of morphine dependence symptoms. Due to the increasing awareness of medicinal plants, *troxerutin* can be possibly considered a new compound to reduce the symptoms of morphine withdrawal syndrome for reducing the incidence of opioid addiction in communities. Anyhow, it might be used after supplementary studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Author contribution: Nasrin Hosseinzad Manie proposed the main idea and gathered data and Ramin Ghasemi Shayan critically revised the article.

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FABAD J. Pharm. Sci., 48, 2, 201-218, 2023
Doi: 10.55262/fabadeczacilik.1201555
RESEARCH ARTICLE

An Ethnomedicine Study of Traditional Healers as Joint Pain Therapy in Bantul District, Yogyakarta

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SUMMARY

Joint pain is a sign of disorders of the musculoskeletal system. In joint pain, there is usually discomfort, swelling, inflammation, and stiffness that cause movement restrictions. Bantul is the district with the lowest prevalence of joint disease in Yogyakarta, Indonesia. It related to the community culture in using traditional herbs to treat the disease. This study aims to determine the types of plants, the process of making traditional herbal medicine, and ways to use herbs to treat joint pain complaints. Traditional knowledge about using local plants was collected through field surveys. The 48 healers from 3 hamlets in Bantul District were found by snowball sampling technique and 47 traditional herbal medicines were recorded as being applied for joint pain treatment. Among traditional herbal medicines are identified that they use 33 plants belonging to 18 families, and the most widely used family is Zingiberaceae. Raw materials were detected to process by washing, boiling, pounding, grinding, pulverizing, and squeezing and used orally and topically (compress). This study showed that most of people in the Bantul District still depend on medicinal plants to treat diseases. Nevertheless, there are only a few traditional healers. There is a great danger that traditional knowledge will soon be lost because the young generation is not concerned about continuing this custom.

Key Words: *Traditional herbs, joint pain, Kiringan, Mangunan, Kunden*

Yogyakarta, Bantul Bölgesinde Eklem Ağrısı Tedavisi Olarak Geleneksel Şifacıların Etnomedikal Bir Çalışması

ÖZ

Eklem ağrısı, kas-iskelet sistemi bozukluklarının bir işaretidir. Eklem ağrılarında genellikle hareket kısıtlılığına neden olan rahatsızlık, şişlik, iltihaplanma ve sertlik vardır. Bantul, Yogyakarta'da eklem hastalığı prevalansının en düşük olduğu bölgedir. Eklem hastalığının düşük prevalansı, hastalığı tedavi etmek için geleneksel bitkileri kullanan toplumun kültürü ile ilgili olabilir. Bu çalışmanın amacı, eklem ağrısı şikayetlerini tedavi etmek için geleneksel bitkisel ilaç yapımında kullanılan bitki türlerini, kullanılan bitkilerin nasıl işlendiğini ve eklem ağrılarını tedavi etmek için şifalı bitkilerin nasıl kullanıldığını belirlemektir. Kullanılan yerel bitkiler hakkında geleneksel bilgiler saha araştırmaları ile toplanmıştır. Yogyakarta, Bantul Mahallesi'ndeki 3 mezradan kartopu örnekleme tekniği ile 48 şifacı bulunmuştur. Eklem ağrısı tedavisi için uygulanan 47 adet geleneksel bitkisel ilaç kaydedilmiştir. Geleneksel bitkisel ilaçlar arasında tespit edilenler 18 familyaya ait 33 bitki kullanılmaktadır ve en çok kullanılan familya Zingiberaceae'dir. Ham materyallerin yıkanarak, kaynatılarak, dövülerek, öğütülerek, toz haline getirilerek ve sıkılarak işlendiği ve dahilen ve haricen (kompres) kullanıldığı tespit edilmiştir. Bu çalışma, Bantul Mahallesi'ndeki insanların çoğunun hastalıkları tedavi etmek için hala şifalı bitkilere bağımlı olduğunu göstermektedir. Bununla birlikte, sadece birkaç geleneksel şifacı kalmıştır. Genç nesil bu geleneği sürdürmekle ilgilenmediği için geleneksel bilginin yakında kaybolma tehlikesi söz konusudur.

Anahtar Kelimeler: *Geleneksel bitkiler, eklem ağrısı, Kiringan, Mangunan, Kunden*

Received: 09.11.2022
Revised: 25.03.2023
Accepted: 29.03.2023

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INTRODUCTION

Joint pain is a sign of disorders of the musculoskeletal system. Joint pain is caused by immune mechanisms, metabolic factors, genetics, environment, and age (Sunaringtyas et al. 2019). In joint pain, there will usually be discomfort, swelling, inflammation, and stiffness that can cause movement restrictions. Musculoskeletal diseases that can cause joint pain include osteoarthritis, gouty arthritis, rheumatoid arthritis, and infectious arthritis (Noviyanti and Azwar, 2021). Joint pain management involves pharmacological and non-pharmacological therapies (Sunaringtyas et al. 2019). Pharmacological therapy is carried out to reduce pain by administering non-steroidal anti-inflammatory drugs (NSAIDs). Besides that, bone surgery therapy (joint replacement) is a therapy that is widely used as a treatment option (Rachmawati et al. 2018). One of the non-pharmacological therapies can be done with warm compresses. With the application of heat, the blood vessels will widen to improve blood circulation in the tissue, and the process of distributing food to cells will increase. Increased cell activity will reduce pain/pain and will support the wound-healing process and the inflammatory process (Hannan et al. 2019).

The low prevalence of joint disease can be influenced by the distinctive culture that is owned and still carried out by the people of the Special Region of Yogyakarta, who are full of noble values including the culture of using traditional herbs to prevent and treat diseases (Yogyakarta Health Office, 2021). In Indonesia, using of traditional herbs has become a culture and tradition since time immemorial. The people of Bantul Regency, Yogyakarta Province, used traditional herbs such as 'Jamu Gendong,' 'Jamu Simplicia,' and 'Jamu Instant' to maintain their health. Some examples of herbs to treat joint pain are 'Jamu Cabe Puyang' which consists of Javanese long pepper (*Piper retrofactum* Vahl.) fruit, bitter ginger (*Zingiber zerumbet* (L.) J.E. Smith) rhizome, turmeric (*Curcuma longa* L.) rhizome, kedawung (*Parkia roxburghii* G. Don) seeds, ginger (*Zingiber officinale* Roscoe) rhizome, tamarind (*Tamarindus indica* L.) fruit, coconut (*Cocos nucifera* L.), sugar, salt, and water (Sukini, 2018). The use of traditional herbal medicine in Bantul District can be seen in several traditional herbal medicine industry centers, namely Kiringan, Mangunan, and Kunden hamlets.

Traditional products derived from plants and natural materials have the potential to develop as an alternative medicine to treat joint pain. Treatment using traditional herbs is in great demand because it is considered more affordable and has lower side effects compared to treatment methods using conventional drugs (Abat et al. 2017). An in-depth study needs to be carried out to find out the concept of the people of Kiringan, Mangunan, and Kunden hamlets, Bantul district in treating joint pain therapy.

The purpose of this study was to identify the traditional medicinal plants in traditional herbal medicine and their use in the Bantul district of Yogyakarta. This research is the first study on the local wisdom of the Bantul ethnic about osteoarthritis (OA) and its treatment methods that they have practiced from generation to generation. In particular, this study is intended to invent and document herbals applied by healers in their joint pain therapy.

MATERIAL AND METHODS

Study Area

This research was conducted in the Bantul district, Yogyakarta, as shown in Figure 1 shows is one of the districts in the special region of Yogyakarta, Indonesia. This district has an area of 508.85 km² and a population of 954,706 people. Bantul district is located between the coordinates of 07° 44′ 04″ – 08° 00′ 27″ south latitude and 110° 12′ 34″ – 110° 31′ 08″ east longitude. Bantul district has a tropical

climate, the rainy season is usually from November to April, and the dry season is usually from May to October. The type of climate influences the diversity of medicinal plants in this area. The rainfall in Bantul is 1942 mm per year with rainy days ranging from 100–130 rainy days, and the months with the highest rainfall are January and February. Temperatures are consistent throughout the year, with average temperatures ranging from 22° to 31°C. Specifically, data collection was carried out in three hamlets in Bantul District, namely Kiringan, Mangunan, and Kunden hamlets.

Demographic Profile of Healers

The demographic profiles of healers are shown in Table 1. More than 50% of healers are over 50 years old. The final education of the healer is described as follows, as much as 8% are illiterate, 40% have an absolute education in elementary school, and the rest have the last education in junior high school, high school, and bachelor's degree. Almost all the healers are entrepreneurs (selling herbs). In addition, most of them are natives of Bantul District, Yogyakarta.

Figure 1. Map of Yogyakarta; Bantul District

Table 1. Demographic profile of healers (N=48)

Parameters	Group	Amount of Informant	
		N	%
Domicile	Kiringan hamlets	44	92
	Mangunan hamlets	2	4
	Kunden hamlets	2	4
Gender	Male	0	0
	Female	48	100
Age	< 49 years old	23	48
	50-69 years old	22	46
	> 70 years old	3	6
Education Levels	Illiterate	4	8
	Elementary School	19	40
	Junior High School	9	19
	Senior High School	14	29
	Diploma/Bachelor Degree	2	4
Main Profession	Entrepreneur (Selling herbal medicine)	48	100

Knowledge of Healers

The knowledge of healers about traditional medicinal plants was obtained in several ways, namely: (1) learning from parents as a legacy of family knowledge, (2) observing traditional medical practices carried out by parents or traditional healers in the hamlets, (3) experiencing illness and being treated with traditional medicine, (4) attending training and learning from books was shown in Table 2. Around 73% of the informants obtained the potions from heredity or learning from parents as a legacy of family knowledge.

Table 2. The knowledge of healers about traditional medicinal plants (N=48)

Local Concept	Group	Amount of Informant	
		N	%
Knowledge & experimental	Hereditary	35	73
	Learn from Training	6	13
	Learn from friends/neighbors	4	8
	Personal Experience	2	4
	Learn from Books, etc.	1	2
Attending of Training	Yes	5	10
	Never	43	90

Interview Methods with Healers

Before conducting the research, we obtained an ethical test license from the health research ethics committee of Universitas Airlangga with certificate number 04/LE/2022. An amount of 48 female healers involved in this study, as shown in Table 1. The informants are residents of the hamlets of Kiringan, Mangunan, and Kunden who work as an herbalists and are at least 21 years old. Informants who know about ingredients for complaints of joint pain are willing to become informants in the study. The selection of informants was made by purposive sampling and snowball sampling. Data was collected using field research, and data collection methods were conducted by interviewing informants who know joint pain treatment, observation, and documentation. This research was conducted in Bantul District, Yogyakarta. In the snowball sampling technique, the information of the second informant is received by asking the first informant, and so on.

Plant Materials

Traditional herbal medicines used by healers were collected during interviews and they were identified at the Materia Medika Institute, Batu. The traditional herbal medicines were deposited in the order of voucher specimen numbers from BY-1 to BY-47 . In addition, identification of the plants used in these traditional herbal medicines was also carried out. These plants are given the number code B1-B33 based on the amount of use. All of the specimens deposited at the Natural Products Drug Discovery & Development-Research Group, Faculty of Pharmacy Universitas Airlangga.

Data Analysis

Data analysis in this study was carried out qualitatively and quantitatively. Quantitative analysis is done by calculates the relative frequency of citations (RFC) and Family Important Value (FIV). RFC is used to describe the distribution of community knowledge in using plants to overcome joint pain complaints which are calculated by the formula; RFC = Fc/N, where Fc is the

number of informants who mention the use of plant species and N the total number of informants participating in the survey. The RFC value is "0" if there is no use mentioned by the informant and 1 if a type is proper (Agbodeka et al. 2016). FIV distinguishes the most influential families based on the number of informants quoted and the number of plant species used for treatment. FIV is calculated using the formula FIV = (Fc/N) x 100, Where Fc is the quoting frequency of the plant family, and N is the number of total informants. The FIV value indicates the local importance of medicinal plant families ranging from 0 to 100, where the most essential family has a value close to 100 (Dapar et al. 2020).

RESULTS AND DISCUSSION

Jamu is traditional herbal medicine from Indonesia and is made from natural materials, such as roots, barks, flowers, seeds, leaves, and fruits of plants. It is also acquired from animals, such as honey, royal jelly, milk, and native chicken eggs are often used as well. Jamu is one of the identities of the people in Central Java, one of which is the people in the Bantul District, because they drink it not only for healing but also for disease prevention. The discussion of herbal medicine also includes the ethnicity of healers. This ethnic group believes in the value and efficacy of jamu, so they influenced other groups involved or not in traditional medicine culture. This value and effectiveness make a group of people who believed in it consume jamu every day as human life mandatory, and providing cultural interpretation as a cognitive map (Spradley, 1997).

Ethnobotany and ethnopharmacology studies have an essential role discovering of new drugs and compounds (Yadav et al.2015). Indonesia is a country with a tropical climate and has a diversity of plants that play an essential role in the discovery of new medicinal plants. Each plant produces varying amounts of secondary metabolites, depending on its defense system against herbivores and pathogens (Rani and Jyothsna, 2010; Adenubi et al. 2016). These compounds work synergistically to increase potency, reduce side effects, and develop resistance (Adenubi et al. 2018).

Most of the patients who went treatment at traditional healers in Bantul district come from the same area (85%), the rest are from outside (15%), and the traditional healers give the potion directly. Healers included in this study had a long experience of which there were working as herbalists for less than 10 years (33%), for 10-30 years (29%), and for more than 30 years (38%). It shows that the informant has had much practical experience regarding traditional ingredients. This experience comes from the habit of the informant to observing or even learning about compounding by using traditional herbal medicine carried out by previous generations. Healers have several ways of selling or marketing their herbal products, including selling products by walking around (79%), selling permanently in a place (15%) such as markets and herbal medicine stalls, and marketing products through online media (6%). The herbal products sold are herbal medicine according to the wishes of consumers. Usually, these herbs are served in coconut shells or "Batok" and consumed individually direct. Special herbs sold online are herbal products that can be stored for an extended of time.

From the survey data in this study, we got 47 traditional herbal medicines consisting of 33 types of plants used by traditional healers in the Bantul district to treat joint pain. Distribution knowledge of healers in utilizing plants to overcome joint pain complaints is described through the RFC. Based on the calculation of the RFC shows that the plant species that are most widely used in traditional herbal medicine for joint pain by traditional healers in the Bantul district include *Kaempferia galanga* L (0.778), *Zingiber officinale* Roescoe (0.667), *Oryza sativa* L. (0.352), *Curcuma xanthorrhiza* Roxb (0.241), and *Piper retrofractum* Vahl (0.204). Joint Pain treatment carried out by the healer consists of consuming the concoction orally (89%) and topical therapy utilizing compresses (11%) as shown in Table 3.

Table 3. Plants used in traditional herbal medicines for joint pain therapy by healers in Bantul District

Family	Plant Number	Plant name	Local name	Part(s) used	Preparations	Applications	RFC
Zingiberaceae	B-1	*Kaempferia galanga* L.	Kencur	Rhizome	Grounded and squeezed with warm water	Drink directly, 2-3 times a week	0.778
					Mixed with Oryza Seed, then mashed and applied to the part that feels painful	Compress-topical used	
Zingiberaceae	B-2	*Zingiber officinale* Roscoe	Jahe	Rhizome	Pounded and squeezed with warm water	Drink directly, 2-3 times a week	0.667
					Mashed and applied to the part that feels painful	Compress-topical used	
Poaceae	B-3	*Oryza sativa* L.	Beras	Seed	Mixed with Kaempferia Rhizome, then mashed and applied to the part that feels painful	Compress-topical used	0.352
Zingiberaceae	B-4	*Curcuma xanthorrhiza* Roxb.	Temulawak	Rhizome	Dried and pulverized. Put in hot water when consuming	Drink directly, 2-3 times a week	0.241
					Mashed and applied to the part that feels painful	Compress-topical used	
Piperaceae	B-5	*Piper retrofractum* Vahl	Cabe Jawa	Fruit	Pounded and boiled	Drink directly, 2-3 times a week	0.204
Zingiberaceae	B-6	*Zingiber zerumbet* (L.) J.E.Smith	Lempuyang	Rhizome	Dried and pulverized. Put in hot water when consuming	Drink directly, 2-3 times a week	0.185
Zingiberaceae	B-7	*Curcuma longa* L.	Kunyit	Rhizome	Grounded and squeezed with warm water	Drink directly, 2-3 times a week	0.111
Acanthaceae	B-8	*Andrographis paniculata* Nees	Sambiloto	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.056
					Leaves and lime betel, then mashed and applied to the part that feels painful	Compress-topical used	
Myrtaceae	B-9	*Syzygium polyanthum* (Wight) Walpers	Daun salam	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.056
Zingiberaceae	B-10	*Curcuma aeruginosa* Roxb.	Temu Ireng	Rhizome	Grounded and squeezed with warm water	Drink directly, 2-3 times a week	0.037
Poaceae	B-11	*Cymbopogon nardus* Rendle	Sereh	Leaf	Pounded and boiled	Drink directly, every day	0.037
Myrtaceae	B-12	*Syzygium aromaticum* (L.) Merrill & Perry	Cengkeh	Leaf	Pounded and boiled	Drink directly, a times a week	0.037
Zingiberaceae	B-13	*Amomum compactum* Soland.ex Maton	Kapulaga	Fruit	Pounded and boiled	Drink directly, times a week	0.037
Meliaceae	B-14	*Swietenia macrophylla* King	Mahoni	Seed	Pounded and boiled	Drink directly, 2-3 times a week	0.056

Zingiberaceae	B-15	*Curcuma mangga* Valenton & van Zijp	Temu mang-ga	Rhizome	Grounded and squeezed with warm water	Drink directly, 2-3 times a week	0.037
Apiaceae	B-16	*Foeniculum vulgare* Mill	Adas	Fruit	Pounded and boiled	Drink directly, times a week	0.037
Annonaceae	B-17	*Annona muricata* L.	Sirsak	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.037
Poaceae	B-18	*Imperata cylindrica* (L.) Raeusch.	Alang-alang	Root	Pounded and boiled	Drink directly, daily until the pain is gone	0.037
Apocynaceae	B-19	*Alstonia scholaris* L. R. Br.	Kayu pule	Bark	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Solanaceae	B-20	*Capsicum annuum* L.	Cabai	Fruit	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Fabaceae	B-21	*Parkia roxburghii* G.Don.	Kedawung	Seed	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Leguminose	B-22	*Tamarindus indica* L	Asam Jawa	Fruit	Grounded and squeezed with warm water	Drink directly, daily until the pain is gone	0.019
Rutaceae	B-23	*Justicia gendarussa* Burm.f.	gandarusa	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.019
					Leaves and Ginger Rhizome, then mashed and applied to the part that feels painful	Compress-topical used	
Lauraceae	B-24	*Cryptocarya massoia* (Oken) Kosterm	Mesoyi	Bark	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Caricaceae	B-25	*Carica papaya* L.	Pepaya	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.019
Sterculiaceae	B-26	*Helicteres isora* L.	Kayu ules	Fruit	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Solanaceae	B-27	*Physalis angulata* L.	Cipluk	Fruit, Leaf	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Fabaceae	B-28	*Caesalpinia sappan* L.	Secang	Bark	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Lamiaceae	B-29	*Orthosiphon stamineus* (Blume) Miq.	Remujung	Leaf	Washed and boiled	Drink directly, daily until the pain is gone	0.019
Myrtaceae	B-30	*Eucalyptus alba* Reinw. Ex Blume	Ceplik	Seed	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Oleaceae	B-31	*Jasmimum sambac* (L.) Sol. ex Aiton	Melati	Flower	Washed and boiled	Drink directly, every day	0.019
Lauracae	B-32	*Cinnamomum burmannii* (Nees & T.Nees)	Kayu manis	Bark	Pounded and boiled	Drink directly, 2-3 times a week	0.019
Piperaceae	B-33	*Piper cubeba* L.	Kemukus	Fruit	Pounded and boiled	Drink directly, 2-3 times a week	0.019

Several parts of the plants are used by healers, such as leaves (9 species), rhizome (7 species), fruits (8 species), seeds (4 species), bark (4 species), roots, and flowers. Leaves are the most widely used plant parts in traditional herbal medicines to treat joint pain. Processing plants into ingredients is also done simply, namely by boiling all or part of the plant. Leaves are the primary photosynthetic organs of plants and are the most commonly used plant parts because they contain helpful bioactive compounds. In this study, it was stated that the use of herbs traditionally could be done orally 2-5 times a week or up to complaints of pain felt better. After the pain has improved, this herb is still carried out as a preventive effort for the emergence of back pain. In addition to the use of herbal medicine, to prevent joint pain complaints as well must be accompanied by a healthy lifestyle.

Another way to be applied is by crushing one or a mixture of plant parts into a paste and then squeezing it into fruit juice. The following are 47 traditional herbal medicines produced by traditional healers in the Bantul district, Yogyakarta, as shown in Table 4.

Table 4. Traditional herbal medicines for joint pain and their applications

Voucher Speciment No.	Compotition of Traditional Herb	Preparation Methode	Application of Traditional Herb
BY-1	Mixed of plants B-2, B-4, B-5, B-6, and B-7	Washed, grounded, and squeezed with warm water	consumed orally
BY-2	Mixed of plants B-1, B-2, B-10, and B-15	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-3	Mixed of plants B-7, B-7, B-8, and B-9	Washed, pounded, crushed, and squeezed. Then the squeezed water is boiled	consumed orally
BY-4	Mixed of plants B-2 and B8	Mashed and applied to the part that feels painful	Used topically (compress)
BY-5	Mixed of plants B-1 and B-2	Washed, pounded, crushed ,and squeezed with warm water.	consumed orally
BY-6	Mixed of plants B-1, B-2, B-4, and B-22	Washed, boiled, drained, and take the boiled water	consumed orally
BY-7	Mixed of plants B-1, B-2, B-5, B7, and B-8	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-8	Mixed of plants B-1, B-2, B-3, and B-21	Washed, pounded, and squeezed	consumed orally
BY-9	Mixed of plants B-1, B-2, B-5, and B-6	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-10	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-11	Mixed of plants B-1 and B-3	Mashed and applied to the part that feels painful	Used topically (compress)
BY-12	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-13	Mixed of plants B-1, B-2, B-3, B-5, and B-6	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-14	Mixed of plants B-1 and B-22	Washed, pounded, and squeezed	consumed orally
BY-15	Mixed of plants B-1, B-2, and B-3	Washed, pounded, crushed, and squeezed with warm water.	consumed orally

BY-16	Mixed of plants B-1, B-3, and B-7	Washed, boiled, drained, and take the boiled water	consumed orally
BY-17	Mixed of plants B-1 and B-3	Washed, pounded, and squeezed	consumed orally
BY-18	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-19	Mixed of plants B-1, B-3, and B-8	Washed, pounded, crushed ,and boiled	consumed orally
BY-20	Mixed of plants B-4, B-5, B-6, B-7, B-12, and B-24	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-21	Mixed of plants B-1 and B-2	Mashed and applied to the part that feels painful	Used topically (compress)
BY-22	Mixed of plants B-7, B-22, and B-25	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-23	Mixed of plants B-1, B-2, and B-7	Washed, pounded, crushed , and squeezed with warm water.	consumed orally
BY-24	Mixed of plants B-1, B-2, B-13, and B-16	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-25	Mixed of plants B-1, B-4, B-5, B-6, and B-7	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-26	Mixed of plants B-1, B-3, B-7	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-27	Mixed of plants B-1, B-2, andB-3	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-28	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-29	Mixed of plants B-1, B-2, B-4, B-9, and B-16	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-30	Mixed of plants B-1, B-2, and B-3	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-31	Mixed of plants B-1, B-3, B-4, B-7 and B-22	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-32	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-33	Mixed of plants B-1, B-3, B-5, and B-6	Washed, pounded, drained, and boiled.	consumed orally
BY-34	Mixed of plants B-1 and B-2	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-35	Mixed of plants B-1 and B-3	Mashed and applied to the part that feels painful	Used topically (compress)
BY-36	Mixed of plants B-1, B-3, B-4, B-5, B-6, and B-15	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-37	Mixed of plants B-2 and B-23	Mashed and applied to the part that feels painful	Used topically (compress)
BY-38	Mixed of plants B-2, B-5, B-6, and B-18	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-39	Mixed of plants B-4, B-8, B-10, B-14, B-19, B-29, B-30, B-31, and B-32	Washed, pounded, drained, and boiled.	consumed orally

BY-40	Mixed of plants B-5, B-6, B-10, B-12, B-13, and B-33	Washed, dried, pounded, and brewed with warm water.	consumed orally
BY-41	Mixed of plants B-4, B-8, B-10, B-11, B-14, B-19, B-26, B-27, and B-28	Washed, pounded, drained, and boiled.	consumed orally
BY-42	Mixed of plants B-1, B-2, B-8, B-17, and B-18	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-43	Mixed of plants B-1, B-2, B-8, and B-14	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-44	Mixed of plants B-1, B-3, B-5, and B-6	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-45	Mixed of plants B-1, B-3, B-4, B-5, B-6, and B-15	Washed, grounded, pounded, crushed, and boiled	consumed orally
BY-46	Mixed of plants B-1, B-2, B-5, B-20, and B-21	Washed, pounded, crushed, and squeezed with warm water.	consumed orally
BY-47	Mixed of plants B-1, B-2, B-3, B-5, and B-6	Washed, pounded, crushed, and squeezed with warm water.	consumed orally

Through FIV analysis, 33 plant species were grouped into 18 families, the most common being Zingiberaceae (9 species) (26.47), followed by Poaceae (4 species) (8.82). Piperaceae (2 species) (8.82) (Table 5):

Table 5. Family Important Value (FIV)

Family	Plant name	Local name	FIV
Zingiberaceae	*Zingiber officinale* Roscoe	Jahe	26.47
	Curcuma longa L.	Kunyit	
	Curcuma aeruginosa Roxb.	Temu Ireng	
	Amomum compactum Soland.ex Maton	Kapulaga	
	Curcuma mangga Valenton & van Zijp	Kunir mangga	
	Kaempferia galanga L.	Kencur	
	Curcuma xanthorrhiza Roxb.	Temulawak	
	Zingiber zerumbet (L.) J.E.Smith	Lempuyang	
Poaceae	*Oryza sativa* L.	Beras	8.82
	Cymbopogon nardus Rendle	Sereh	
	Imperata cylindrica (L.) Raeusch.	Alang-alang	
Myrtaceae	*Syzygium polyanthum* (Wight) Walp.	Daun salam	8.82
	Syzygium aromaticum (L.) Merrill & Perry.	Cengkeh	
	Eucalyptus alba Reinw. Ex Blume	Ceplik	
Piperaceae	*Piper retrofractum* Vahl.	Cabe Jawa	5.88
	Piper cubeba L.	Kemukus	
Lauraceae	*Cryptocarya massoia* (Oken) Kosterm	Mesoyi	5.88
	Cinnamomum burmannii (Nees & T.Nees)	Kayu manis	

Fabaceae	*Parkia roxburghii* G.Don.	Kedawung	5.88
	Caesalpinia sappan L.	Secang	
Solanaceae	*Capsicum annuum* L.	Cabai	5.88
	Physalis angulata L.	Cipluk	
Acanthaceae	*Andrographis paniculata* Nees	Sambiloto	2.94
Apocynaceae	*Alstonia scholaris* L. R. Br.	Kayu pule	2.94
Apiaceae	*Foeniculum vulgare* Mill.	Adas	2.94
Annonaceae	*Annona muricata* L.	Sirsak	2.94
Caricaceae	*Carica papaya* L.	Pepaya	2.94
Lamiaceae	*Orthosiphon stamineus* (Blume) Miq.	Remujung	2.94
Leguminose	*Tamarindus indica* L.	Asam jawa	2.94
Meliaceae	*Swietenia macrophylla* King	Mahoni	2.94
Oleaceae	*Jasmimum sambac* (L.) Sol.ex Aiton	Melati	2.94
Sterculiaceae	*Helicteres isora* L.	Kayu ules	2.94
Rutaceae	*Justicia gendarussa* Burm.f.	Gendarusa	2.94

The most widely used plant species for joint pain therapy in the Bantul regency are from the Zingiberaceae family. This family is widely used in traditional medicine medicine in Indonesia, and about 700 species are widely distributed globally (Christenhusz & Byng, 2016). This family members usually contain essential oils and flavonoids. Flavonoids represent the best-known and widespread group of plant phenolics and their biological actions include protection against allergies, platelet aggregation, microbes, tumors, ulcers, and inflammation (Okwu & Okwu, 2004).

One of the plants belonging to Zingiberaceae and widely used in traditional herbal medicines for treatment of joint pain by healers in the Bantul district is *Kaempferia galanga* was shown in Table 3. It is known in Indonesia as "Kencur" and it is a medicinal plant used empirically and occupies 16th place as the most widely used medicinal plant in traditional medicinal herbal formulas (Subositi et al. 2020). This plant is traditionally used as a medicine because it has anti-inflammatory, analgesic, antioxidant, and antimicrobial properties (Subositi et al. 2020). According to Indonesian Herbal Pharmacopoeia, a thick extract of *Kaempferia galanga* rhizome contains essential oil not less than 7.93% v/w and ethyl-p-methoxycinnamate not less than 4.30%. Ethyl-p-methoxycinnamate is an anti-inflammatory constituent that can inhibit inflammation by suppressing interleukin-1, tumor necrosis factor (TNF), and angiogenesis by blocking endothelial function (Shetu et al. 2018).

In recent years, several studies have been conducted on the biological activity of ginger, including antioxidant, antimicrobial, anti-inflammatory, and anticancer (Mao et al. 2019). The content of phytochemical compounds that have been identified in ginger is zingerone, gingerdiol, gingerol, zingiberene, and shogaol. In addition, the content obtained from others includes essential oils, diarylheptanoids, paradol, zerumbone, 1-dehydro-(10) gingerdione, terpenoids, and flavonoids (Nutakor et al. 2020). In particular, shogaol and gingerols exhibit therapeutic activity in bone disease by inhibiting rheumatic

inflammation and autoimmune response (Hwang et al. 2017). The 6-gingerol and 6-shogaol have a sharp odor in the rhizome (Shahrajabian et al. 2019). Several *in vivo* and *in vitro* studies have been carried out to describe the activity of 6-shogaol which is the main bioactive constituent in dry rhizomes. *In vivo* studies, 6-shogaol significantly reduced inflammatory markers such as leukocyte infiltration or edema formation and demonstrated neuroprotective effects. Whereas *in vitro* studies, 6-shogaol inhibited proinflammatory compounds such as cyclooxygenase-2 (COX-2), weakened inducible nitric oxide synthase (iNOS) levels, and resulted in reduced nitric oxide (NO) levels, weakened the release of proinflammatory cytokines such as interferon, TNF, interleukins, and chemokines (Bischoff-Kont et al. 2021).

Curcuma xanthorriza known as "Temulawak" is also a species of the family Zingiberaceae which has many health benefits. The active compounds reported to have activity in temulawak are curcuminoids, curcumene, tumerone, xanthorhizol (Rahmat et al. 2021). The secondary metabolite with the highest number in temulawak rhizome is xanthorrizol (Rahmat et al. 2021). Xanthorrizol is one of the anti-inflammatory agents with a mechanism of inhibiting interleukin-6 (IL-6) and TNF-α and suppressing the expression of COX-2 and iNOS through the nuclear factor kappa B (NF-kB) pathway resulting in reduced prostaglandin E2 (PGE2) and NO (Oon et al. 2015).

In addition to these plants, other plants are used by traditional healers in Bantul for joint pain therapy, such as *Piper retrofractum, Zingiber zerumbet*, *Curcuma longa*, and *Andrographis paniculata*. *Piper retrofractum* from the Piperaceae family is a medicinal plant because it contains several secondary metabolites including piperine, essential oil, piperidine, benzene, sesamine, palmitic acid, and acid hydropiperic (Boangmanalu et al, 2018). In a study using the model arthritis animals, piperine exhibits anti-inflammatory activity by inhibiting IL-6, matrix metalloproteinase-13 (MMP-13), activator protein (AP-1), and reduced PGE2 by significantly reducing rheumatism in mice (Bang et al. 2009).

Zingiber zerumbet known as "Lempuyang" is also a plant that comes from the Zingiberaceae family. The main compound in lempuyang is zerumbone which is a sesquiterpene with one ring of atoms in the molecule and having a double bond. Lempuyang extract has activity as an anti-inflammatory and analgesic (Ahmadabadi et al. 2019). Zerumbone has been reported to have acted as an anti-inflammatory and analgesic because it can prevent inflammation through lambda carrageenan and dinoprostone which are comparable to non-steroidal anti-inflammatory drugs from the oxicam class (Somchit et al. 2012; Hosseinpour et al. 2014).

Curcuma longa (turmeric) is a plant that belongs to the Zingiberaceae family. There are three main compounds found in turmeric, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcuminoids are the main secondary metabolites found in turmeric rhizomes. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin can inhibit the activation of TNF-induced NF-kB (Fuloria et al. 2022).

"Sambiloto" (*Andrographis paniculata*) is a plant that belongs to the family Acanthaceae. The main compound is andrographolide which acts as an anti-inflammatory, antipyretic, antimalarial, and antidiabetic agent (Prihatini et al. 2020). In a study using a collagen-induced arthritis model in rodents, andrographolide may reduce arthritis clinical scores, joint damage, and NO and TNF-α production (Gupta et al. 2018). Based on these, andrographolide can be used as an anti-inflammatory agent naturally or in the form of more powerful derivatives (Burgos et al. 2020).

In the study that has been accomplished, mahogany seeds (*Swietenia macrophylla*) contain secondary

metabolites, including flavonoids, alkaloids, and saponins, which are often used in traditional medicine and have anticancer, neuroprotective, anti-hyperglycemic, anti-inflammatory, antioxidant, and anti-viral activities (Moghadamtousi et al. 2013). These compounds can not only inhibit NO production, but can also downregulate the production of proinflammatory compounds such as IL-1β, TNF-α, IFN-γ, IL-6, COX-2, and NF-kB (Mak et al. 2021). In addition, bay leaves (*Syzygium polyanthum*) contain flavonoids and tannins are two compounds that were suspected to be responsible for anti-inflammatory effects (Hasan et al. 2020). In a study using the ethanol extract of secang (*Caesalpinia sappan)*, brazilin compound was found that can inhibit the expression of proinflammatory cytokines IL-1β and TNF-α in IL-1β stimulated chondrocytes and LPS-stimulated THP-1 macrophages (Jung et al. 2015). In jasmine, anti-inflammatory activity was found in root and leaf extracts, there has been no further research on compounds that have activity as analgesic and anti-inflammatory in jasmine flower extract (Bhangale et al. 2012).

Based on this study, 47 traditional herbal medicines consisting of 33 plant species from 18 families. 42 herbal medicine were used orally and 5 of them were used topically. The most widely used herbal medicine orally consists of *Kaempferia galanga* (Kencur) and *Zingiber officinale* (Ginger). The oldest healer (79 years) treats joint pain by giving *Kaempferia galangal* rizhome, *Oryza sativa* seed, *Curcuma xanthorrhiza* rhizome, and *Zingiber officinale* rhizome. All part plants were washed, peeled, pounded, squeezed and consumed 2 to 4 times a week. She also provides additional therapy through ginger compresses by mashing and attaching ginger to the painful part. Furthermore, the longest-practice healer of 50 years uses *Kaempferia galanga* rhizome and *Zingiber officinale* rhizhome. A mixture of these plants is pounded and squeezed using warm water. She also suggests to compressing the painful part with salt warm water. So Zingiberaceae plants are always present in every traditional herbal medicine that mentioned by the healer in the Bantul district, Yogyakarta.

In addition to the use herbal medicine orally, the healers in the Bantul district also recommended using of the herbal medicine topically by means of compress. The plants or ingredients used for compress are *Kaempferia galanga*, *Zingiber officinale*, *Curcuma xanthorrhiza*, *Andrographis paniculata*, *Justicia gendarussa*, salt, warm water, and cold water. They are prepared by smoothing each or a mixture of materials, then affixed to the painful area. For example, ginger rhizome as a warm compress is placed on the painful site and has a hot and spicy taste which relieves pain, stiffness, muscle spasms, or vasodilation of blood vessels (Fatmawati and Ariyanto, 2021).

In general, each herb from a healer in Bantul District consists of more than three plants that are believed to be efficacious in the prevention and treatment of various diseases, one of which is joint pain. The traditional herbal medicine is made by washing, boiling, pounding, grinding, crushing, and squeezing. The boiling process is to boil some ingredients with water then filter and drink regularly at a particular time. The boiling process is considered an effective process for extracting plant material and maintaining its stability in microbes (Kamatensi et al. 2011). However, this method has a weakness, namely if the heating is high enough and for a long time it can damage the active compounds contained in plants. Besides boiling, another method is pounding. The pounding process damages the tissue and breaks down the cell wall making it easier to remove active compounds present in the cells (Taek et al. 2019). The practice of traditional medicine by healers in the Bantul district has existed for a long time since the

treatment was passed down from one generation to the next. This knowledge is still known by parents and gradually disappears among young people because it is slowly being abandoned by society. The main reason is that many young people think that traditional medicine is primitive while modern medicine is practiced.

CONCLUSION

A total of 47 traditional herbal medicine consisting of 33 types of plants from 18 families were documented as medicinal plants for the treatment of joint pain by healers in the Bantul district, Yogyakarta. These plants are a resource that plays an essential role in the development of new drugs in the future. Many local pearls of wisdom and traditional experiences have disappeared. Therefore, ethnomedical studies like this need to be done to document valuable knowledge before it is lost from society.

ACKNOWLEDGEMENTS

The authors appreciate the support and encouragement provided by PUF research of the Faculty of Pharmacy Universitas Airlangga and the authors are very thankful to the respondents for sharing their valuable knowledge.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHOR CONTRIBUTIONS

R.W., research concepts, design, data analysis, preparation and editing manuscript; N.P., literature search, statistical analysis, and manuscript review; W.E., validated questions for informants, and definition of intellectual content; M.A., research concepts, design, guarantor and manuscript review; R.K.S., guarantor, corrected and reviewed manuscript writing; Z.B.A., Experimental studies, literature search, data acquisition, and statistical analysis; I.S., data acquisition, preparation and editing manuscript.

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FABAD J. Pharm. Sci., 48, 2, 219-230, 2023
Doi: 10.55262/fabadeczacilik.1268029
RESEARCH ARTICLE

Evaluation of Mechanical and Mucoadhesive Properties of Polyvinyl Alcohol Nanofibers As Vaginal Drug Delivery System

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Evaluation of Mechanical and Mucoadhesive Properties of Polyvinyl Alcohol Nanofibers As Vaginal Drug Delivery System

SUMMARY

Electrospinning is a versatile and inexpensive technique to produce nanofibers. Nanofibers can be an excellent alternative to classical dosage forms in vaginal applications due to high surface area/volume ratio, high encapsulation efficiency, and mucoadhesive properties. Polyvinyl alcohol (PVA) is a biocompatible, easily degradable, and flexible polymer with mucoadhesive properties used in industrial, commercial, and medical applications. The scope of this study is to characterize electrospun nanofibers produced with different PVA types for vaginal use. PVA nanofibers were produced using the electrospinning method. Nanofiber formulations were prepared by dissolving PVA in dimethylformamide (DMF): distilled water (1:1) solvent system. Nanofibers were produced with three different types of PVA at 5%, 7.5%, and 10% concentrations. The surface tension, viscosity, and conductivity properties of the polymer mixtures were measured for the electrospinning process and these parameters were found suitable for nanofiber production. While the viscosity increased with increasing polymer concentration, the surface tension values were found to be close to each other since the solvent system was the same. The mechanical and mucoadhesive properties of nanofibers were examined and compared. Mucoadhesive and mechanical properties of nanofiber formulations differed depending on molecular weight and electrospinning process. The nanofiber formulations produced with Polyviol 13/140 were found suitable for vaginal applications in terms of their mechanical and mucoadhesive properties. PVA nanofibers can be a good alternative as a drug delivery system in vaginal applications.

Key Words: *Nanofiber, Polyvinyl alcohol, Vaginal application, Electrospinning.*

Vajinal İlaç Taşıyıcı Sistem Olarak Polivinil Alkol Nanoliflerinin Mekanik ve Mukoadezif Özelliklerinin Değerlendirilmesi

ÖZ

Elektroçekim, nanoliflerin üretilmesi için çok yönlü ve ucuz bir tekniktir. Nanolifler, yüksek yüzey alanı/hacim oranı, yüksek kapsülleme etkinliği ve mukoadezif özellikleri nedeniyle vajinal uygulamalarda klasik dozaj formlarına iyi bir alternatif olabilir. Polivinil alkol (PVA), endüstriyel, ticari ve tıbbi uygulamalarda kullanılan, biyouyumlu, kolay parçalanabilen ve mukoadezif özelliklere sahip esnek bir polimerdir. Bu çalışmanın kapsamı vajinal kullanım için farklı PVA tipleri ile üretilen elektropsun nanoliflerini karakterize etmektir. PVA nanolifleri, elektroeğirme yöntemi kullanılarak üretildi. Nanolif formülasyonları, PVA'nın dimetilformamid (DMF):saf su (1:1) solvent sisteminde çözülmesiyle hazırlandı. Nanolifler, %5, %7,5 ve %10 konsantrasyonlarında üç farklı tipte PVA ile üretilmiştir. Elektroçekim işlemi için polimer karışımlarının yüzey gerilimi, viskozite ve iletkenlik özellikleri ölçülmüş ve bu parametreler nanolif üretimi için uygun bulunmuştur. Artan polimer konsantrasyonu ile viskozite artarken, solvent sistemi aynı olduğu için yüzey gerilimi değerlerinin birbirine yakın olduğu görülmüştür. Nanoliflerin mekanik ve mukoadezif özellikleri incelenmiş ve karşılaştırılmıştır. Nanolif formülasyonlarının mukoadezif ve mekanik özellikleri moleküler ağırlık ve elektroeğirme işlemine bağlı olarak farklılık göstermiştir. Polyviol 13/140 ile üretilen nanolif formülasyonları, mekanik ve mukoadezif özellikleri açısından vajinal uygulamalar için uygun bulunmuştur. PVA nanolifleri, vajinal uygulamalarda ilaç taşıyıcı sistem olarak iyi bir alternatif olabilir.

Anahtar Kelimeler: *Nanolif, Polivinil alkol, Vajinal uygulama, Electroçekim.*

Received: 20.03.2023
Revised: 05.04.2023
Accepted: 05.04.2023

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INTRODUCTION

Vaginal drug administration provides unique properties for women in local or systemic administration of drugs to achieve the desired therapeutic effects (Vermani, 2000). Several drugs, such as antibacterial, antifungal, antiprotozoal, antiviral, spermicidal agents, and hormones have been administered through the vaginal route (Das Neves, 2006). Various drug delivery systems are used vaginally, including tablets, capsules, gels, suppositories, creams, ointments, rings, films, and foam (Bachhav, 2009; Dobaria, 2009; Ya, 2010; Woodsong & Holt, 2015; Tunpanich, 2019). Conventional drug delivery systems such as creams, foams, and gels stay in the vaginal cavity for a relatively short time due to the self-cleaning effect and leakage in vaginal use (Acartürk, 2009). Nanofibers provide potential advantages for vaginal drug delivery with their large surface area, increased solubility, stability, good mechanical properties, high flexibility, soft, non-abrasive, and rapid or controlled drug release (Stojanov, 2022).

Electrospinning is an easy, versatile, economical, productive, and applicable in different areas method used to manufacture fibers sizes from nanometers to micrometers (Kanjwal, 2022). The electrospinning method is a system consisting of a nozzle, collector, and high-voltage source. A cone called a Taylor cone is formed by the effect of the electric field applied to the polymer solution at the nozzle tip. After a critical voltage value, the first jet is formed, and the solvent of the solution evaporates and collects on the collector (Nematpour, 2020). Electrospinning technique is preferred for biomedical applications such as filtration and shielding materials, electrical and optical applications, and sensors (Agarwal, 2008).

Different bioadhesive systems have been developed using mucoadhesive polymers to prolong the residence time in the vaginal application. For this purpose, different mucoadhesive polymers such as alginate, gum arabic, chitosan, polyvinyl pyrrolidone, poly (ethylene oxide), pullulan, hydroxypropyl cellulose, and pectin have been studied (Tuğcu-Demiröz, 2020; Cazorla-Luna, 2021; Martín-Illana, 2021). Products developed for vaginal application must be safe, effective, and acceptable enough for women to use for a long time. Polyvinyl alcohol (PVA) is hydrophilic, non-toxic, biocompatible, and water-soluble. Excellent chemical resistance, physical properties, biodegradability, high swelling in water, and biological fluids and elastic structure enable PVA to be used in different areas such as contact lenses, skin, artificial cartilage, and drug release systems (Ekrem, 2017). PVA nanofibers can be produced by electrospinning, but their applications are limited due to their high hydrophilicity (Park, 2010). Many studies have investigated the effects of PVA fibers on manufacturing conditions, molecular weight, and solution composition, but they have not been evaluated for vaginal use (Park, 2010; Ekrem, 2017). In this study, we aimed to produce different types and concentrations of PVA fibers and to determine the appropriate nanofiber formulation for vaginal application. Contact angles, mucoadhesive and mechanical properties of PVA nanofibers produced with electrospinning were evaluated for vaginal use.

MATERIALS AND METHODS

Materials

Different molecular weight PVA were used for the preparation of nanofibers. Polyviol 13/140 (49.000 Da), Polyviol 26/140 (80.000 Da), and Polyviol 40/140 (100.000 Da) were purchased from Wacker Chemie AG. N, N-Dimethylformamide (DMF) was purchased from Sigma Aldrich (St. Louis, MO). All chemicals were of analytical grade. Distilled water was used for all studies.

Preparation of the polymer mixture for electrospinning

PVA was dissolved in hot water at 90 °C stirring at 500 rpm. Afterward, DMF was added and mixed until a homogeneous mixture was obtained. Before electrospinning, solutions were kept in an ultrasonic bath to remove air bubbles. Different types of PVA and different concentrations were used for formulation production. Polyviol 13/140, Polyviol 26/140 and Polyviol 40/140 were coded as A, B, and C, respectively. The content and codes of formulations are shown in Table 1.

Table 1. Concentration and codes of PVA nanofiber formulations

Concentration	Polyviol 13/14	Polyviol 26/140	Polyviol 40/140
5%	A1	B1	C1
7.5%	A2	B2	C2
10%	A3	B3	C3

Characterization of electrospinning solutions

Viscosity, conductivity, and surface tension of the polymer mixtures were characterized to determine the electrospinnability of the polymer mixtures. The viscosities of the polymer solutions were measured using a cone-plate viscometer (Brookfield, DV-III Rheometer with spindle type CPE-41, USA). Rheological experiments were performed with 0.5 mL of polymer with spindle 52 at room temperature. The viscosities of polymer mixtures obtained at 20 rpm were compared. The conductivity meter was used for conductivity measurements by immersing the probe in the polymer solution at room temperature (Seven2Go Cond meter S3, Mettler Toledo, UK). The conductivity values of the solutions were measured as μs/cm. Measurements of surface tension of polymer solutions were measured using an optical tensiometer (Attension-Theta Lite, Biolin Scientific, Finland). Surface tension was calculated by the device software using the Young-Laplace equation.

Production of nanofibers by electrospinning method

Nanofiber formulations were prepared using a single nozzle equipment electrospinning process (Inovenso Ltd, NE300, Turkey). Polymer solutions were drawn into a syringe. Fibers were collected on a rotary cylinder at 500 rpm rotation speed. The process parameters such as feed rate, voltage, and distance tip to the collector were adjusted for each polymer solution and these are given in Table 2. All processes were performed at room temperature.

Table 2. Process parameters of nanofibers produced via the electrospinning method

Formulation Code	Voltage (kV)	Feed Rate (mL/h)	Distance (mm)	Rotating Speed (rpm)
A1	18.2	1	90	500
A2	18.5	0.9	95	500
A3	17.7	0.5	95	500
B1	14.5	1.5	115	500
B2	15.9	1.2	132	500
B3	14	0.5	145	500
C1	16.5	1	125	500
C2	15	0.7	148	500
C3	18	1	130	500

Morphological studies

Fiber morphology and average fiber diameter of the PVA fibers were characterized using scanning electron microscopy (FEI Company, Quanta 400 F, USA). The average diameter of PVA nanofiber was calculated with the ImageJ (National Institute of Health) program. SEM images of nanofibers were taken at 20000X and 40000X magnifications.

Thermal analysis of nanofibers

Thermal analysis of pure PVA and PVA nanofiber was performed using a differential scanning calorimeter (Shimadzu, DSC-60, Japan). For DSC analysis, 2 mg samples were weighed and heated 300 °C at the heating rate of 10 °C/min under a nitrogen atmosphere. The flow rate of nitrogen gas was 20 mL/min.

Fourier transform infrared (FT-IR) spectroscopy studies

Fourier Transform Infrared Spectroscopy (FT-IR) was performed to examine the chemical changes and interactions occurring in PVA nanofiber formulations. FT-IR analyses were conducted from 350 to 4400 cm^{-1} at room temperature with an ATR probe (Perkin Elmer, Spectrum 400, USA).

Mechanical properties of nanofibers

Tensile strength and elongation at break values of the formulations were investigated using a texture analyzer (TA.XTPlus Texture Analyzer, Stable Micro Systems, UK). Mechanical properties were analyzed using a tensile grip apparatus. Nanofibers were cut into rectangles of 3 cm x 1 cm and attached to the apparatus. Elongation at break (%) and tensile strength (MPa) values were determined using stress-strain graphs. Elongation at break and tensile strength are calculated according to the values on the stress-strain graphs at the point where the elongation is maximum and the rupture does not occur (Figure 1.). The value on the x-axis is elongation at break, and the value on the y-axis is tensile strength.

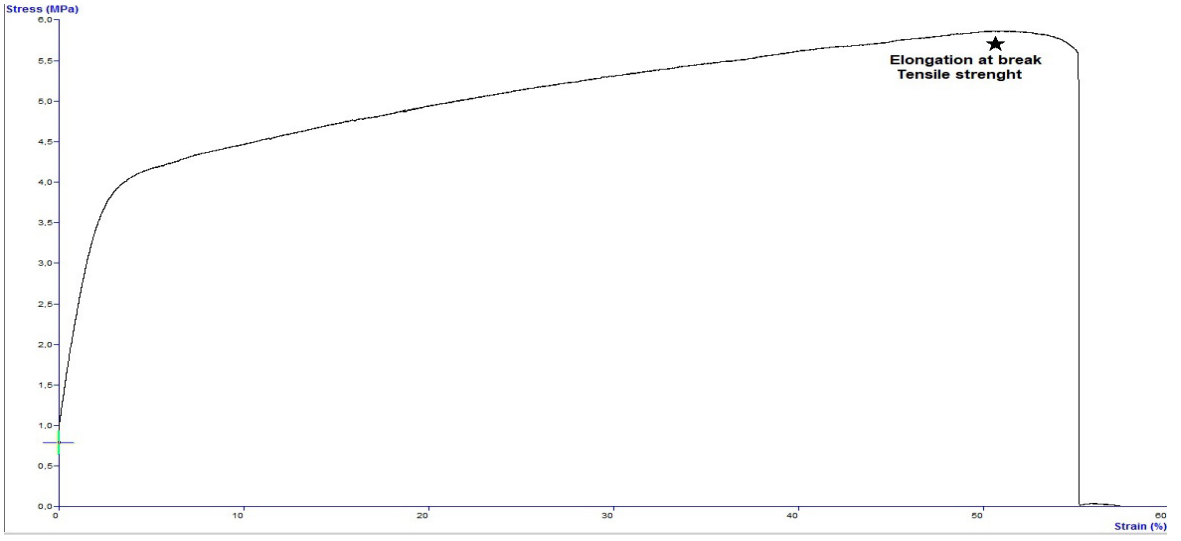

Figure 1. Stress-strain graphs of the tensile strength and elongation at break values obtained from texture analysis

Contact angle measurements

The Wettability of nanofiber was determined through contact angle measurements. Contact angles were measured by optical tensiometer using distilled water (Attension, Theta Lite, Finland). The nanofibers were stretched on a convex sample holder, and distilled water was dripped onto the nanofiber. The shape of the drop formed on the nanofiber and the contact angles were calculated using the software of the device.

Ex-Vivo Mucoadhesion studies

Mucoadhesion properties were determined by using the TA-XT Plus Texture Analyzer. The cow vagina was used as a model tissue for mucoadhesion studies. Nanofibers were attached to the upper probe of the device with double-sided tape, and vaginal tissue was placed on the platform at the bottom. In the mucoadhesion study, a probe speed of 1 mms^{-1}, a probe force of 0,2 N, and a contact time of 150 s with the mucosa were studied (Tuğcu-Demiröz, 2013; Tuğcu-Demiröz, 2015). The work of mucoadhesion was calculated from the area under the force-distance curve, and mucoadhesive properties were compared. Work of mucoadhesion values per cm^2 was calculated according to the formula (mJ/cm^2) = AUC/(πr^2) (Cevher, 2008).

Statistical analysis

GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of polymer solutions

The concentration of the polymer solution affects parameters such as viscosity, surface tension, and conductivity (İlbasmıs-Tamer, 2022). The physicochemical properties of the solutions directly affect the electrospinning process. As polymer concentration increased, the viscosity values of the solutions increased in all solutions. Selection of the appropriate solution concentration is one of the critical parameters to obtain nanofibers. High viscosity makes it extremely difficult for solutions to flow through the syringe needle to form nanofibers under an electrostatic force (Ding, 2010). Surface tension is influenced by the solvent system, and this parameter affects fiber formation and properties (Birer and Acartürk, 2022). Surface tension values of all polymer solutes were found to be similar, as seen in Table 3. The conductivity value was sufficient for the electrospinning process in all formulations. The highest conductivity value was found in A1, A2, and A3 formulations. Differences vin conductivity may have been observed due to the different molecular weights of the polymer used. Viscosity, conductivity, and surface tension measurements are important for the preliminary evaluation of polymer solutions in electrospinning method, but these parameters are not sufficient to decide on the optimum polymer solution properties. Many different parameters such as the evaporation of solvents and solvent systems affect the electrospinning process (Birer and Acartürk, 2022).

Table 3. The characterization results of viscosity, surface tension, and conductivity measurements (n = 3, mean ± SD).

Formulation Code	Viscosity (cP.s)	Conductivity (µS/cm)	Surface Tension (mN/m)
A1	41.42±2.87	174.73±4.97	43.74±0.20
A2	349.67±2.89	152.27±0.51	43.00±0.03
A3	542±0	129.47±2.26	42.11±0.08
B1	183.89±4.97	18.46±0.11	46.01±0.10
B2	699.11±2.87	32.31±0.30	43.01±0.02
B3	1740.16±93.91	36.49±0.23	44.78±0.07
C1	805.14±24.85	67.51±0.14	44.92±0.04
C2	1960±5.739	97.82±0.35	42.72±0.13
C3	2909±80.29	95.61±0.74	45.67±0.03

Morphological studies

SEM images demonstrated the successful production of nanofibers in formulations A3 and B3 using the electrospinning method (Figure 2.). Because the high molecular weight and concentration of PVA increase the viscosity, the solutions do not flow well from the injector and continuous fiber production becomes difficult. This situation was observed in group C formulations, and it was observed that high concentration nanofiber production did not occur. It was observed that the fibers were obtained wet due to the high-concentration and production parameters of the C3 formulation. No fiber structure was observed in C3. The average fiber diameter was found to be 159.416±65.015 nm for A3 and 167.171±39.016 nm for B3 formulation.

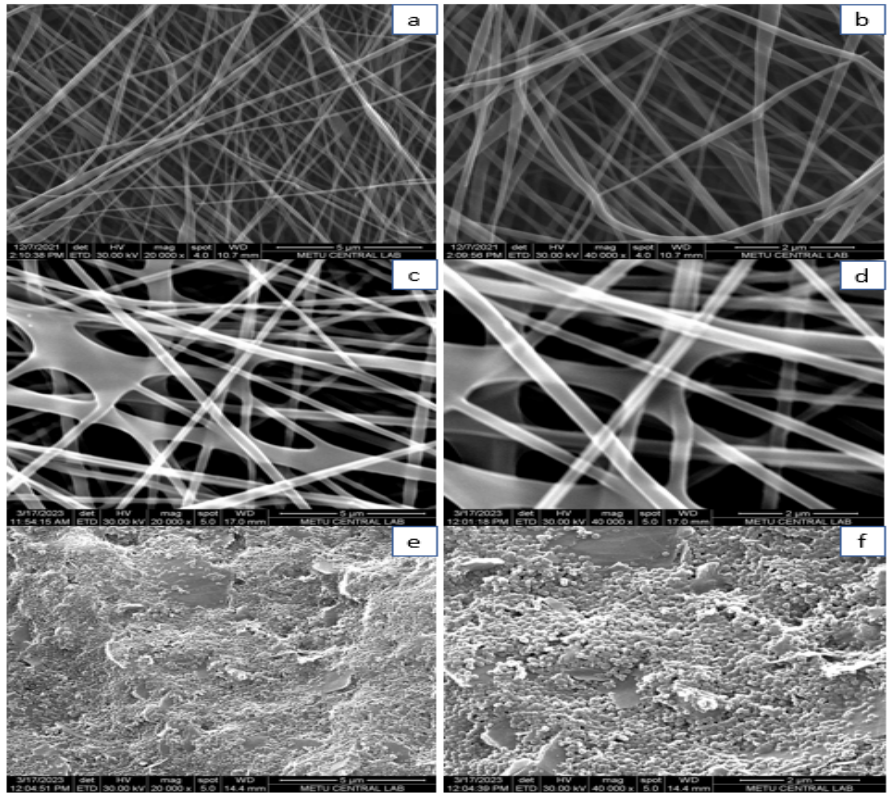

Figure 2. SEM images of A3 (magnifications a: 20000X, b: 40000X) and B3 formulation (magnifications c: 20000X, d: 40000X) and C3 formulation (magnifications e: 20000X, f: 40000X)

DSC analyses

We performed DSC measurements to investigate thermal behaviors such as melting, crystallization, and crystal structure formation. DSC thermograms of polymers and nanofibers are shown in Figure 3. DSC analysis showed an endothermic peak at 193,8 °C due to the melting point of PVA. Sudhamani et al. found the melting point of pure PVA as 202 °C in DSC analysis. In a study with PVA nanofibers, it was observed that the melting temperature in electrospun PVA fibers was almost unchanged compared to that of pure PVA (Kim, 2010).

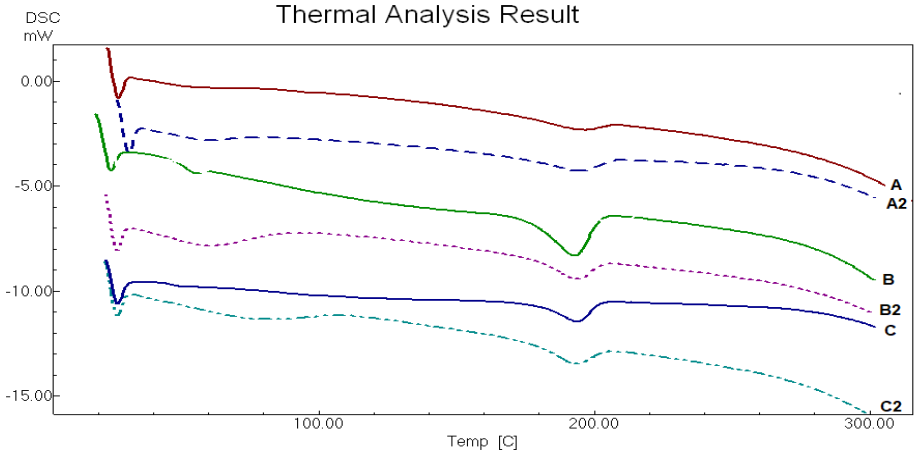

Figure 3. DSC thermogram of different pure PVA polymers (Polyviol 13/140 (A), Polyviol 26/140 (B) and Polyviol 40/140 (C)), and PVA nanofiber formulation

FT-IR analyses

Characteristic peaks of PVA were observed in the FT-IR spectra of the nanofibers. In addition, PVA absorption bands according to the literature (Ding, 2010). The spectrum of PVA and formulations showed a large band at 3268 cm^{-1} attributed to inter- and intramolecular hydrogen bonds in PVA (Reguieg, 2020). The peak at 2940 cm^{-1} is related to antisymmetric CH_2 stretching in the PVA sample, and two peaks at 1377 and 1432 cm^{-1} were CH-OH and CH_2 symmetric bending mode vibrations of PVA (Koosha, 2015). In the FT-IR analysis, specific PVA peaks were observed near 3320, 2940, 1420, and 840 cm^{-1}, consistent with the literature (Figure 4.). The observation of these FTIR bands in the spectra of the pure polymer and PVA nanofibers indicated that the electrospinning technique did not interfere with the chemical integrity of this polymer.

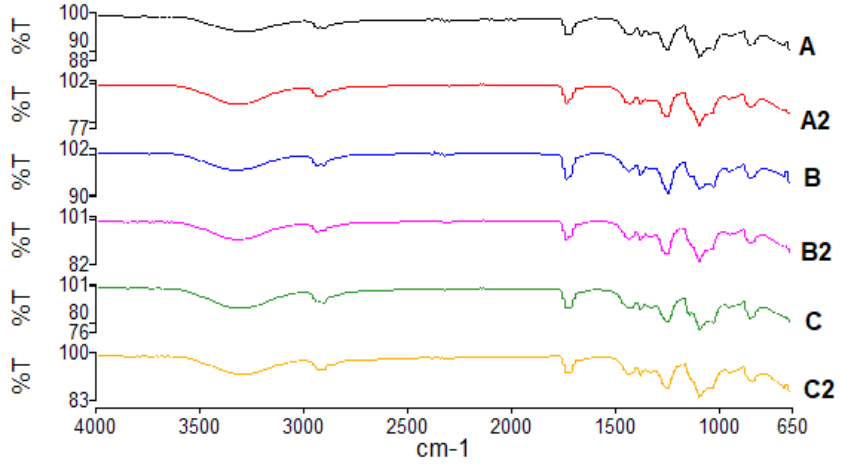

Figure 4. FT-IR spectrum of different pure PVA polymers (Polyviol 13/140 (A), Polyviol 26/140 (B) and Polyviol 40/140 (C)), and PVA nanofiber formulation

Mechanical properties of nanofibers

More flexible and elastic formulations are preferred for comfortable application in vaginal use (Szymańska, 2022). The mechanical properties of nanofibers are affected by different variables such as the rotation speed of the collector, the concentration of the polymers, the addition of crosslinking agents, and the change of production parameters (Sofi, 2020). The increase in the polymer concentration caused a change in the mechanical properties of the nanofibers. There is no increase in tensile strength was observed in formulations B1 and B2 with increasing PVA concentration. This difference was found to be statistically insignificant ($p>0.05$). Similarly, the difference between tensile strength value in A2 and A3 formulations was statistically insignificant ($p>0.05$). The tensile strength between A3 and B3 produced at high PVA concentration was found to be statistically insignificant ($p>0.05$). A3 was more suitable for electrospinning because it showed continuous production in the electrospinning method. The highest elongation at break values was found in the B3 formulation, whereas the highest tensile strength was found in the C3 formulation (Figure 5). Differences in the mechanical properties of nanofibers may be due to differences in the molecular weight of the polymer or changes in the structure of nanofibers by the electrospinning process. In the study of Koski et al., as the solution concentration increases, the fiber diameter and the distance between the fibers increase, and there is a gradual transition from circular to flat fibers (Koski, 2003). In addition, in low molecular weight samples, this transition from circular to straight fibers occurs at a higher concentration value than in high molecular weight polymers. As a result of such a change with the increase of polymer concentration and molecular weight in the fibers, changes in their mechanical properties may have been

observed. Ngadiman et al., in their study with PVA nanofibers, stated that although higher mechanical properties were obtained from high molecular weight PVA at high concentrations, the difficulty during the spinning process also increased (Ngadiman, 2015). It was stated that the high molecular weight and concentration of PVA would increase the viscosity. In this case, the solutions did not flow well, and extra force was required to extrude the solution from the syringe. Similar to these results, flow from the syrınge ıs dıffıcult at high PVA concentrations.

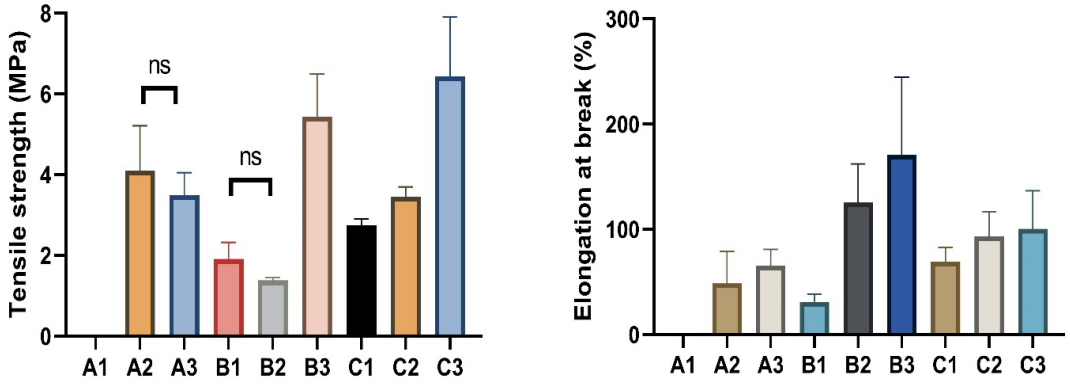

Figure 5. Tensile strength and elongation at break values of formulations (n=3, mean ± standard deviation, A1: Not detected, ns: not significant)

Contact angle measurements of nanofibers

The contact angle of all formulations was found to be less than 90 (Table 4.). These results show that the PVA nanofibers are hydrophilic structure. Among the fibers with high PVA concentration, the lowest contact angle was obtained in the A3 formulation. The difference in the contact angle in the fibers may be due to the changes in the fiber structure. Krogstad et al stated that PVA and PVP nanofibers for vaginal application should be wetted rapidly in order to provide nanoparticle diffusion into the vaginal tissue (Krogstad, 2017). Tissue regeneration and the biodegradation rate of the formulations will increase with their hydrophilic structure. Tuğcu-Demiröz et al. stated that the fibers for vaginal application could provide proper adhesion to the vagina due to their hydrophilic structure (Tuğcu-Demiröz, 2020). In another study shows that the complete wetting of the nanofibers releases the drug with a rapid effect, and this wettability increases the contact of the fibers with the environment (Tuğcu-Demiröz, 2021). The difference in molecular weight and solution concentration does not affect the hydrophilicity of PVA (Ngadiman, 2015).

Table 4. Characterization result of nanofiber formulations (n=3, *Not detected)

Formulation Code	Contact Angle (°)
A1	ND*
A2	17.29±2.69
A3	0±0
B1	0±0
B2	11.07±5.52
B3	19.62±5.21
C1	15.73±1.27
C2	24.78±0.71
C3	38.98±10.62

Mucoadhesion Studies

Work of mucoadhesion values of the nanofiber formulations are shown in Figure 6. Work of mucoadhesion increased by increase polymer concentration in C series formulations. The change in mucoadhesion in the A and B series may have resulted from the change in the structure of the fibers due to the electrospinning method. The highest work of mucoadhesion was found in B3 formulation. The difference between the mucoadhesion values of the A2 and A3 formulations and the B3 formulations was statistically insignificant ($p>0.05$). Mucoadhesion is an important factor for vaginal drug delivery and the use of mucoadhesive polymers such as carbopol, polyvinyl alcohol, hydroxyethyl cellulose, and chitosan provides various advantages such as prolonged residence time, improved location on the vagina, and controlled drug release rate (Zong, 2015). The fibers showed mucoadhesive properties for vaginal application.

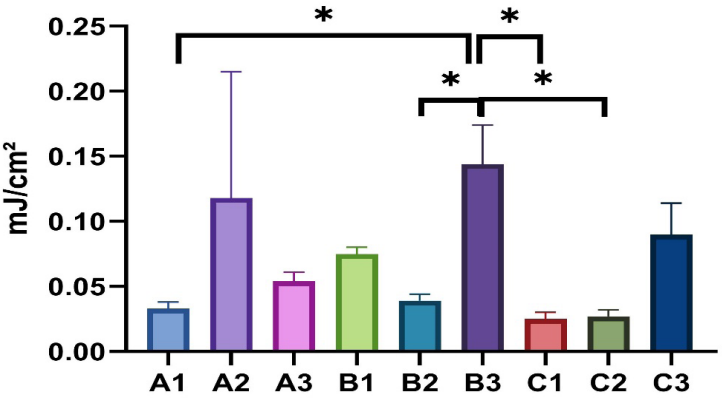

Figure 6. Work of mucoadhesion values of PVA nanofiber with cow vaginal tissue (n=3, mean ± standard deviation, *=$p<0.05$)

CONCLUSION

In this study, we evaluated PVA electrospun fibers as a vaginal delivery system for vaginal use according to their different concentration and different molecular weights. Structural properties, contact angles, mechanical and mucoadhesive properties of PVA nanofibers for vaginal application were investigated. PVA fibers have been shown to have different mechanical and mucoadhesive properties. We showed that the mucoadhesion and mechanical properties of PVA fibers differ depending on the concentration and molecular weight. The nanofiber prepared with PVA moleculer weight with 49.000 Da (Polyviol 13/140) showed continuous fiber production and showed sufficient mechanical and mucoadhesive properties for vaginal application. It was found that the A3 formulation among the vaginal nanofiber formulations prepared with PVA (Polyviol 13/140) has superior properties compared to the others. It was concluded that the A3 nanofiber formulation prepared using PVA (Polyviol 13/140) could be a promising alternative dosage form for the vaginal delivery of different drugs.

ACKNOWLEDGEMENTS

This study was supported by the Scientific Research Fund of Gazi University (BAP Project No: 02/2020-17).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

SS: Conceptualization, Methodology, Writing, Original draft preparation. **FTD:** Corresponding Author, Supervision, Writing, Reviewing and Editing.

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FABAD J. Pharm. Sci., 48, 2, 231-240, 2023
Doi: 10.55262/fabadeczacilik.1200880
RESEARCH ARTICLE

The Potency of Beligo Seeds (*Benincasa hispida* (Thunb.) Cogn.) as Antihyperlipidemic in L-NAME-induced Hyperlipidemic Rats

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The Potency of Beligo Seeds (Benincasa hispida (Thunb.) Cogn.) as Antihyperlipidemic in L-NAME-induced Hyperlipidemic Rats

SUMMARY

Beligo or Bligo is the name of Benincasa hispida (Thunb.) Cogn. in Indonesian, empirically used in the treatment of cholesterol and hypertension. The part of the plant used is the seeds. This study aimed to determine the antihyperlipidemic activity of beligo seeds in hyperlipidemic rats induced by L-NAME. The method of this study, male Wistar albino rats (n = 25) were measured for their initial levels of total cholesterol (TC), high density lipoprotein (HDL), triglyceride (TG), very low density lipoprotein (VLDL) and low density lipoprotein (LDL) using a human analyzer (Thermo Scientific Indico®). All rats were induced by L-NAME 40 mg/kg body weight (BW) for four weeks and then the TC, HDL, TG, VLDL, and LDL levels were measured again. After the discontinuation of L-NAME administration, the treatment was carried out and all rats were divided into five groups consisting of group I as negative control which was given sodium carboxy methyl celulose (CMC) 1%; groups II, III, and IV were given beligo seeds ethanol extract (BSEE) each dose of 100 mg/kg BW, 200 mg/kg BW, 300 mg/kg BW; and group V as the positive control group which was given Simvastatin 10 mg/kg BW. The results showed that the beligo seeds ethanol extract (BSEE) had an antihyperlipidemic activity where doses of 100 mg/kg BW, 200 mg/kg BW, and 300 mg/kg BW could significantly reduce levels of TC, TG, LDL, and VLDL (p<0 .05) and significantly increased HDL levels (p<0.05).

Key Words: *Benincasa hispida (Thunb.) Cogn., Hyperlipidemic, L-NAME, Lipid profile*

Beligo Tohumlarının (Benincasa hispida (Thunb.) Cogn.) L-NAME ile İndüklenen Hiperlipidemik Sıçanlarda Antihiperlipidemik Olarak Potansiyeli

ÖZ

Beligo veya Bligo, Endonezce'de kolesterol ve hipertansiyon tedavisinde ampirik olarak kullanılan Benincasa hispida (Thunb.) Cogn.'un adıdır. Bitkinin kullanılan kısmı tohumlarıdır. Bu çalışma, L-NAME ile indüklenen hiperlipidemik sıçanlarda beligo tohumlarının antihiperlipidemik aktivitesini belirlemeyi amaçlamaktadır. Bu çalışmanın yönteminde, erkek Wistar albino sıçanlarının (n = 25) başlangıç total kolesterol (TK), yüksek dansiteli lipoprotein (YDL), trigliserit (TG), çok düşük dansiteli lipoprotein (ÇDDL) ve düşük dansiteli lipoprotein (DDL) seviyeleri bir insan analizörü (Thermo Scientific Indico®) kullanılarak ölçülmüştür. Tüm sıçanlar, dört hafta boyunca L-NAME 40 mg/kg BW ile uyarılmış ve ardından TK, YDL, TG, ÇDDL ve DDL seviyeleri tekrar ölçülmüştür. L-NAME uygulaması kesildikten sonra tedaviye devam edilmiş ve negatif kontrol olarak sodyum karboksimetilselüloz CMC %1 verilen I. grup; her bir dozu 100 mg/kg vücut ağırlığı (VA), 200 mg/kg VA, 300 mg/kg VA olan beligo tohumları etanol ekstresi (BSEE) verilen gruplar II, III ve IV. grup; Simvastatin 10 mg/kg VA verilen pozitif kontrol grubu V. grup olmak üzere tüm sıçanlar beş gruba ayrılmıştır. Sonuçlar, beligo tohumları etanol ekstraktının (BSEE), 100 mg/kg VA, 200 mg/kg VA ve 300 mg/kg VA dozlarının antihiperlipidemik aktiviteye sahip olduğunu, TK, YDL, TG, ÇDDL, DDL düzeylerini önemli ölçüde azaltabileceğini (p<0.05) ve YDL düzeylerini önemli ölçüde artırabileceğini (p<0.05) göstermiştir.

Anahtar Kelimeler: *Benincasa hispida (Thunb.) Cogn., Hiperlipidemik, L-NAME, Lipit profili*

Received: 08.11.2023
Revised: 15.04.2023
Accepted: 17.04.2023

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INTRODUCTION

Hyperlipidemia is considered to be a major risk factor for cardiovascular diseases. Cardiovascular disease is responsible for the highest disease burden in the world. It is the leading cause of death, morbidity, and health costs in both developed and developing countries, accounting for approximately 30% of annual global deaths and 10% of the world's health burden (Cosenza et al., 2019).

An increase in plasma lipids, including total cholesterol and triglycerides, is one of the main factors causing cardiovascular disease defined as hyperlipidemia. Hyperlipidemia has also been reported as the most widespread marker for susceptibility to atherosclerotic heart disease (Surya et al., 2017).

One of the plants that can be used as a treatment for hyperlipidemia is the seeds of beligo (*Benincasa hispida* (Thunb.) Cogn.). Beligo plant belongs to the Cucurbitaceae family, it is known to be widely available in Asia such as India, China, Malaysia, Japan, and Indonesia, and tropical countries. Beligo is a popular vegetable plant, especially among Asian people to fulfill nutrition and medicine (Al-snafi, 2013).

Beligo or Bligo is the name of *Benincasa hispida* (Thunb.) Cogn. in Indonesian, is empirically used in the treatment of cholesterol and hypertension. The part of the plant used is the seed. Previous research conducted showed that the secondary metabolites contained in beligo seeds are alkaloids, flavonoids, fatty acids, phenolics, and saponins (Aqilah et al., 2010). Meanwhile, according to Samad et al. (2013), beligo seed extract has a high total phenolic and flavonoid content, therefore beligo seed extract can be used as a natural antioxidant. Saponins have broad activities such as the ability to lower cholesterol in the blood. Meanwhile, according to Samad et al. (2013), beligo seed extract has a high total phenolic and flavonoid content, therefore beligo seed extract can be used as a natural antioxidant. Saponins have broad activities such as the ability to lower cholesterol in the blood. Phenolic compounds and flavonoids donate hydrogen to free radicals and thereby break the lipid oxidation chain reaction so that they can act as excellent radical scavengers (Alim et al., 2021, 2022), which is one of the most prominent and medically useful properties, especially in preventing or treating cardiovascular diseases. Fatty acids can lower blood lipid profiles (Burdge & Calder, 2015), namely lowering high levels of cholesterol, triglycerides (TAG), low-density lipoprotein (LDL) and increasing levels of low high-density lipoprotein (HDL) cholesterol (Harris et al., 2018; Kontostathi et al., 2021). Therefore, the content of secondary metabolites can be used in the treatment of hyperlipidemia. And this property has not been evaluated for *Benincasa hispida* (Thunb.) Cogn before.

Beligo seed ethanol extract has a hypoglycemic effect on male Wistar rats (Maryati et al., 2019). The stem alcoholic extract of *Benincasa hispida* has hypoglycemic and antihyperglycemic effects in normal and in alloxan-induced diabetic rats at doses 50,100, 200 mg/kg body weight (BW), per oral (p.o.). The maximum reduction in blood glucose levels with stem extract of *Benincasa hispida* was recorded at a dose of 200 mg/kg BW (Battu et al., 2007). The stem chloroform extract of *Benincasa hispida* has significantly decreased elevated levels of serum glucose, cholesterol, LDL, and triglyceride and increased serum levels of HDL in diabetic rats (Patil et al., 2011). *Benincasa hispida* in a dose of 250 and 500 mg/kg in mice induced a dose-dependent decrease in glucose, triglyceride, and insulin levels in plasma (Al-snafi, 2013). So, this experiment used doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg body weight Wistar rats.

This experiment was conducted to determine the antihyperlipidemic activity of beligo seed ethanol ext-

ract (BSEE) to L-NAME induced hyperlipidemic rats by measurement of increase the levels of HDL and reduction of total cholesterol, triglycerides, very low-density lipoprotein (VLDL) and LDL to compare with positive control of simvastatin which is a synthetic drug.

Simvastatin, a cholesterol-lowering agent, has been widely used in the treatment of hyperlipidemia**.** As a comparison, simvastatin was used, which is included in the statin class and is the first-line drug to treat hypercholesterolemia. Comparison as a positive control was used to get a clearer picture of the effect of beligo seed ethanol extract on hyperlipidemic rats. Simvastatin works by inhibiting cholesterol synthesis in the liver, and by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. As a result of this reduction in cholesterol synthesis, the sterol regulatory element binding proteins (SREBPs) contained in the membrane are then transported to the nucleus. Transcription factors will then bind to the LDL receptor gene, increasing LDL receptor synthesis. Increasing the number of LDL receptors on the hepatocyte cell membrane will reduce cholesterol levels even more. Apart from LDL, VLDL, and IDL also decreased, while HDL increased (Bhattarai et al., 2020; Welty et al., 2016) significantly at 10 mg/kg and 20 mg/kg orally (Verma et al., 2022).

MATERIAL AND METHODS

Chemical Material

N(G)-nitro-L-arginine methyl ester hydrate (L-NAME) (Sigma Aldrich), reagent HDL, triglycerides, total cholesterol for human analyzer (Thermo Scientific Indico®), simvastatin (Sigma Aldrich), 70% ethanol and other chemical material obtained by the official chemical store in Makassar, South Sulawesi, Indonesia.

Plant Collection

Beligo fruit is collected from farmers in South Sulawesi, Indonesia. Determined in the Biology laboratory of Makassar State University. The sample herbarium is stored in the Pharmacognosy-Phytochemical Laboratory of the Islamic University of Makassar.

Extraction

The sample of beligo seeds (as shown in Figure 1) was extracted by maceration method (Rusdi, M. et al., 2017) using 70% ethanol. Beligo seeds that have been separated from the rest of the fruit, are dried and powdered. Extracted using 70% ethanol, evaporated to produce a thick extract, and freeze-dried to obtain a dry extract (Samad et al., 2013). Stored in a refrigerator at 2-8° Celsius until used in the test (Tata et al., 2019).

Figure 1. Beligo fruit and seed

Animal Preparation

Male Wistar albino rats (n=25) aged 2-3 months, weighing 200-250 g were obtained from animal breeders in the City of Bandung, West Java Province, Indonesia, and were declared healthy and free from infectious animal diseases by the Department of Food Security and Agriculture of the City of Bandung, certified with Number: TN.01.01.11 /4543-DKPP/XI/2021. Male Wistar albino rats (n=25) aged 2-3 months, weighing 200-250 grams were obtained from animal breeders in the City of Bandung, West Java Province, Indonesia, and were declared healthy and free from infectious animal diseases by the Department of Food Security and Agriculture of the City of Bandung, certified with Number: TN.01.01.11 /4543-DKPP/XI/2021.

All rats were adapted for seven days before the study was conducted to get used to the experimental environment and placed in animal cages with 12 hours of day and night lighting each. The animals had free access to food and water. All animal protocols were performed by the Guide for the Care and Use of Laboratory Animals. This experiment was carried out after obtaining an ethical approval recommendation from the Health Research Ethics Commission, Faculty of Medicine, Muslim University of Indonesia, and IBNU SINA YW-UMI Hospital with Number: 073/A.1/KEPK-UMI/2021 on February 25, 2021.

Experimental Protocol

This protocol is based on the modification of Tata et al., (2019) and Salam, et al., (2016). After being adapted for seven days, the levels of TC, HDL, TG, VLDL, and LDL were measured. All rats were induced by L-NAME 40 mg/kg BW for four weeks and then the total cholesterol (TC), HDL, triglyceride (TG), VLDL, and LDL levels were measured again. After discontinuation of L-NAME administration, the treatment was carried out.

Animals were randomized and divided into five groups of five animals per group (n = 5) as follows:

Group I: L-NAME + Sodium CMC 1% (Sodium Carboxy Methyl Cellulose 1%)

Group II: L-NAME+BSEE 100 mg/kg BW

Group III: L-NAME+BSEE 200 mg/kg BW

Group IV: L-NAME +BSEE 300 mg/kg BW

Group V: L-NAME + SIMVASTATIN 10 mg/kg BW

All treatment groups were given via the oral route.

SOD. CMC 1% is used as a negative control because the ethanol extract BSEE is insoluble in water, so a suspension is needed so that BSEE is suspended homogeneously. BSEE is a plant extract as a test sample. Simvastatin is a synthetic drug as a positive control.

After four weeks of treatment, TC, HDL, TG, VLDL, and LDL levels were measured. Measurement of lipid profile levels using a human analyzer (Thermo Scientific Indico®).

Measurement of the lipid profile of rat blood

Animals are anesthetized first by inhalation using ether. Blood was drawn from the lateral vein in the rat's tail and through the orbital sinus in the eye with a microhematocrit pipette. Blood was collected in a microtube and allowed to stand for 5 minutes and centrifuged for 20 minutes at a speed of 3000 rpm until serum was obtained.

Measurement of total cholesterol (TC) levels

Pipette blood serum as much as 500 µL into the sample cup, and place the sample according to the position of the data inputted on the Thermo Scientific Indico® instrument. After pressing start, the sample reagent needle will take the total cholesterol reagent after it is incubated and then read at a wavelength of 550 nm.

Measurement of high-density lipoprotein (HDL) level

Pipette blood serum as much as 500 µL into the sample cup, and place the sample according to the position of the data inputted on the Thermo Scientific Indico device. After pressing start, the sample reagent needle will take the HDL reagent after it is incubated and then read at a wavelength of 600 nm.

Measurement of triglyceride (TG) and very low-density lipoprotein (VLDL) levels

Pipette blood serum as much as 500 µL into the sample cup, and place the sample according to the position of the data inputted on the Thermo Scientific Indico device. After pressing start, the sample reagent needle will take the triglyceride reagent after it is incubated and then read at a wavelength of 510 nm.

Measure VLDL levels, it is calculated by the formula of the Friedewald equation as described by Vuilleumier et al., (2010):

VLDL = TG/5.

Measurement of low-density lipoprotein (LDL) levels

Measure LDL levels, it is calculated by the formula of the Friedewald equation as described by Vuilleumier et al., (2010):

TC = HDL + LDL + VLDL

Which, VLDL = TG/5.

So , LDL = TC - HDL - VLDL

Data analysis

The results are presented in the form of mean ± standard error of the mean (SEM). Statistic analysis using the paired T-test (comparing before-induced, and induced-post treatment) to determine differences. Before the data analysis, the normality and homogeneity of the data were first tested. *One-way* analysis of variance (ANOVA) followed by Tukey's HSD *posthoc* test for multiple comparisons was performed to determine differences between treatment groups. Statistical tests were carried out at a 95% confidence level and this difference was significant if the *p*-value was less than 0.05.

RESULTS AND DISCUSSION

Table 1a. Lipid profile of TG and VLDL of rats, before and after L-NAME induced for four weeks, and post-treatment for four weeks

Group	Parameter (mg/dL)					
	TG			VLDL		
	Before induced	L-NAME induced	Post Treatment	Before induced	L-NAME induced	Post Treatment
LN + SOD. CMC 1%	38.40±1.14	86.60±3.78*	79.60±4.83##	7.68±0.23	17.32±0.75*	16.76±0.63##
LN+ BSEE 100 mg/kg BW	46.80±3.83	82.60±3.78*	49.40±1.14**#	9.36±0.08	16.52±0.76*	9.80±0.24**#
LN+ BSEE 200 mg/kg BW	47.80±0.84	89.00±0.00*	48.80±1.48**#	9.56±0.17	16.60±0.00*	9.76±0.29**#
LN+ BSEE 300 mg/kg BW	45.20±1.48	98.40±5.41*	45.20±1.09**#	9.16±0.33	19.68±1.08*	8.68±0.27**#
LN + Simvastatin 10 mg/kg BW	45.40±3.28	97.60±0.55*	46.80±1.48**#	9.12±0.63	19.52±1.11*	8.80±0.25**#

LN= L-NAME, BSEE= Beligo seed ethanol extract, BSEE = Beligo Seed Ethanol Extract; LN + SOD.CMC = L-NAME + Sodium Carboxy Methyl Cellulose control group; LN+Simvastatin = L-NAME + Simvastatin control group;

*p<0.05 compared to before induced and **p<0.05 compared to L-NAME induce by the paired T-Test;

#p< 0.05 compared to LN + SOD.CMC 1% control group and ##p < 0.05 compared to LN + Simvastatin by Tukey's HSD *posthoc*

Table 1b. Lipid profile of LDL and TC of rats, before and after L-NAME induced for four weeks, and post-treatment for four weeks

Group	Parameter (mg/dL)					
	LDL			TC		
	Before induced	L-NAME induced	Post Treatment	Before induced	L-NAME induced	Post Treatment
LN + SOD. CMC 1%	4.72±2.67	39.16±1.12*	37.32±1.78##	58.20±1.30	96.20±1.30*	94.60±1.14##
LN+ BSEE 100 mg/kg BW	5.24±2.29	40.48±4.31*	6.20±3.69**#	56.20±2.39	92.00±1.58*	74.80±3.96**#
LN+ BSEE 200 mg/kg BW	4.04±2.50	43.00±4.09*	5.24±3.69**#	56.40±3.05	98.40±2.97*	79.20±5.54**#
LN+ BSEE 300 mg/kg BW	5.44±1.56	38.92±2.41*	1.52±1.05**#	58.20±1.64	95.60±2.79*	73.00±2.44**#
LN + Simvastatin 10 mg/kg BW	7.48±2.30	37.48±1.49*	1.20±0.51**#	57.60±1.82	92.00±1.00*	74.40±1.51**#

LN= L-NAME, BSEE= Beligo seed ethanol extract, BSEE = Beligo Seed Ethanol Extract; LN + SOD.CMC = L-NAME + Sodium Carboxy Methyl Cellulose control group; LN+Simvastatin = L-NAME + Simvastatin control group;

*p<0.05 compared to before induced and **p<0.05 compared to L-NAME induce by the paired T-Test;

#p < 0.05 compared to LN + SOD.CMC 1% control group and ##p < 0.05 compared to LN + Simvastatin by Tukey's HSD *posthoc*

Table 1c. Lipid profile of HDL of rats, before and after L-NAME induced for four weeks, and post-treatment for four weeks

Group	Parameter (mg/dL)		
	HDL		
	Before induced	L-NAME induced	Post Treatment
LN + SOD. CMC 1%	45.80±1.30	39.60±0.55*	40.00±1.00##
LN+ BSEE 100 mg/kg BW	42.40±0.55	37.00±1.00*	58.60±0.55**#
LN BSEE 200 mg/kg BW	44.40±0.55	39.80±1.09*	65.00±1.00**#
LN+ BSEE 300 mg/kg BW	43.60±0.55	37.00±2.74*	65.40±0.55**#
LN + Simvastatin 10 mg/kg BW	41.00±1.00	35.00±1.08*	60.60±1.00**#

LN= L-NAME, BSEE= Beligo seed ethanol extract, BSEE = Beligo Seed Ethanol Extract; LN + SOD.CMC = L-NAME + Sodium Carboxy Methyl Cellulose control group; LN+Simvastatin = L-NAME + Simvastatin control group;

*p<0.05 compared to before induced and **p<0.05 compared to L-NAME induce by the paired T-Test;

#p < 0.05 compared to LN + SOD.CMC 1% control group and ##p < 0.05 compared to LN + Simvastatin by Tukey's HSD *posthoc*

Table 2. The percentage of decrease of TG, VLDL, LDL, TC, and increase of HDL post-treatment in L-NAME-induced hyperlipidemic rats

GROUP	Parameter (mg/dL)				
	TG	VLDL	HDL	LDL	TC
LN + SOD. CMC 1%	8.80%	5.42%	1.00%	4.69%	1.66%
LN+ BSEE 100 mg/kg BW	67.20%	40.68%	36.86%	84.68%	18.70%
LN+ BSEE 200 mg/kg BW	82.38%	41.20%	38.77%	87.81%	19.51%
LN+ BSEE 300 mg/kg BW	117.70%	55.89%	43.43%	96.09%	23.64%
LN + Simvastatin 10 mg/kg BW	100.00%	54.92%	45.48%	96.80%	19.13%

Plant Sample

This study used a sample of beligo seeds. The health benefits of the *Benincasa hispida* (Thunb.) Cogn. seeds may be related to their rich fatty acids, flavonoid, phenolic, and saponin contents. Indeed, studies have demonstrated the usefulness of plant fatty acids, flavonoids, and phenolic content in the prevention of hyperlipidemia (Ramchoun et al., 2020) and these properties have been evaluated in this experiment.

Lipid profile

Administration of L-NAME for four weeks in this experiment can improve lipid profile. L-NAME is an inhibitor of endothelial nitric oxide synthase (eNOS), which is widely used as an inducer of the hypertension model but also has an effect on increasing lipid profiles so that it can be used as an inducer of the hyperlipidemia model (Aluko et al., 2020; Tata et al., 2019). Treatment with L-NAME resulted in decreased HDL significantly ($p<0.05$) and increased TC, LDL, TG, and VLDL significantly ($p<0.05$) in all groups compared to baseline as shown in (Table 1a., Table 1b., and Table 1c.), thus corroborating the findings of (Salam, et al., 2016) who showed that L-NAME treatment harmed lipid profiles in treated rats. L-NAME treatment raised the concentration of TC, LDL, TG, and VLDL and reduced the concentration of HDL which in turn was capable of interfering with eNOS activity. Under normal conditions, eNOS is associated with cholesterol-enriched caveolae in endothelial cells, where its activity can be carefully regulated (Shu et al., 2015). However, in hyperlipidemia, LDL, especially oxidized LDL (ox-LDL) negatively affects the activity and subcellular distribution of eNOS hence leading to a decrease in NO bioavailability (Förstermann & Münzel, 2006; Shaul, 2003) which is generated by eNOS. On the other hand, HDL causes the activation of eNOS within the caveolae, with the resultant generation of NO (Talas et al., 2014). The result of our experiment showed that BSEE at the dose of 100 mg/kg BW, 200 mg/kg BW and 300 mg/kg BW reduced significantly ($p<0,05$) levels of TC, TG, LDL, and VLDL and increased significantly ($p<0,05$) HDL levels compared to simvastatin as a positive control and Sod. CMC 1% is used as a negative control (shown in Table 1a., Table 1b. and Table 1c.). The percentage reduction of all groups is shown in Table 2. These results indicate that BSEE can be reducing of TC, LDL, TG VLDL, and increasing of HDL by increasing eNOS activity compared to simvastatin (Tata et al., 2019).

CONCLUSION

The results showed that administration of BSEE has an antihyperlipidemic activity which at a dose of 100 mg/kg BW, 200 mg/kg BW, and 300 mg/kg BW can reduce significantly ($p<0,05$) levels of TC, TG, LDL, and VLDL and can increase significantly ($p<0,05$) HDL levels.

ACKNOWLEDGEMENTS

The authors would like to thank profusely the supervisors and all parties and stakeholders who have helped so that this research runs smoothly until it becomes an article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

NA as the research leader is responsible for compiling and conceptualizing the research flow, carrying out research, interpreting data, and compiling and revising the manuscript. RH is responsible for extracting, collecting research data, and conducting research. HR, AB, ND, and YYD are responsible as advisors and directors for conducting research, data interpretation, and manuscript revision.

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FABAD J. Pharm. Sci., 48, 2, 241-254, 2023
Doi: 10.55262/fabadeczacilik.1213447
RESEARCH ARTICLE

Antibiofilm Effect of *Moringa oleifera* Leaf Extract Against *Staphylococcus aureus*, Cytotoxicity, Biochemical aspects, Anti-Inflammatory potential, and Interference on the Activity of Antimicrobial Drugs

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Antibiofilm Effect of Moringa oleifera Leaf Extract Against Staphylococcus aureus, Cytotoxicity, Biochemical aspects, Anti-Inflammatory potential, and Interference on the Activity of Antimicrobial Drugs

SUMMARY

There is a growing technical difficulty in treating infectious diseases due to bacterial resistance to antimicrobial drugs, such as biofilm formation. Here we provide evidence of the antimicrobial potential of the hydroethanolic extract of Moringa oleifera leaves, traditionally used to treat disorders like cardiovascular and endocrine diseases, on clinical isolates of Staphylococcus aureus. The plant extract was chemically characterized using classic techniques and by ultra-performance liquid chromatography (UPLC). We carried out minimum inhibitory concentration tests, minimal bactericidal concentration and minimal biofilm eradication concentration tests. Moreover, we tested the anti-inflammatory potential and assessed the toxicity of the extract on buffalo green monkey (BGM) cells. We also investigated the effects of combining the extract with clinically relevant antimicrobial drugs (i.e., synergistic or antagonistic interactions). The extract was active at 8 μg/mL and 16 μg/mL for planktonic cells and biofilms, respectively. Its anti-inflammatory potential was confirmed, and it lacked cytotoxicity. No significant interference of the extract on antimicrobial drugs was observed. Flavonoids, tannins, proteins, carbohydrates and vitamin C were detected in the extract. Our data open doors for further studies with isolated molecules of the extract in order to conduct in vivo antimicrobial tests.

Key Words: *Moringa oleifera, Staphylococcus aureus, antimicrobial, biofilms.*

Moringa oleifera Yaprağı Ekstresinin Staphylococcus aureus'a Karşı Antibiyofilm Etkisi, Sitotoksisitesi, Biyokimyasal Yönleri, Anti-inflamatuar Potansiyeli ve Antimikrobiyal İlaçların Aktivitesi Üzerinde Etkileşimi

ÖZ

Biyofilm oluşumu gibi antimikrobiyal ilaçlara karşı bakteriyel direnç nedeniyle infeksiyon hastalıklarının tedavisinde teknik zorluklar artış göstermektedir. Burada, geleneksel olarak kardiyovasküler ve endokrin hastalıklar gibi bozuklukları tedavi etmek için kullanılan Moringa oleifera yapraklarının sulu etanol ekstresinin Staphylococcus aureus'un klinik izolatları üzerindeki antimikrobiyal potansiyeline dair kanıtlar sunuyoruz. Bitki ekstresi, klasik teknikler ve ultra performanslı sıvı kromatografisi (UPLC) kullanılarak kimyasal olarak karakterize edilmiştir. Minimum inhibitör konsantrasyon testleri, minimum bakterisidal konsantrasyon ve minimum biyofilm eradikasyon konsantrasyon testleri gerçekleştirilmiştir. Ayrıca, anti-inflamatuar potansiyeli test ettik ve ekstrenin bufalo yeşil maymunu (BGM) hücreleri üzerindeki toksisitesini değerlendirdik. Ekstreyi klinik olarak ilgili antimikrobiyal ilaçlarla kombine etmenin etkilerini (yani sinerjistik veya antagonistik etkileşimler) de araştırdık. Ekstre, planktonik hücreler ve biyofilmler için sırasıyla 8 μg/mL ve 16 μg/mL'de aktif bulundu. Anti-inflamatuar potansiyeli doğrulandı ve sitotoksik değildi. Ekstrenin antimikrobiyal ilaçlar üzerinde önemli bir etkileşimi gözlenmedi. Ekstrede flavonoidler, tanenler, proteinler, karbonhidratlar ve C vitamini tespit edildi. Verilerimiz, in vivo antimikrobiyal testler yapmak için ekstraktın izole edilmiş molekülleri ile daha ileri çalışmalar için kapılar açmaktadır.

Anahtar Kelimeler: *Moringa oleifera, Staphylococcus aureus, antimikrobiyal, biyofilmler.*

Received: 02.12.2022
Revised: 17.04.2023
Accepted: 24.04.2023

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterial species that can be both commensal on the mucosa and skin of humans and an opportunistic pathogen (Krismer et al., 2017). It causes foodborne infections and diseases such as otitis, osteomyelitis, endocarditis, septicemia and mastitis, which also affect milking animals (Abril et al., 2020; Miller et al., 2020). *S. aureus* is among the most common pathogens detected in hospital and community infections, and is associated to elevated mortality rates due to antimicrobial resistance (Kwiecinski et al., 2020; Guo et al., 2020). The main mechanisms of resistance found in this species include enzymatic inactivation of antimicrobials, biochemical alterations that decrease the affinity of microbial molecular targets to the drugs, and biofilm formation (Guo et al., 2020; Miller et al., 2020).

Biofilms are microbial microcolonies that develop in an adhesive matrix of extracellular polymeric substances (EPS), generally composed of carbohydrates, proteins and lipids (Penesyan et al., 2021). Biofilms are polymicrobial in humans, animals, and in most ecosystems. The biochemical composition of the EPS is influenced by factors that comprise the amount and diversity of adhered microbial species, physical and chemical characteristics of the surface where the biofilm is developing, and nutrient availability (Rumbaugh and Sauer, 2020; Penesyan et al., 2021). Biofilms act as physical and chemical barriers to the diffusion and action of antimicrobials (Koo et al., 2017; Brito, 2021). As a result, large amounts of drugs are necessary for prolonged periods, posing risks of adverse reactions such as nephrotoxicity, ototoxicity, and extended bacterial resistance (Rumbaugh and Sauer, 2020; Maslova et al., 2021). Curiously, despite the exposed, the antibiofilm effect of potential antimicrobials of natural sources are not explored as for planktonic (free) cells.

Natural products are relevant sources of antimicrobial molecules, for which reports of microbial resistance are rare. *Moringa oleifera* is traditionally used to treat different disorders such as hypertension and diabetes (Mabrouki et al., 2020). The leaves of the plant are the most consumed part in several countries, due to their high levels of carotenoids, vitamins and minerals like calcium and iron (Leone et al., 2015; Stohs and Hartman, 2015; Dhakad et al., 2019). Different biological properties of *M. oleifera* have been described, including immunomodulatory, hepatoprotective and neuroprotective (Dhakad et al., 2019; Fernandes et al., 2021). Nevertheless, some questions remain unanswered concerning the antimicrobial potential of *M. oleifera* extracts, such as antibiofilm potential and effects when combined to antimicrobial drugs.

Here we show that *M. oleifera* leaf extract (MOLE) is effective against planktonic cells and biofilms of clinical isolates of *S. aureus* and investigated its anti-inflammatory potential. We also investigated its cytotoxicity and its influences on the activity of antimicrobial drugs. Biochemical and ultraperformance liquid chromatography (UPLC) analysis confirmed the presence of polyphenols, proteins, carbohydrates and vitamin C. Our study opens doors for more investigations towards the use of *M. oleifera* for staphylococcal diseases.

MATERIALS AND METHODS

Preparation of the extract

We used a fresh powder (20 g) of Brazilian *M. oleifera* fresh leaves (obtained by spray drying) produced and provided by RVN (MG, Brazil). An authenticity certificate supplied by the manufacturer confirmed the botanical identity of the plant. The leaves are collected during the early hours in the city of Carlos Chagas (Minas Gerais state, Brazil).

The powder was sifted before being suspended in 70% cold ethanol (150 mL), with slow maceration. The powder remained in the solvent for 48 h at 4 °C. Following, this system was centrifuged (5000 *g*, 10 min.), and the supernatant was concentrated by a rota evaporator (40 °C) until a dark paste was obtained. The

final product was weighed (275.38 mg) and stored at 4 °C until used. A stock solution of 4 mg/mL of MOLE was prepared using sterile 0.9% saline (pH 7.2).

Biochemical characterization assays

We conducted the following biochemical analyzes for the leaf powder and MOLE in triplicate. For the leaf powder, the American Association of Cereal Chemists (AACC) Micro-Kjeldahl 46-13.01 method was used to detect and quantify total protein content (AACC, 2010). Calcium, iron and vitamin C levels were determined using the titration methods standardized by the Adolfo Lutz Institute (2008). Ashes and total fibers were analyzed following the Association of Official Analytical Chemists (AOAC) 923.03 method (AOAC, 2005), and the ISO 5498 method (ISO, 1981), respectively.

For MOLE (at 4 mg/mL), we used the biuret reaction to quantify total proteins (Gornall et al., 1949), with a calibration curve prepared using bovine serum albumin (BSA, Thermo Fisher, USA). Total carbohydrates were quantified by using the phenol-sulphuric method (Dubois et al., 1956), with a calibration curve prepared with glucose. The presence of vitamin C was confirmed using the molybdenum blue reaction (Bajaj and Kaur, 1981). Flavonoids and tannins were qualitatively detected in the extract using the Shinoda method and the ferric chloride reaction, respectively (Harborne, 1988).

Ultra-performance liquid chromatography

The stock solution of the extract was diluted to 1 mg/mL in ultra-purified Mili-Q water and filtered through a 220 nm PVDF membrane. An aliquot of 1 µL of the filtrate was injected on a Shimadzu Shim-Pack XR-ODS-III column (C18, 2.2 µm, 2.0×150 mm) of a Nexera UPLC-system (Shimadzu). The analysis was conducted at 220 nm, with a flow rate of 400 µL/min, at 40 °C. The mobile phase A consisted of a 0.1% formic acid aqueous solution, and phase B was a 0.1% solution of formic acid prepared in acetonitrile. An isocratic run was performed using 5% B and 95% A for 5 min, followed by a linear gradient to 100% B in 40 min and a hold at 100% B for 5 min.

Bacterial strains

Ten strains of *S. aureus* were used in this study. They are part of the microorganisms collection from Pitágoras College and were isolated from hemodialysis catheter tips. Their identity was confirmed using Gram-positive cards for VITEK 2 system (version R04.02, bioMérieux) following the manufacturer's instructions. Following, we used two different approaches to assess the isolates' ability to produce β-lactamase: the zone-edge method described by Gill et al. (1981), which uses antimicrobial disks of penicillin and oxacillin, and the iodometric test described by Jarløv and Rosdahl (1986), to confirm the zone-edge results.

Antimicrobial assays

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal biofilm eradication concentration (MBEC) of the extract were determined in triplicate following CLSI standards and a protocol standardized by our group (Dias-Souza et al., 2017; CLSI, 2018). The extract was prepared as an aqueous solution to reach final concentrations ranging from 1024 to 8 µg/mL, and the final concentration of the bacterial suspensions was $5x10^4$ CFU/mL. Before the MBEC assay, biofilm formation was induced overnight as described (Dias-Souza et al., 2013a). We used resazurin staining (0.1 g/L, 50 µL) in MIC and MBEC assays to analyze the results. MBC was determined by the direct plate count of colonies.

Interference of the extract on the activity of antimicrobial drugs

We investigated the effect of combining the extract to antimicrobial drugs against *S. aureus* isolates using the Dias-Souza method (Dias-Souza et al., 2013b) in duplicate. MOLE was prepared in MIC and MBEC values in sterile saline, and was added to antimicrobial disks (all from Sensifar, Brazil) of cephalothin (30 µg), ceftriaxone (30 µg) and chloramphenicol (30 µg). Interpretation parameters for statistically significant

(or the tendency of) synergism or antagonism were applied to the results as described (Dias-Souza et al., 2013b). Disks without the addition of MOLE were used as controls.

Anti-inflammatory assay

We used the BSA denaturation assay in triplicate to assess the anti-inflammatory potential of MOLE. This method is based on the ability of a determined compound to inhibit molecular events that unfold upon protein denaturation by heat, such as the formation of aggregates and exposure of chromophore groups. We followed the method and calculations described by Marius et al. (2020), with slight modifications. BSA (ThemoFisher, USA) denaturation was conducted for 15 minutes at 70 °C, using Tenoxicam (Sigma) as a standard non-steroidal anti-inflammatory drug (NSAID) (positive control, 500 µg/mL). Pure BSA exposed to heat was used as a negative control. MOLE was tested at the same concentrations used for antimicrobial activity assays.

Cytotoxicity assay

MOLE was tested for cytotoxic effects in triplicate using BGM cells as described by our group (Dias-Souza et al., 2018). Cells were exposed to the extract in concentrations ranging from 600 to 4.687 µg/mL for 18h. Neutral red uptake was used to assess cell viability as described (Siqueira et al., 2018).

Statistics

Homocedacisty of data was assessed using the Bartlett test. Normality was assessed using the Shapiro-Wilk test. For data with parametric distribution, we used ANOVA followed by the Tukey test. For non-parametric data, we used Kruskal-Wallis followed by the Dunn test. All analyses were carried out using Bioestat 5.0 for Windows.

RESULTS and DISCUSSION

Biochemical characteristics of the extract

The main characteristics of the leaf powder are shown in Table 1. The levels of vitamin C and proteins were higher than the other nutrients. Total ashes content corresponded to approximately 9%. Iron levels were superior to calcium levels.

Table 1: Biochemical parameters of the leaf powder

Total proteins	Total fibers	Ashes	Total iron	Total calcium	Vitamin C
31.74 mg/100g	7.19 g/100g	9.11 g/100g	17.62 mg/100g	1.32 mg/100g	293 mg/100g

The characteristics of MOLE are presented in Table 2. The qualitative data suggested the presence of flavonols in the extract. The UPLC chromatogram indicated the presence of flavonoids in the extract (Figure 1). Retention times of the proeminent peaks were suggestive of gallic acid (~1 min), caffeic acid (~10 min), rutin (~12 and 52 mins), quercetin (~14, 15 min), kaempferol (16 min), isoquercetin (~22 min), and apigenin (~26 min), as described elsewhere (Makita et al., 2016; Zhu et al., 2021).

Table 2: Biochemical parameters of MOLE (Flavonoids, tannins, and Vitamin C were analyzed using qualitative assays)

Total proteins	Total carbohydrates	Flavonoids	Tannins	Vitamin C
6.83 µg/mL	415.15 µg/mL	Positive	Positive	Positive

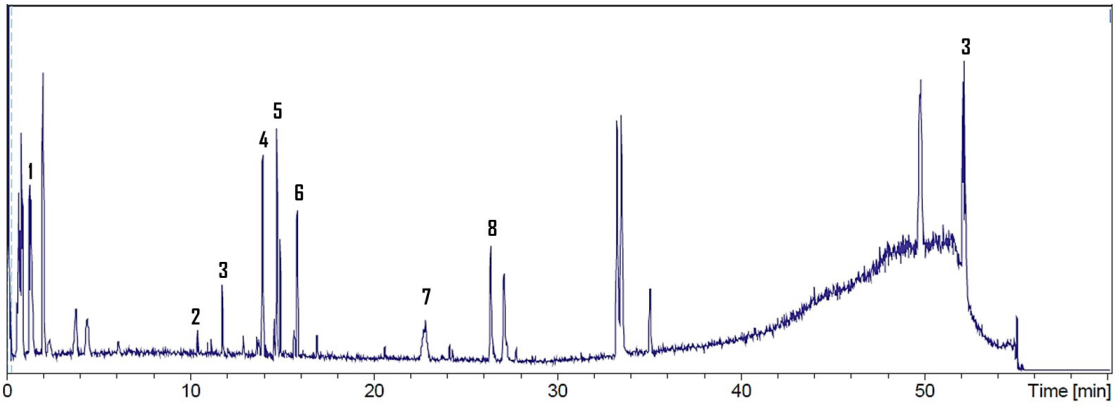

Figure 1. UPLC chromatogram for *M. oleifera* leaf extract (C18 column, 220 nm) for polyphenolics. Peaks: gallic acid (1), caffeic acid (2), rutin (3), quercetin (4, 5) kaempferol (6), isoquercetin (7), apigenin (8). Y-axis represent arbitrary units of readings.

Antimicrobial activity of MOLE

Using the zone-edge and iodometric tests, we confirmed that from the 10 tested isolates, the isolates n#1 to 7 are producers of β-lactamases. MOLE was active against all the bacterial isolates as planktonic cells and biofilms (Table 3). Given that the MIC and MBC values were equally the same for the isolates, we suggest that the extract presents a bactericide effect (Levinson et al., 2009).

Table 3. Results of the antimicrobial assays

MIC	8 µg/mL
MBC	8 µg/mL
MBEC	16 µg/mL

Results are referent to all tested strains

We investigated if combinations of MOLE to clinically relevant antimicrobials in disks would result in synergistic or antagonistic interactions. When comparing the antimicrobials without the addition of MOLE, ceftriaxone was the most effective drug (29.2±1.22 mm, $p<0.05$). Surprisingly, the interference of MOLE on the antimicrobials was very discrete (Table 4, $p>0.05$), and did not meet the criteria to be characterized as synergistic or antagonistic (i.e., sizes of the inhibition zones did not change ±2mm when compared to control). This suggests its safety in an eventual simultaneous use with these drugs, but further *in vivo* experiments are still necessary to confirm this observation.

Table 4. Inhibition zones of antimicrobials with or without addition of *M. oleifera* extract.

Isolates	CEPH	CEPH +E	CEFT	CEFT +E	CHLO	CHLO +E
S1	23	24	30	31	25	25
S2	22	21	28	29	23	24
S3	20	21	30	30	24	25
S4	25	26	29	28	25	25
S5	24	23	27	28	26	27
S6	22	23	28	29	28	29
S7	21	22	29	30	23	24
S8	22	22	31	31	25	24
S9	25	25	30	29	24	25
S10	24	25	30	31	27	27

Data are expressed as averages of duplicates in mm. CEPH: cephalothin. CEFT: ceftriaxone. CHLO: chloramphenicol. +E: addition of the extract at the MIC. *S. aureus* isolates are identified as S+number.

Anti-inflammatory potential and cytotoxicity

The extract presented anti-inflammatory effect at MIC and MBEC values, with no statistical difference between them (Figure 2). Thus, antimicrobial and anti-inflammatory properties of MOLE might be observed simultaneously. Tenoxicam (500 µg/mL) and the highest tested concentration of MOLE (1024 µg/mL) were significantly (although numerically slightly) less effective than MOLE in MIC (8 µg/mL) and MBEC (16 µg/mL) in preventing BSA denaturation ($p<0.05$). The extract had no cytotoxic effect on BGM cells in any tested concentration (data not shown).

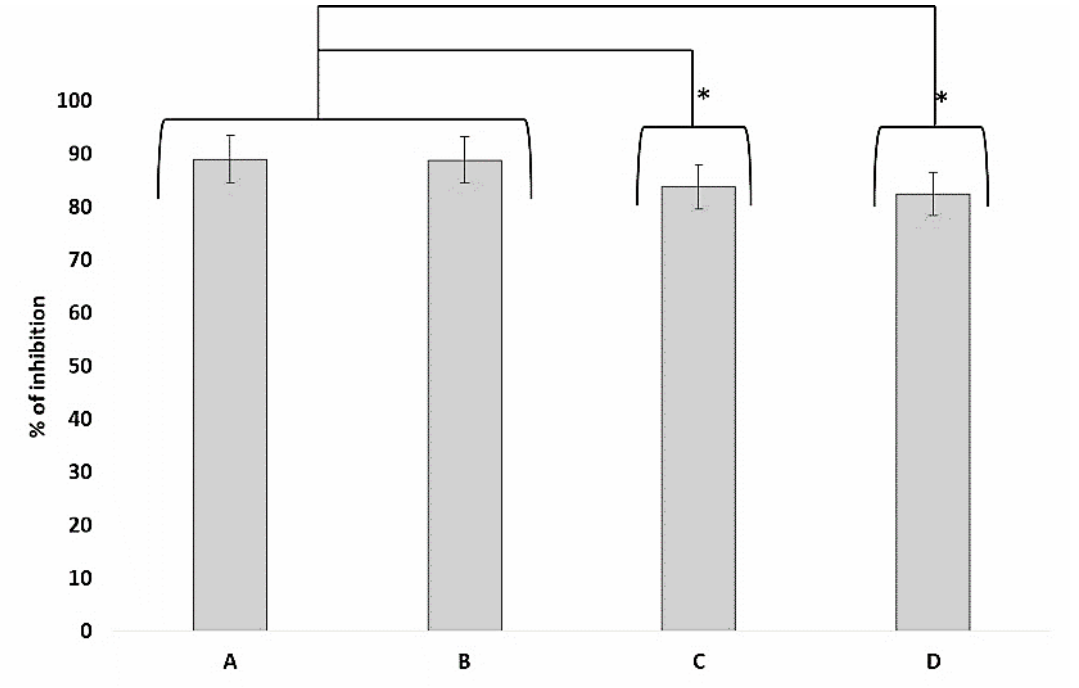

Figure 2. Anti-inflammatory potential of the extract was assessed as inhibition of BSA denaturation by heat. A: extract at MIC (8 µg/mL). B: extract at MBEC (16 µg/mL). C: extract at 1000 µg/mL. D: Tenoxicam (500 µg/mL). *: $p<0.05$

Discussion

M. oleifera leaves are explored as plant food for their varied nutritional benefits (Leone et al., 2015), but data concerning its antimicrobial and antibiofilm effects remain scarce. In the present study, the hydroethanolic extract of *M. oleifera* leaves was active at 8 µg/mL against planktonic cells of *S. aureus* isolates, and at 16 µg/mL against overnight-formed biofilms. This antibiofilm potential is especially relevant as MBEC might be more than 1000 times superior to MIC, due to biofilms' ability to hamper the diffusion and action of antimicrobials (Dias-Souza et al., 2017).

To the best of our knowledge, the MIC, MBC and MBEC values reported here are the lowest described for *M. oleifera* leaf extract against *S. aureus*. MOLE was prepared with a fine particles leaf powder obtained by spray drying. Compared to leaves used in laboratory scale experiments, the powder used in this study has an increased content of phytomolecules per unit of mass. Most of the available works on the antimicrobial activity of *M. oleifera* tested aqueous extracts of parts such as leaves and seeds. However, water is not as efficient as organic solvents like alcohols in extracting bioactive molecules from plants (Jwa, 2019). Furthermore, variations on soil nutrition,

endophytic microbiota, frequency and intensity of the rains (or irrigation), collection and storage procedures, and of methods of harvest and extraction, may all profoundly impact the diversity and level of phytomolecules in plant extracts (Cock, 2011; Masota et al., 2021). Moreover, the sources of bacterial strains are variable and their susceptibility to antimicrobial drugs and plant extracts is poorly predictable. Taken together, these reasons help to explain our remarkable results on the antimicrobial activity of MOLE, which are consistent with the existing studies on the activity of different types of *M. oleífera* leaf extracts against bacterial planktonic cells and biofilms.

The acetone extract prepared with *M. oleifera* leaves was active at 420 µg/mL against planktonic (free) cells of a *S. aureus* reference strain (Ratshilivha et al., 2014). Flavonoids of *M. oleifera* seed coat were active at 50 µg/mL against planktonic cells and biofilms of a reference *P. aeruginosa* strain, and at 80 and 240 µg/mL against planktonic cells and biofilms of *S. aureus,* respectively (Onsare and Arora, 2015). More recently, a study found that the MIC of the leaf extract against clinical isolates of *S. aureus* ranged from 800 to 2000 µg/mL (Zubair, 2020). Biofilms of an environmental strain of *S. aureus* were susceptible to the aqueous leaf extract at 20, 40, and 60 mg/mL (Nasr-Eldin et al., 2017). MIC and MBC of an aqueous leaf extract against an environmental strain of *S. aureus* were 250 and 1000 µg/mL, respectively, and inhibition of biofilm formation required concentrations higher than 250 µg/mL (De Oliveira et al., 2020).

A common (and often dangerous) practice among patients in several countries seeking to increase or accelerate antimicrobial therapy outcomes is combining antimicrobials to phytoextracts. This practice is supported by an inaccurate assumption that combining antimicrobial drugs and phytoextracts with antimicrobial activity results in synergistic interactions (Dias-Souza et al., 2013b). Here we combined MOLE to chloramphenicol, which is widely used in topical medication for infected wounds (Livingston et al., 2013), and to cephalosporins, which are naturally more resistant to enzymatic hydrolysis (Bush et al., 2016). These drugs were selected considering that β-lactamase production was confirmed in most of the isolates. MOLE did not interfere significantly with the activity of these drugs (Table 4), as observed using the method standardized by our group (Dias-Souza et al., 2013b). Likewise, a previous study described that *Vaccinium myrtillus* extract, caused no interference on the activity of antimicrobial drugs against *S. aureus* isolates (Costa et al., 2017). We also described synergistic behavior (i.e., increased activity of the drugs) for combinations of antimicrobial drugs to black tea (*Camellia sinensis*) and açaí (*Euterpe oleracea*) extracts (Dias-Souza et al., 2018; Dos Santos et al., 2018), and antagonistic behavior for cashew (*Anacardium occidentale*) pulp and stembark extracts, and for some carotenoids and flavonoids against Gram-negative bacterial species (Dias-Souza et al., 2013; Dos Santos et al., 2015; Dias-Souza et al., 2016). Therefore, synergistic or antagonistic behaviors of such interactions are poorly predictable, can only be determined experimentally. Patients should be advised to avoid such combinations.

We used the BSA denaturation assay to investigate the anti-inflammatory potential of MOLE. The extract at MIC and MBEC values was more effective than the highest concentration of the extract and tenoxicam, a potent NSAID that inhibits cyclooxygenase (COX) 1 and COX 2 (Valentovic, 2007; Barbosa et al., 2021). Flavonoids are major compounds in *M. oleifera* leaves and are the main anti-inflammatory molecules of the plant (Luetragoon et al., 2021). Additionally, minerals and proteins were found in the powder, and they can interfere with the anti-inflammatory and antioxidant potentials of flavonoids by complexing to them (Selvaraj et al., 2013; Swieca et al., 2013). The level of such interferents in concentrations of MOLE such as MIC was expected to be very low, due to their removal by serial dilutions. Thus, biological properties of flavonoids remained active in MOLE. *In vivo* anti-inflammatory mechanisms described for *M. oleifera* extracts

include decreasing the expression of pro-inflammatory cytokines and blockage of toll-like receptors (Luetragoon et al., 2021; Wuryandari et al., 2021).

The biochemical characteristics of the extract are in agreement with the observation of others, specially concerning the presence of polyphenolics, mostly flavonoids, which are major compounds in the leaves (Hassan et al., 2021). UPLC analysis suggested that quercetin, kaempferol and rutin are among the polyphenolics detected in MOLE. There is evidence indicating that these are the main flavonoids present in the leaves, and have a role in both antimicrobial and anti-inflammatory properties of the plant extract (Abd et al., 2018; Dhakad et al., 2019; Zhu et al., 2021). Here, quercetin was detected in two peaks, possibly due to the different glycone (sugar group) to which they might be bounded to. Similarly, a study found these same molecules in a 80% methanolic extract of the leaves (Makita et al., 2016). Also in line with our observations, quercetin, kaempferol and chlorogenic acid, bounded to different glycones, as well as glucosinolates, where found in leaves and other parts of *M. oleifera*, prepared as 70% methanolic extracts (Bennett et al., 2003). A series of compounds, including phytol, hexadecanoic acid and 4-(1-aminoethyl)-3,3-dimethylazetidin-2-one, were found in the leaves of *M. oleifera* by gas chromatography coupled to mass spectrometry (Bhalla et al., 2021). These compounds are described to be anti-inflammatory as some of the flavonoids detected in the leaves (Bhalla et al., 2021; Zhu et al., 2021). Thus, it is possible flavonoids and these compounds had a role in protecting BSA from denaturation in this study.

The presence of vitamin C, iron and calcium in leaf extracts is well described, what supports their safe use as nutritional supplements (Abd et al., 2018; Fernandes et al., 2020). Such elements are extremely valuable for human nutrition, and could be of interest for use as a nutritional supplement, due to the low cost of the plant compared to traditional (non-phytotherapeutic) products. Recent clinical trials have provided evidence of varied benefits of *M. oleifera* leaves as a complementary treatment for bone healing (Singh et al., 2011), control of blood glucose levels (Leone et al., 2018), management of HIV patients (Gambo et al., 2021), and even as a mouthwash (Buakaew et al., 2021). Thus, more clinical trials considering laboratorial parameters and health benefits would be of interest.

There are no reference values in the Brazilian legislation for the biochemical parameters assessed in *M. oleifera* powder and hydroethanolic extract at the moment (proteins, carbohydrates, Vitamin C and minerals). However, they were within acceptable ranges adopted by international standards of quality control and of recommended daily intake of nutrients such as the codex nutrient reference values of the Food and Agriculture Organization of the United Nations, and dietary reference intake values from the USA Food and Nutrition Board of the National Academies of Sciences Engineering and Medicine (Caballero, 2012; Raymon and Morrow, 2021).

CONCLUSIONS

In this study we investigated the antimicrobial and antibiofilm activities of MOLE, and its anti-inflammatory potential and its cytotoxicity. MOLE was effective against planktonic cells and biofilms of β-lactamase producing *S. aureus* clinical isolates. To the best of our knowledge, this is the lowest effective concentration described to date. MOLE also presented anti-inflammatory potential and lacked cytotoxicity. Taken together, our results open doors for developing formulations to treat topical staphylococcal diseases, and anti-inflammatory and antimicrobial effects can be expected. Further studies with *in vivo* models of staphylococcal diseases are necessary to determine safe concentrations for clinical use and assess the effectiveness of the extract considering metabolic and immunological issues.

ACKNOWLEDGEMENTS

We are thankful to Tito Pechir (head of RVN, Brazil), for kindly providing the leaf powder used in this study, and to Arthur Menezes (Pitágoras College, Brazil), for the technical support during the experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

LKSA: performed antimicrobial and anti-inflammatory activities experiments, drafted the manuscript. **LFCM:** performed antimicrobial activity and interference assays. **IPC:** performed cytotoxicity experiments, drafted the manuscript. **RMDS:** co-supervision of the research, critical review of the manuscript. **MVDS:** conceptualization, project administration, supervision, statistical analysis, preparation of the final version of the manuscript.

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FABAD J. Pharm. Sci., 48, 2, 255-264, 2023
Doi: 10.55262/fabadeczacilik.1293118
RESEARCH ARTICLE

Determination of Antioxidant Activity of *Salvia sclarea* L. and Its Inhibitory Effects on Acetylcholinesterase and Monoamine Oxidase A

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Determination of Antioxidant Activity of Salvia sclarea L. and Its Inhibitory Effects on Acetylcholinesterase and Monoamine Oxidase A

SUMMARY

Over the past two to three decades, there has been a significant increase in research focused on the treatment of neurodegenerative disorders. In this study, our aim was to determine some biological activities of the ethanolic and methanolic extracts of Salvia sclarea L. The extracts were first assessed for their capacity to scavenge DPPH radicals, then their total phenolic content (TPC) were determined. Afterward, the extracts were evaluated for their effects on acetylcholinesterase (AChE) and monoamine oxidase-A (MAO-A). These two enzymes play a crucial role in the treatment of neurodegenerative disorders. It has been found that, the DPPH activity of the methanolic extract was higher than that of ethanolic extracts; while TPC was higher for the ethanolic extract. For AChE, the IC50 values for ethanolic extract and methanolic extract were 0,27±0,005 mg/mL and 1,19±0,037 mg/mL, respectively. And for MAO-A, the IC50 values for ethanolic extract and methanolic extract were 6,53±0,72 μg/mL and 3,03±0,05 μg/mL, respectively. As the result of this study, the antioxidant property of Salvia sclarea was determined, and it was observed that this property changed in accordance with the total phenolic content of the plant. It has been shown that the extracts have inhibitory effects on both enzymes. This means, the obtained data are promising for further drug development studies.

Key Words: *Salvia sclarea L., DPPH, total phenolic content, acetylcholinesterase, monoamine oxidase-A, neurodegenerative disorders.*

Salvia sclarea L.'nin Antioksidan Aktivitesi ile Asetilkolinesteraz ve Monoamin Oksidaz-A Üzerindeki İnhibitör Etkilerinin Belirlenmesi

ÖZ

Son yıllarda nörodejeneratif hastalıkların tedavisine odaklanan araştırmalarda önemli bir artış yaşanmıştır. Bu çalışmada amacımız, Salvia sclarea L.'nin etanolik ve metanolik ekstrelerinin bazı biyolojik aktivitelerini belirlemektir. Ekstreler elde edildikten sonra, öncelikle DPPH radikal süpürücü aktiviteleri test edilmiş ve sonra ekstrelerin toplam fenolik içerikleri (TPC) tespit edilmiştir. Daha sonra ekstreler hem asetilkolinesteraz (AChE) hem de monoamin oksidaz-A (MAO-A) üzerindeki etkileri açısından değerlendirilmiştir. Bu iki enzim nörodejeneratif rahatsızlıkların tedavisinde çok önemli bir rol oynamaktadır. Metanolik ekstrenin DPPH aktivitesi etanolik ekstreninkinden daha yüksek bulunmuştur; TPC ise etanolik ekstre için daha yüksek olarak tespit edilmiştir. AChE için etanolik ekstre ve metanolik ekstre için IC50 değerleri sırasıyla; 0,27±0,005 mg/mL ve 1,19±0,037 mg/mL; MAO-A için ise etanolik ekstre ve metanolik ekstre için IC50 değerleri sırasıyla; 6,53±0,72 μg/mL ve 3,03±0,05 μg/mL olarak hesaplanmıştır. Yapılan çalışama sonucunda Salvia sclarea'nın antioksidan özelliği belirlenmiş; bu özelliğin total fenolik içerik ile uyumlu olarak değiştiği görülmüş; ekstrelerin her iki enzim üzerine de inhibitör etkileri olduğu gösterilmiş ve bu elde edilen verilerin ilerideki ilaç geliştirme çalışmaları için ümit verici oldukları tespit edilmiştir.

Anahtar Kelimeler: *Salvia sclarea L., DPPH, total fenolik içerik, asetilkolinesteraz, monoamin oksidaz-A, nörodejeneratif bozukluklar.*

Received: 05.05.2023
Revised: 06.06.2023
Accepted: 12.06.2023

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INTRODUCTION

A significant rise in the number of non-contagious diseases, which now account for nearly 64% of global deaths, has been seen in recent years (Institute for Health Metrics and Evaluation,2019). Alzheimer's disease, a non-contagious condition characterized in late adulthood, is defined by progressive memory loss and cognitive impairment (Alzheimer's Association, 2017). Acetylcholine is a neurotransmitter released into the interneuron space and is shown to be one of the causes of the illness by the decline in its levels (Martorana, 2010). It has also been suggested that some diseases of the central and peripheral nervous systems may be linked to alterations in monoamine oxidase (MAO) activity (Riederer, 2004). On the other hand, oxidative stress brought on by reactive oxygen species (ROS) is known to damage cells by causing cellular and biomolecule oxidation. In addition, oxidative stress is implicated in the development of several neurological illnesses, such as Alzheimer's and Parkinson's (Bastianetto, 2002; Viña, 2004). By stopping or postponing free radicals from oxidizing other molecules, antioxidants reduce the harm they cause in the body (Namiki, 1990). It is seen that the consumption of antioxidant-containing plants as food or food additives not only has a therapeutic but also a preventive effect. According to the World Health Organization (WHO), there is a considerable demand for alternative or complementary medicine practices and practitioners globally (WHO, 2013). Numerous studies have examined the pharmacological uses of phytochemicals and found that they have beneficial effects on human health (Fernando, 2017).

Salvia sclarea L. (Clary sage) has been used from ancient times that belongs to the Lamiaceae family. The Lamiaceae family is of great importance in the fields of medicine, food, and cosmetics because it is rich in essential oils, aromatic compounds, and secondary metabolites, and the members of this family are frequently used in ethnobotanical practices (Baser, 1992; Matkowski, 2008). *Salvia* genus has 100 species in Türkiye and 1049 species in the world. Fifty-eight of the *Salvia* taxa found in Türkiye are endemic (Celep, 2012; World Flora Online). It is known in Aladağlar, Niğde (Türkiye) as "Misk adaçayı, Yağlı kara"; flowers are used as a digestive, branches and leaves are used as a sedative and for diarrhea in tea preparation (Özdemir, 2015). In Antalya, its infusion is used for throat ache (Fakir, 2016). The leaf of *Salvia sclarea*, which is known as "Dağ çayı" is used for cold as a tea in the East Anatolia (Altundag, 2011). *Salvia* species have been shown to contain considerable amounts of flavonoid derivatives and have significant bioactivities. According to a study conducted in 2017, ethanolic extract from *Salvia sclarea*'s aerial part was characterized by HPLC, and the active ingredients found in the result were phenolic acids (rosmarinic acid 165.30 μg/mg, caffeic acid 0.95 μg/mg), flavonoid aglycones (luteolin 0.50 μg/mg, apigenin 0.22 μg/mg), flavonoid glycosides (luteolin-7-O-glucoside 5.55 μg/mg, apigenin-7-O-glucoside 8.5 μg/mg) (Kostić, 2017). Acacetin, one of the major components studied in *Salvia* species, has been identified as a powerful compound with substantial anti-inflammatory and anti-cancer activity. However, further research is required to understand these benefits fully (Singh, 2020). Lutein, a derivative of flavones, has beneficial antioxidant, anti-cancer, anti-inflammatory, and neuroprotective properties (Nabavi, 2015). Apigenin has comparatively potent therapeutic effects in boosting health (Salehi, 2019).

In this study, *Salvia sclarea* was chosen to be tested for some of its biological activities. We aimed to show its DPPH activity and the correlation between this activity and its phenolic content. We also wanted to see the effects of its extracts on acetylcholinesterase (AChE) and MAO-A; those are two important enyzmes in the focus of science due to their roles in the treatment of neurodegenerative disorders.

MATERIAL AND METHODS

Plant Materials

The *Salvia sclarea* was collected from Gemlik (Bursa, Türkiye) in the June of 2021. The plant sample

was identified by Asst. Prof. Dr. Ebru Özdemir Nath from the Department of Pharmaceutical Botany, Faculty of Pharmacy, Altınbaş University, İstanbul/Türkiye, and the herbarium specimen is deposited at the Herbarium of Altınbaş University Faculty of Pharmacy (HERA) with the HERA1037 herbarium number.

Extraction

The plant parts were air-dried in a dark, shaded area at room temperature, pounded into a fine powder using a mechanic's grinder, and weighed roughly using a digital balance. The aerial parts of *Salvia sclarea* were weighed and powdered. The plant samples were extracted via maceration, occasionally shaking for 24 hours using ethanol and methanol with the volume-to-mass ratio of the solvent to the sample was 10/1 (v/w). The solvent evaporated to dryness under a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator) and 2.8 g ethanolic extract and 7.84 g methanolic extract was obtained from 100 g plant. The plant extract of *Salvia sclarea* was stored at +4 C° until biological activity studies.

Biological Activities

DPPH Radical Scavenging Activity

The capacity to scavenge the stable free radical (DPPH) was evaluated according to the method of Brand-Williams (Brand-Williams, 1995). 10 µL of plant extracts (10, 5, 1, and 0,5 mg plant extracts dissolved in 1 mL DMSO) were mixed with 240µL of 1mM DPPH radical containing methanolic solution. The well plates were incubated at room temperature for 10 minutes in the dark. As a standard, quercetin from Sigma-Aldrich (Germany) was employed. The following formula (1) was used to calculate the radical scavenging activity (Inh %) as a proportion of DPPH discoloration:

$$\text{Inh \%} = [1 - (\text{Abs}_{\text{extract}} / \text{Abs}_{\text{DPPH}})] \times 100 \qquad (1)$$

Total Phenolic Content

A modified Folin Ciocalteu method was used to quantify the extracts' total phenolic content spectrophotometrically (Slinkard, 1977). At 760 nm, the reagent's reduction, which produced the development of blue color, was seen. The plant extracts (10, 5, 1, and 0,5 mg plant extracts dissolved in 1 mL DMSO) were mixed with 225 µL of distilled water, then 5 µL of Folin Ciocalteu reagent was added. The mixture was incubated at room temperature. After 3 min, 15 µL of 2% Na_2CO_3 was added. A multimode microplate reader, BioTek Synergy H1 (Agilent), was used to detect the absorbance at 760 nm following a two-hour incubation period at room temperature in a dark area. The standard solution was gallic acid from Sigma-Aldrich (Germany). The average values were used to calculate equivalent gallic acid amounts in the extracts.

Acetylcholinesterase (AChE) Inhibitory Activity

The effects of the extracts on acetylcholinesterase activity were carried out spectrophotometrically. Briefly, the activity of the AChE of *Electrophorus electricus* (electric eel) was determined by the Ellman method (Ellman, 1961). Acetylthiocholine iodide (ATC) was used as the substrate in the study. AChE activity was determined in 0.4 mM ATC and 0.125 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 100 mM Tris HCl (pH 8.0) buffer at 25 °C. AChE was added in amounts of ~0.05 U/mL to start the reactions. The hydrolysis of acetylthiocholine was monitored over the increase in absorbance at 412 nm using a spectrophotometer (Carry 60 Single Beam Spectrophotometer, Agilent Technologies, USA). Enzyme activity was calculated using the linear portions of the absorbance-time curve over the first 60 seconds. Runs for each ligand were repeated at least three times.

Monoamine oxidase A (MAO-A) Inhibitory Activity

According to a previously published protocol, monoamine oxidase-A (MAO-A) inhibition assays were performed (Krajl, 1965; Urban, 1991). Monoamine oxidase-A activities of the extracts were determined spectrofluorometrically and carried in triplicate. 100 mM at a pH of 7.4 potassium phosphate buffer was used to carry out each reaction. Kynuramine, a suitable non-fluorescent substrate that is converted

into the fluorescent metabolite 4-Hydroxyquinoline (4-HQ) during the process, was used as substrate in a method to examine the test inhibitors' ability to inhibit MAO-A. Before testing, all extracts dissolved in their organic solvents. Buffer, substrate, and extracts were preincubated for 10 min at 37 °C. Reactions were started by adding the enzyme and the reactions were then kept at 37 °C for another 20 minutes. Following the addition of NaOH (2 N) to stop the reactions, 1000 mL of distilled water were added. Using a multimode microplate reader, BioTek Synergy H1 (Agilent) with an excitation wavelength of 310 nm and an emission wavelength of 400 nm, the amount of fluorescence was measured. The associated IC_{50} values were to express the inhibitory potencies of the extracts.

RESULTS AND DISCUSSION

DPPH radical scavenging activity and total phenolic content (TPC)

The results of the DPPH radical scavenging activity and total phenolic content assays of methanolic and ethanolic extracts of *Salvia sclarea* are given in Table 1. Different concentrations of ethanolic and methanolic *Salvia sclarea* extracts were tested at various doses. It has been found that the total amount of quercetin equivalent to the methanolic extract was quite higher than that of the ethanolic extract. At low doses, there does not appear to be any significant distinction between the effects of the different percentages of radical scavenging of both extracts. At low concentrations, the gallic acid equivalent TPCs were also similar, which is parallel to the radical scavenging effect, as well.

Table 1. DPPH radical scavenging activity and TFC of the methanolic and ethanolic extracts of *Salvia sclarea*.

Extract	Concentration. (mg/mL)	DPPH. Equivalent Quercetin (mg/mL)	DPPH. %	TFC. Equivalent Gallic Acid (mg/mL)
Ethanolic Extracts	10	0.24±0.02	82.21±1.79	0.87±0.005
	5	0.16±0.01	73.86±1.51	0.42±0.35
	1	0.05±0.001	50.55±1.18	0.09±0.003
	0.5	0.03±0.0004	38.34±0.72	0.04±0.00
Methanolic Extracts	10	0.33±0.11	88.49±2.63	0.81±0.041
	5	0.20±0.006	78.30±0.98	0.40±0.017
	1	0.06±0.004	53.80±2.59	0.09±0.0016
	0.5	0.03±0.001	39.45±2.47	0.04±0.0016

Antioxidants are molecules that often include a phenolic group and work to prevent the development of free radicals or harm to the cell caused by radicals. They do this by removing radicals. In general, it is known that even in extremely low concentrations, antioxidants are capable of protecting biomolecules in their surroundings from oxidative damage that can be caused by free radicals (Halliwell, 1986). Because of this, it is vital to investigate the antioxidant capabilities and the phenolic content of potential therapeutic plants like *Salvia*. There are a variety of other procedures that may be used to determine the antioxidant capabilities of plant extracts. Still DPPH is selected as a method that is commonly used with its advantages like its simple application, high accuracy, and speedy response (Buyuktuncel, 2013). In the DPPH technique, typically, violet-colored DPPH radical is changed into yellow DPPH-H form by the antioxidants (compounds with phenolic groups) in the extract. In other words, the DPPH radical becomes less reactive. This color shift can be evaluated spectrophotometrically at 517 nm, at which point one additionally can determine the number of radicals that have been reduced (Brand-Williams, 1995).

In research with essential oils and extracts from *Salvia officinalis* L., it was shown that plant oil had a low antioxidant content, while methanolic extracts had the highest antioxidant content (DPPH IC_{50}=37.29 g/g) (Abdelkader, 2015). There hasn't been a lot of research done on *Salvia sclarea* L., another significant species in the *Salvia* genus. Both chloroform and acetone extracts of the plant were investigated, and the results showed that 100 mg of chloroform extract had a greater effect on scavenging radicals than acetone extract; nevertheless, this effect was less potent than the other standard compounds that were investigated (Gülçin. 2004). The TPC of plant extracts containing 1000 mg of dried extracts was also tested in the same study. The results showed that the chloroform extract had 28.91 µg of pyrocatechol equivalent phenolic compound, while the acetone extract contained 35.24 µg of this molecule. In a study carried out in Turkey using six distinct species of *Salvia*, non-polar subfractions of methanol extracts of *Salvia sclarea* were analyzed, and the IC_{50} value for DPPH was found to be 23.4 mg/mL. DPPH radical scavenging activity was found to be 79.48% in another study that used 10 mL of methanol extract of 1 gram of dry *Salvia sclarea* plant material, while in our study, a similar radical scavenging activity was observed in the extract sample that was prepared at a dose of 5 mg/mL (Pop (Cuceu), 2016). In a different investigation, methanolic extract of *Salvia sclarea* was employed, and BHT was used as the standard. The quantity of IC_{50} was calculated to be 58.20 g/mL, and the TPC was 24.38 mg per gram of dry weight. Comparing the levels of TPC and IC_{50} DPPH in plants that grew in environments with varying concentrations of salt was the purpose of that investigation. According to the findings of the research carried out by Taarit, DPPH activity and the overall quantity of phenolic compounds were proportionate to one another (Taarit, 2012). In our research, just as in the study conducted by Taarit, it was demonstrated that DPPH activity increased with increasing concentrations of phenolic content. In another study comparing the methanolic contents of *Salvia sclarea* samples collected at different times of the day, it was found that plant collection time had no effect on phenolic content and DPPH radical scavenging activity (Tulukcu, 2009). The HPLC method was used to assess the TPC of aqueous extracts of different *Salvia* species that were collected in the region of Salento in Southern Italy. According to the findings of the study, *Salvia sclarea* has phenolic content of 55.60 mg/g DW (Vergine, 2019).

AChE and MAO-A Inhibitory Activity

The results of the inhibitory effects of both ethanolic and methanolic extracts of *Salvia sclarea* on acetylcholinesterase and MAO-A are given in Table 2. A wide range of concentrations of the extracts were tested with different doses, and for both AChE and MAO-A, IC_{50} values were calculated. While the effect of the ethanolic extract of *Salvia sclarea* on AChE was nearly four times higher, on the contrary, the effect of the methanolic extract of *Salvia sclarea* on MAO-A was found higher.

Table 2. AChE and MAO-A inhibitory activity of the methanolic and ethanolic extracts of *Salvia sclarea*.

Extracts	IC_{50}, AChE (mg/mL)	IC_{50}, MAO-A (mg/mL)
Ethanolic Extracts	0.27 ± 0.005	6.53 ± 0.72
Methanolic Extracts	1.19 ± 0.037	3.03 ± 0.05

A deficiency of the neurotransmitter acetylcholine, which is essential for cognitive functioning, is thought to be a sign of Alzheimer's disease (AD), an irreversible neurodegenerative condition. Cholinesterase inhibitors are now the medications used to treat AD the most frequently. *Salvia* species are used in traditional European medicine for their memory-enhancing properties (Bahadori, 2017; Orhan, 2013). Because

of this, we have determined the effects of the extracts of *Salvia sclarea* on AChE. In a study, the IC_{50} value of the ethanolic extracts of the leaves of *S. fruticosa* and *S. officinalis* were found to be 287.02 and 268.45 µg/mL, respectively (Mervić, 2022). These are nearly the same results for our extracts. In another study, the IC_{50} value of AChE for acetone extract obtained from the roots of *S. syriaca* was found as 500 µg/mL (Bahadori, 2016). In one of the most comprehensive studies about *Salvia* ethyl acetate, dichloromethane, and methanol extracts were studied and, dichloromethane extract of *S. fruticosa* was shown to have the highest inhibitory effect on AChE compared with the others in the same study (Senol, 2010). Also, essential oil of *Salvia sclarea* has been studied and found to have a very low inhibitory effect on AChE (Orhan, 2008).

Due to its crucial function, MAO inhibitors serve as an effective therapeutic option for several mental and neurological conditions and have become the focus of scientists (Rudorfer, 1989; Gökhan-Kelekçi, 2007; Gökhan-Kelekçi, 2009). On the other hand, some medicinal plants utilized in traditional medicine have been used as a significant source for the treatment of depression, Parkinson's disease, and other neuropsychiatric and neurological illnesses (Akhondzadeh, 2003, Saki, 2014). In a study, *S. miltiorrhiza* Bunge has been shown to have some inhibitory effects on MAO-A (Dittmann, 2004). According to our searches, the effects of other types of *Salvia* species on monoamine oxidases have not been studied yet.

Here in this study, we have determined the effects of the ethanolic and methanolic extracts of *Salvia sclarea* on AChE and MAO-A. Although the IC_{50} values were higher when compared to their specific inhibitors, galantamine and clorgiline, the results were encouraging in comparison to other plant species.

CONCLUSION

Here in this study, we have determined some of the biological activities of ethanolic and methanolic extracts of *Salvia sclarea.* Although various studies have explored the properties of *Salvia* species, our research is the first to demonstrate the impact of the methanolic extract of *Salvia sclarea* on monoamine oxidase-A. Additionally, ethanolic extract of *Salvia sclarea* has been evaluated for its DPPH, TPC, AChE, and MAO-A activities for the first time. The data obtained is quite encouraging and promising. Further studies can be done to use *Salvia sclarea* as a natural source to cure for many neurodegenerative disorders.

ACKNOWLEDGEMENTS

This research project was supported by Altınbaş University Scientific Research Fund. Project Number: PB2020-ECZ-3.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing the hypothesis, literature research, writing the original draft (YYY, EON), collection, identification, and extraction of the plant material (EON), analysis of the biological activities and interpretation of the data (YYY).

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FABAD J. Pharm. Sci., 48, 2, 265-278, 2023
Doi: 10.55262/fabadeczacilik.1263949
RESEARCH ARTICLE

Toxicological Evaluations of *Smilax myosotiflora* Methanol Extract and its Effect on Testosterone Level of Male Rats in Subacute Study

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Toxicological Evaluations of Smilax myosotiflora Methanol Extract and its Effect on Testosterone Level of Male Rats in Subacute Study

SUMMARY

S. myosotiflora A. DC., the horny little devil, is a tropical creeping plant which popularly consumed as a male aphrodisiac, energy booster, and lumbago reliever in the old traditional medicine. The scientific studies showed that the plant able to increase sexual behaviors and testosterone levels in male rats. However, its toxicity effect still remained unknown. Therefore, this study aimed to investigate the toxicity effects of S. myosotiflora methanol extract (SMME) through in vitro and in vivo studies. The SMME was subjected to the brine shrimp lethality test (BSLT) to determine the LC50. Acute and subacute toxicity studies according to the Limit Test of OECD guidelines no. 425 and 407 were carried out through oral gavage accordingly. It was found that the LC50 of SMME was 674.4ppm while its LD50 via acute test was more than 5000 mg/kg. Neither sign of toxicity nor significant difference in food intake, weight gain, gross necropsy, hematological and biochemical analyses, and histological evaluation were recorded between the subacute of control and treated groups except the levels of AST and testosterone in male and sodium and triglycerides in female rats. The increase of testosterone in male rats might occur through a specific pathway as the SMME did not increase the hormone level in the female's. According to Globally Harmonized System (GHS) classification, SMME in this study can be classified as Category 5 (Safe) and nontoxic. Data from this study can be served as a primary predictive guide for future research in assessing the efficiency and safety of S. myosotiflora consumption for human trials.

Key Words: *Smilax myosotiflora, aphrodisiac, acute, subacute, toxicity, BSLT.*

Smilax myosotiflora Metanol Ekstresinin Toksikolojik Değerlendirmeleri ve Subakut Çalışmada Erkek Sıçanların Testosteron Düzeyine Etkisi

ÖZ

S. myosotiflora A. DC., popüler bir erkek afrodizyağı, enerji artırıcı ve geleneksel tıpta bel ağrısını giderici olarak tüketilen tropikal bir sürünücü bitkidir. Bilimsel çalışmalar, bitkinin erkek sıçanlarda cinsel davranışları ve testosteron seviyesini artırabildiğini göstermiştir. Bununla birlikte, toksisitesi hala bilinmemektedir. Bu nedenle, bu çalışmada S. myosotiflora metanol ekstresinin (SMME) toksisitesinin in vitro ve in vivo çalışmalarla araştırılması amaçlanmıştır. SMME, LC50'yi belirlemek için tuzlu su karidesi ölüm testine (BSLT) tabi tutuldu. Akut ve subakut toksisite çalışmaları OECD yönergelerinin 425 ve 407 nolu testleri ile oral gavaj kullanılarak gerçekleştirildi. SMME'nin LC50'sinin 674.4ppm olduğu, akut toksisite testi ile LD50'sinin ise 5000 mg/kg'dan fazla olduğu bulundu. Subakut kontrol ve tedavi edilen gruplar arasında erkek sıçanlarda AST ve testosteron ve dişi sıçanlarda sodyum ve trigliseritler dışında, ne toksisite belirtisi ne de gıda alımı, kilo alımı, makroskopik otopsi, hematolojik ve biyokimyasal analizler ve histolojik değerlendirmelerde anlamlı fark kaydedilmedi. SMME kadınlarda hormon seviyesini artırmadığı için, erkeklerde testosteron artışı, belirli bir yolakla gerçekleşiyor olabilir. Küresel Uyumlaştırılmış Sistem (GHS) sınıflandırmasına göre SMME, bu çalışmada Kategori 5 (Güvenli) ve toksik olmayan olarak sınıflandırılabilir. Bu çalışmadan elde edilen veriler, insan denemeleri için S. myosotiflora tüketiminin etkinliğini ve güvenliğini değerlendirmede gelecekteki araştırmalar için birincil tahmin kılavuzu olarak kullanılabilir.

Anahtar Kelimeler: *Smilax myosotiflora, afrodizyak, akut, subakut, toksisite, BSLT.*

Received: 12.03.2023
Revised: 28.06.2023
Accepted: 05.07.2023

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INTRODUCTION

In the history of Greek, Aphrodite was referred to a daughter of Zeus which embodied the meaning of goddess of love, beauty, allure, and procreation. Today, adapting from the word, aphrodisiac is defined as a substance or component that can boost sexual desire or increase sexual enjoyment. Its comprehension has been widened to include any material that is able to aid sexual function or improve the systems of sexual operation either in males or females (Sharma et al., 2017). The substance is designated to any form which able to increase sex performances, for example foods, vitamins, beverages, or natural/chemical compounds. Inability to perform in the intimate event may create huge problems and socially, and physiologically affects the one and their partners (Capogrosso et al., 2021; Sharma et al., 2016). For men, this indicates that they may potentially have the male sexual dysfunction (MSD) problem, one of the most common health threats other than heart and diabetes diseases among them (Andrea et al., 2021). The growing incidences and the demand for better therapeutic drugs on the men's sexual incompetence issue has led to the discoveries of natural substances from aphrodisiac plants, organisms, or microbes as an alternative to treat the MSD. Natural constituents from aphrodisiac plants received a great deal and global attention from scientists as they are cheaper, more accessible, lesser toxic, have no physical suffer and safer than synthetic drugs or other conventional clinical treatments. Among these natural and traditional love potions is a plant from *Smilax* sp., *Smilax myosotiflora* A. DC., which known as the 'horny little devil'.

S. myosotiflora is a plant that habited throughout the tropical climate regions in Southeast Asia (SEA), such as Peninsular Malaysia, the Indonesian Island of Java, and southern Thailand. The plant was popularly consumed by the indigenous people and local folks as a male aphrodisiac, energy booster, and lumbago reliever in the old medicinal practice in the regions (Nurraihana et al., 2016; Rao et al., 2016). It also was widely used by the ancient medicine practitioners to treat rheumatism, diabetes, syphilis, fever, sore throat, and virility (George et al., 2010; Lin, 2005; Ong & Azliza, 2015). The leaves are dark green, heart-shaped, and deciduous from 5-15cm long, while the tubers are dark brown rough surface, irregular round shape, and slightly sweet in odor. The tuber was reported to be the most functional part of the *S. myosotiflora* plant as it composed many bioactivities, especially as a male sexual enhancer and other medicinal benefits (Chyang et al., 2018; Dasuki et al., 2012; George et al., 2010; Mustaffar Bakri, 2013; Rahman et al., 2010; Wan Ghazali et al., 2016; Wan et al., 2013). Previously, it was found to have a comparable peak of protein to *E. longifolia*, *Rafflesia sp*., and *Labisia pumila*, which is responsible to increase the expression of testosterone levels in the Leydig cells, the 4.3kDa peptide (A. Osman et al., 2007). This finding was relatable to the *in vivo* test elsewhere, where the intake of the plant was able to significantly elevate the level of testosterone in male rats (Hilmi et al., 2015; Hoon et al., 2005; Wan et al., 2013, 2016)commonly known as ubi jaga in Malaysia, from the family of Liliaceae. The optimum dose of *S. myosotiflora* as an aphrodisiac was found between 400-800mg/kg where aqueous and methanol were the most active forms (Hilmi et al., 2015; Hoon et al., 2005; Wan et al., 2013).

Despite its broad use in folk medicine and significant contributions to male reproductive studies, data on its toxicity effect remains unknown. Thus, this study aimed to investigate the toxicity effects of *S. myosotiflora* methanol extract (SMME) through acute and subacute tests *in vivo*. The cytotoxicity profile of the plant was also evaluated *in vitro* through the brine shrimp lethality test (BSLT). The findings from this study can be a primary predictive guide for future research in assessing the efficiency and safety of *S. myosotiflora* consumption for human trials.

MATERIALS -METHODS

Sample Preparation

Tubers of *S. myosotiflora* were collected from the Titiwangsa range in the state of Perak, Malaysia (5°29'31.6"N, 101°26'26.6"E) from May to August

2018 with the help of the aboriginal people. The plant material was identified by a botanist and was deposited at the Herbarium Universiti Kebangsaan Malaysia Bangi of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia, with an authentication no. PIIUM0018-1. Tubers were washed under running water and dried in a circulating air oven at 50°C for approximately five days. The grinding process was performed on the dry material using a power grinder machine (Golden Bull, Malaysia) to obtain the powdery sample. Plant powder was subjected to Soxhlet extraction, where methanol was the solvent using a 1:10 ratio. The methanolic solution was filtered and concentrated in a rotary evaporator under reduced pressure continued with oven-drying to yield the sticky paste of SMME. The SMME was tightly sealed in 4°C storage prior to future use.

Toxicity Test in vitro - BSLT

BSLT is one of the effective *in vitro* assays to evaluate the cytotoxicity substance using a biological model, *Artemia salina*. The assay was conducted according to Laurentius et al. (2018) with slight modifications. Briefly, the cysts were initially hatched in the artificial seawater for 48 hours. A set of SMME concentrations (1000, 800, 600, 400 , and 200ppm) was prepared using distilled water and 1% DMSO as the emulsifier. Later, 10 active brine shrimps were transferred into each 6-well plate before 2.5mL of an SMME concentration, and seawater were added to every well. Each SMME concentration was prepared in triplicate in three independent experiments. Controls were set by 10 brine shrimps in 5mL seawater as the negative and 10 brine shrimps with 2.5mL of pure ethanol and seawater as the positive. Using a magnifying glass, the dead shrimps were counted after 24 hours of incubation. The percentage of mortality was calculated for every concentration by summing the dead over total brine shrimp tested. The median lethality concentration (LC_{50}) of SMME was determined by plotting a graph of mortality percentage against SMME concentrations using the linear regression method. The obtained data explained the concentration of the SMME, which can cause to half death of total shrimps in a certain time exposure.

Toxicity Test in vivo - Acute , and Subacute Tests

In this study, the *in vivo* toxicity tests were performed according to the Limit Test of the Organization of Economic Co-Operation and Development (OECD) guidelines no. 425 (Up-and-down Procedure) for acute toxicity and Limit Test of no. 407 (Repeated dose 28-days) for subacute toxicity (OECD/OCDE, 2008a, 2008b). The animal ethic of the tests was obtained from Universiti Sains Malaysia (USM) Institutional Animal Care and Use Committee (Approval no.: USM/IACUC/2018/113-936) while the rats were supplied and placed in the Animal Research and Service Centre (ARASC), USM, Malaysia. In the tests, healthy, nulliparous with 8-10 weeks old of Sprague Dawley rats were first acclimatized for a minimum of five days and were maintained under the standard laboratory condition at ambient temperature 22±2°C and relative humidity of 60-70%. The photoperiod was consistent throughout the study, with 12 hours light and 12 hours dark. Their food supply was freely accessible, where a standard rodent diet pellet and reverse osmosis (RO) water *ad libitum* were given. Rats were grouped in 2-3 per standard cage filled with wood shaving as the bedding.

In the acute toxicity through the Limit Test method, five female rats received a single dose of 2000mg/kg SMME on their first day and were observed within a fortnight for any sign of toxicity or mortality. The test proceeded with a new batch of five female rats treated with a single dose of 5000mg/kg and monitored within the same period accordingly. In the Limit Test of subacute, 12 rats of both sexes which were randomly selected into control and treated groups, were treated with distilled water or 1000mg/kg SMME daily for 28 days. Weight gain, food intake, signs of toxicity, and mortality of the rats were recorded during the period. Then, all rats were anesthetized and humanely sacrificed on the

following day through intraperitoneal injection of pentobarbital for blood collection (only for subacute rats), and dissection of organs such as liver, kidney, heart, spleen, brain, lungs and sex organs to perform the gross examination and relative organ weight calculation.

Hematological , and biochemical analyses

The blood samples collection taken through cardiac puncture were subjected to the heparin lithium tubes for hematological test and the non-EDTA coated tubes for biochemical analyses at a commercial laboratory. Other than the normal biochemical parameters such as total protein (TP), albumin (ALB), aspartate transaminase (AST), aminotransferase (ALT), total bilirubin (TBIL), triglyceride (TG), total cholesterol (TC), creatinine (CRE), and so forth, the reproductive hormones namely testosterone, estradiol, and progesterone were also evaluated.

Histological analysis

After euthanasia, the liver, kidney, and testis from the subacute rats were preserved in a fixation medium of 10% buffered formalin for histopathological examination. The organs were routinely processed, embedded in paraffin wax, sectioned into 3–5µm with a microtome and stained with hematoxylin and eosin (H&E) stain according to a standard laboratory method. Finally, the stained sections were analyzed for any modifications in the morphology of particular tissues under a microscope fluorescence and an image analyzer (Olympus, Japan).

Statistical analysis

Data was first determined for their normality using D'Agostino-Pearson Omnibus and, or Shapiro-Wilk normality tests. All values were expressed as the mean ± standard deviation (SD), and the results were analyzed statistically by One-way Analysis of Variance (ANOVA) or Kruskal Wallis test followed by Mann-Whitney when not normally distributed using statistical software GraphPad PRISM Version 6.0 by GraphPad Software Incorporated Company, California. $P < 0.05$ compared to control was considered to be statistically significant.

RESULTS AND DISCUSSION

BSLT is an early cytotoxicity screening of the bioactive compounds in plant extracts using the *A. salina* as a bioindicator. Figure 1 displayed the graph of mortality percentage of the brine shrimp over SMME concentrations with an equation y = 0.1075x-22.5 and $R^2 = 0.938$. The mortality percentage in the graph was the average number from triplicates in the three independent experiments. According to the graph, the mortality percentage would increase if the SMME concentration increased. At the maximum of SMME concentration, 1000 ppm, 90% of the brine shrimp was killed. From the graph, it was found that the LC_{50} of crude methanol extract of *S. myosotiflora* was 674.4 ppm.

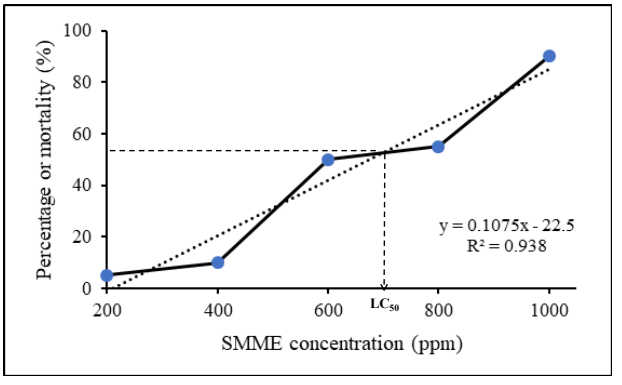

Figure 1. Mortality percentage of *A. salina* over SMME concentrations in BSLT toxicity study. *Abbreviation*: LC_{50} - Median lethality concentration, SMME - *S. myosotiflora* methanol extract.

The acute toxicity effect of the SMME was determined as per OECD guideline no. 425, where the Limit Test single doses of 2000 and 5000 mg/kg were applied. At the high doses of SMME administration, the female rats did not induce any lethality or mortality effect till their necropsy day. All rats displayed normal behavior and no visible signs of toxicity; for example, salivation, aggression, rising furs, and writhing were noticed throughout the study, except half of 5000 mg/kg SMME-treated rats experienced frequent head rubbing in the first few hours. The color stools and urine of all rats were also normal. Food and water consumption in all groups were perceived as normal within the period. Through the gross necropsy, no pathological abnormalities were discovered in the groups. On top of that, no significant difference was noted in the relative organ weight (ROW) in all groups (Table 1). According to the OECD guideline no. 425, since no irreversible or remarkable toxicity or mortality effect occurred in the treated rats at 2000 mg/kg and 5000mg/kg dosages, hence, the median lethality dose (LD_{50}) of the SMME was greater than 5000 mg/kg.

Table 1. Absolute and relative organ weight of the female rats in acute toxicity test.

Organs	Control		2000mg/kg		5000mg/kg	
	AOW (g)	ROW (%)	AOW (g)	ROW (%)	AOW (g)	ROW (%)
Brain	2.32 ± 0.16	0.98 ± 0.08	1.98 ± 0.15	0.84 ± 0.05	1.94 ± 0.23	0.88 ± 0.13
Thyroid	0.51 ± 0.14	0.22 ± 0.07	0.34 ± 0.19	0.21 ± 0.00	0.47 ± 0.04	0.21 ± 0.02
Trachea	0.25 ± 0.14	0.10 ± 0.05	0.16 ± 0.03	0.07 ± 0.01	0.17 ± 0.08	0.08 ± 0.04
Lungs	2.13 ± 0.47	0.89 ± 0.15	11.3 ± 0.23	0.78 ± 0.12	1.47 ± 0.28	0.66 ± 0.14
Thymus	0.40 ± 0.08	0.17 ± 0.04	0.47 ± 0.03	0.20 ± 0.01	0.45 ± 0.17	0.21 ± 0.08
Heart	0.93 ± 0.06	0.39 ± 0.03	0.87 ± 0.11	0.37 ± 0.03	0.91 ± 0.07	0.41 ± 0.03
Stomach	2.41 ± 0.39	1.02 ± 0.15	2.72 ± 1.37	1.00 ± 0.37	2.07 ± 0.43	0.93 ± 0.19
Intestines	18.09 ± 3.32	8.08 ± 1.83	13.4 ± 3.45	6.41 ± 0.76	17.33 ± 1.42	7.80 ± 0.60
Spleen	0.85 ± 0.16	0.37 ± 0.06	0.63 ± 0.20	0.30 ± 0.03	0.61 ± 0.04	0.28 ± 0.03
Liver	9.36 ± 1.97	3.94 ± 0.79	8.29 ± 1.05	3.55 ± 0.55	8.09 ± 1.50	3.68 ± 0.63
Adrenals	0.07 ± 0.00	0.03 ± 0.00	0.05 ± 0.02	0.03 ± 0.00	0.06 ± 0.01	0.03 ± 0.01
Kidneys	2.36 ± 0.48	0.97 ± 0.14	1.52 ± 0.41	0.72 ± 0.07	1.60 ± 0.15	0.72 ± 0.05

Values are expressed as mean ± SD; n = 5-6 rats per group. No significant difference between the organs of control and SMME-treated groups in AOW or ROW. P-value < 0.05 is considered significantly different. *Abbreviations*: AOW - absolute organ weight, ROW - relative organ weight.

In the subacute test, the Limit Test of the OECD guideline no. 407 was implemented. The daily oral administration of SMME at a high dose, 1000 mg/kg, for 28 days did not induce any symptoms of toxicity either in male or female rats. No deaths or obvious clinical signs were found in any groups throughout the study. None of the rats showed signs of toxicity on their skins, fur, eyes, sleep, salivation, diarrhea, and behavior. Overall, their daily food intake, and body weight gain in the male and female groups were found no significant difference between the control and treated rats (Figures 2). The average daily food intake for control and treated groups in male were 14.4 and 17.9g/day, while in the female were 16.1 and 17.1g/day accordingly. The body weight gain in male control rats was 15.3%, while for the treated group was 18.5% during the interval. For females, the body weight gain was 14.0% in the control group and 14.2% for the treated group. The gross observations have revealed no abnormalities either in the control or treated groups. The ROW recorded on the necropsy day also did not show any significant difference between both

groups of genders, as displayed in Table 2, except there were significant differences on the absolute weight of thyroid in male and trachea in female groups. Meanwhile, in the study, the cervix, uterus body, uterine horns, oviduct, and ovary were weighed as a set of female sex organs, and they were found to have no significance between the control and treated groups. Concurrently, no significant difference was also displayed in the sex organs of male rats namely, the vesicle, penile, and testis.

The effects of the subacute oral administration of the SMME on the hematological and biochemical parameters are represented in Table 3. The hematological test was evaluated through full blood count, while the biochemical test was determined by renal function test, liver function test, and lipid profile. All of the tested hematological parameters were within comparable range in both genders. The SMME also caused no significant effect on the biochemical parameters except for the sodium and triglycerides of female rats, where they were detected significantly higher in the treated rats. Meanwhile, the treated male rats demonstrated a significantly reduced in the enzyme of AST but a significantly increased in testosterone levels ($P < 0.0001$). Likewise, the thyroid-stimulating hormone (TSH) and follicle-stimulating hormone (FSH) levels were lower than 0.008mIU/L and 0.3IU/L in the control and treated male rats. There were no significant differences in the testosterone, oestradiol, and progesterone hormone levels among female rats, with the TSH value being equal in both groups, 0.006 ± 0.002 mIU/L.

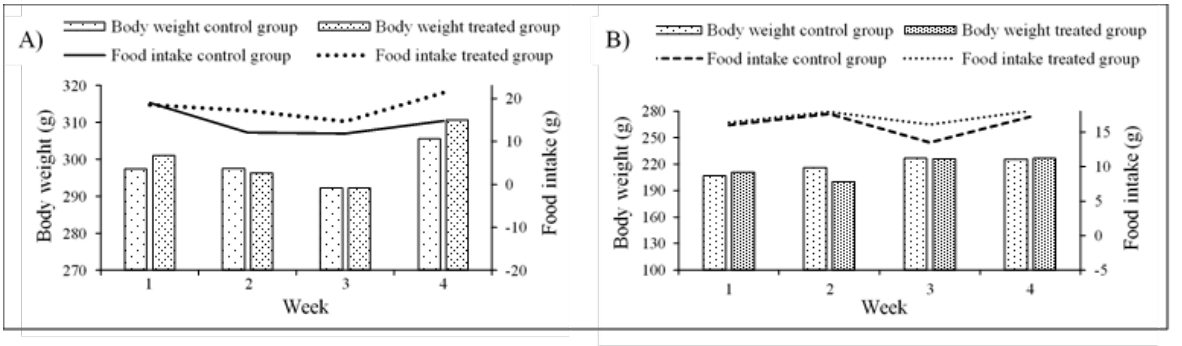

Figure 2. Body weight and food intake in male (A) and female (B) groups in the subacute test.

Table 2. AOW and ROW of male and female rats in the subacute test.

Organs	AOW (g)				ROW (%)			
	Control (M)	Treated (M)	Control (F)	Treated (F)	Control (M)	Treated (M)	Control (F)	Treated (F)
Brain	2.25 ± 0.09	2.25 ± 0.20	2.29 ± 0.18	2.22 ± 0.18	0.75 ± 0.06	0.76 ± 0.10	1.02 ± 0.12	0.98 ± 0.08
Thyroid$^{\alpha}$	0.89 ± 0.19	0.71 ± 0.16	1.04 ± 0.25	0.84 ± 0.34	0.29 ± 0.06	0.24 ± 0.05	0.46 ± 0.12	0.37 ± 0.15
Trachea$^{\beta}$	0.42 ± 0.36	0.29 ± 0.13	0.30 ± 0.02	0.25 ± 0.04	0.14 ± 0.14	0.10 ± 0.04	0.13 ± 0.01	0.11 ± 0.02
Lungs	2.23 ± 0.83	2.14 ± 0.81	2.84 ± 0.26	2.45 ± 0.66	0.73 ± 0.28	0.71 ± 0.24	1.26 ± 0.26	1.07 ± 0.27
Thymus	0.45 ± 0.08	0.45 ± 0.18	0.66 ± 0.59	0.42 ± 0.14	0.15 ± 0.03	0.15 ± 0.05	0.30 ± 0.15	0.18 ± 0.05
Heart	1.24 ± 0.17	1.15 ± 0.20	1.02 ± 0.19	0.94 ± 0.16	0.41 ± 0.03	0.39 ± 0.04	0.45 ± 0.07	0.41 ± 0.04
Stomach	3.00 ± 1.02	3.47 ± 1.23	2.63 ± 0.86	3.08 ± 0.48	1.01 ± 0.44	1.16 ±0.33	1.18 ± 0.42	1.65 ± 0.39
Intestines	7.25 ± 1.03	7.02 ± 1.21	19.79 ± 1.76	19.69 ± 2.08	7.25 ± 1.03	7.02 ± 1.21	8.77 ± 0.48	8.63 ± 0.62
Spleen	1.00 ± 0.08	0.92 ± 0.15	0.83 ± 0.15	0.76 ± 0.11	0.33 ± 0.02	0.31 ± 0.03	0.37 ± 0.06	0.33 ± 0.03
Liver	12.18 ± 0.78	12.08 ± 2.30	8.51 ± 0.86	8.27 ± 1.41	4.07 ± 0.50	4.05 ± 0.40	3.77 ± 0.28	3.60 ± 0.33
Adrenals	0.05 ± 0.01	0.06 ± 0.02	0.10 ± 0.14	0.08 ± 0.01	0.007 ± 0.001	0.007 ± 0.003	0.02 ± 0.03	0.02 ± 0.002
Kidneys	3.01 ± 0.54	2.70 ± 0.49	1.59 ± 0.70	1.65 ± 0.24	0.50 ± 0.11	0.46 ± 0.07	0.40 ± 0.06	0.36 ± 0.02
Vesicle	2.19 ± 0.64	1.78 ± 0.52	-	-	0.71 ± 0.18	0.60 ± 0.16	-	-
Penile	0.39 ± 0.14	0.36 ± 0.24	-	-	0.13 ± 0.05	0.13 ± 0.08	-	-
Testis	7.79 ± 1.78	6.08 ± 0.69	-	-	2.38 ± 0.54	1.97 ± 0.34	-	-
Female sex organs	-	-	1.20 ± 0.29	1.50 ± 0.86	-	-	0.53 ± 0.14	0.66 ± 0.38

Values are expressed as mean ± SD; n = 5-6 rats per group. *Abbreviations*: M - male, F - female, AOW - absolute organ weight, ROW - relative organ weight, $^{\alpha}$ - Significant difference in AOW of male groups, $^{\beta}$- Significant difference in AOW of female groups. P-value < 0.05 is considered significantly different.

Table 3. Effects of SMME on hematological and biochemical parameters in subacute toxicity study.

Parameters	Male		Female	
	Control	Treated	Control	Treated
Full Blood Count				
Hemoglobin (g/L)	143.00 ± 22.11	142.70 ± 4.08	136.40 ± 9.24	137.80 ± 6.62
RBC (x10^12 L)	8.53 ± 1.54	8.19 ± 0.29	7.84 ± 0.59	8.04 ± 0.43
PCV (L/L)	0.48 ± 0.10	0.45 ± 0.03	0.46 ± 0.03	0.45 ± 0.02
MCV (fL)	56.00 ± 3.16	55.17 ± 2.40	58.40 ± 2.30	56.50 ± 1.05
MCH (pg)	16.67 ± 0.82	17.33 ± 0.82	17.60 ± 1.34	17.33 ± 0.52
MCHC (g/L)	302.00 ± 28.02	317.00 ± 24.60	298.60 ± 16.9	303.70 ± 8.1
RDW (%)	19.75 ± 3.42	19.08 ± 1.86	16.70 ± 2.08	16.72 ± 1.30
White cell (x10^9L)	4.70 ± 3.47	5.25 ± 2.59	4.08 ± 1.03	3.67 ± 1.41
Neutrophils				
%	28.33 ± 13.97	34.33 ± 15.85	17.20 ± 4.9	16.33 ± 4.37
x 10^9 L	1.50 ± 1.27	1.93 ± 1.31	0.72 ± 0.33	0.62 ± 0.31
Lymphocytes				
%	64.50 ± 14.63	59.50 ± 14.38	72.00 ± 7.21	72.50 ± 7.12
x 10^9 L	2.85 ± 2.04	3.07 ± 1.57	2.92 ± 0.63	2.70 ± 1.11
Monocytes				
%	4.67 ± 3.32	3.50 ± 2.43	8.80 ± 4.44	8.00 ± 4.90
x 10^9 L	0.28 ± 0.26	0.15 ± 0.12	0.38 ± 0.22	0.27 ± 0.19
Eosinophils				
%	2.33 ± 2.33	2.67 ± 3.67	1.80 ± 0.84	2.83 ± 0.98
x 10^9 L	0.07 ± 0.05	0.08 ± 0.08	0.06 ± 0.05	0.10 ± 0.06
Platelet (x10^9 L)	489.80 ± 370.9	832.50 ± 403.3	723.20 ± 401.3	834.00 ± 329.4
Renal Function Test				
Sodium (nmol/L)$^{\beta}$	144.60 ± 3.91	142.00 ± 1.89	140.00 ± 1.41	142.00 ± 0.89
Potassium (mmol/L)	6.13 ± 1.49	5.17 ± 0.73	4.92 ± 0.84	4.72 ± 0.70
Chloride (mmol/L)	106.80 ± 12.26	102.10 ± 2.13	101.80 ± 1.30	100.80 ± 1.47
Urea (mmol/L)	8.04 ± 1.52	7.83 ± 1.16	7.28 ± 1.41	8.02 ± 1.36
Creatinine (µmol)/L	44.36 ± 8.57	38.28 ± 8.61	42.40 ± 11.67	36.67 ± 5.72
Uric acid (mmol/L)	0.17 ± 0.10	0.09 ± 0.05	0.06 ± 0.01	0.07 ± 0.03
Liver Function Test				
Total protein(g/L)	61.67 ± 4.41	58.00 ± 4.47	59.40 ± 4.40	62.67 ± 2.58
Albumin (g/L)	35.00 ± 2.53	33.00 ± 0.00	34.20 ± 1.92	35.50 ± 1.64
Globulin (g/L)	26.67 ± 3.01	25.00 ± 4.47	25.20 ± 2.78	27.17 ± 1.60
Albumin/Globulin ratio	1.33 ± 0.14	1.34 ± 0.22	1.36 ± 0.11	1.30 ± 0.09
Alkaline phosphatase (U/L)	164.50 ± 80.02	141.60 ± 43.88	158.20 ± 58.53	170.50 ± 47.45
AST (U/L)$^{\alpha}$	197.00 ± 40.08	144.40 ± 14.26	177.60 ± 54.98	178.30 ± 98.95
ALT (U/L)	99.33 ± 34.63	62.20 ± 13.83	63.40 ± 12.10	60.83 ± 14.70
Lipid Profile				
Total Chol. (mmol/L)	1.65 ± 0.26	1.58 ± 0.29	1.54 ± 0.17	1.70 ± 0.17
Triglycerides (mmol/L)$^{\beta}$	0.70 ± 0.31	0.54 ± 0.19	0.56 ± 0.09	0.77 ± 0.10
HDL Chol. (mmol/L)	0.47 ± 0.18	0.39 ± 0.07	0.46 ± 0.07	0.50 ± 0.14
LDL Chol. (mmol/L)	0.86 ± 0.27	0.94 ± 0.21	0.82 ± 0.14	0.84 ± 0.02
Total Chol/ HDL Ratio	3.75 ± 0.92	4.14 ± 0.80	3.39 ± 0.27	3.53 ± 0.60
Testosterone (nmol/L)$^{\alpha}$*	5.62 ± 3.61	21.40 ± 8.70	0.53 ± 0.23	0.48 ± 0.22
Estradiol (pmol/L)	-	-	151.00 ± 30.55	122.8 ± 57.00
Progesterone (nmol/L)	-	-	26.88 ± 17.90	48.77 ± 27.68

Values are expressed as mean ± SD; n = 5-6 rats per group. *Abbreviations*: M - male, F - female, P-value < 0.05 is considered significantly different. $^{\alpha}$ - Significantly different in male groups, $^{\beta}$- Significantly different in female groups, *- P-value < 0.0001, RBC-Red blood cell, PCV-Polycythemia vera, MCV-Mean corpuscular volume, MCH-Mean corpuscular hemoglobin, MCHC-Mean corpuscular hemoglobin concentration, RDW-Red cell distribution width, AST-Aspartate transaminase, ALT-Alanine transaminase, Chol.-Cholesterol, HDL-High density lipoprotein, LDL-Low density lipoprotein.

The histological evaluation of liver, kidney, and testis (the male) from rats of the subacute test was featured in Figure 3. Those photomicrographs represented the organs of both genders. The histology of the liver manifested normal architecture of visible central vein, hepatocyte, and sinusoids with no apparent congestion, inflammation, or cytoplasmic inclusion in all groups. While the kidney showed a normal histological structure of glomerulus, Bowman's space, and convoluted tubules in control and treated groups. For testis, the spermatogenesis process was normal in male rats where spermatogonia, spermatocytes, and spermatids exhibited normal arrangement in the respective stages. The seminiferous epithelium of SMME-treated rats appeared similarly to the control's. Normal features of Sertoli cells were also observed in the 1000mg/kg SMME-treated group.

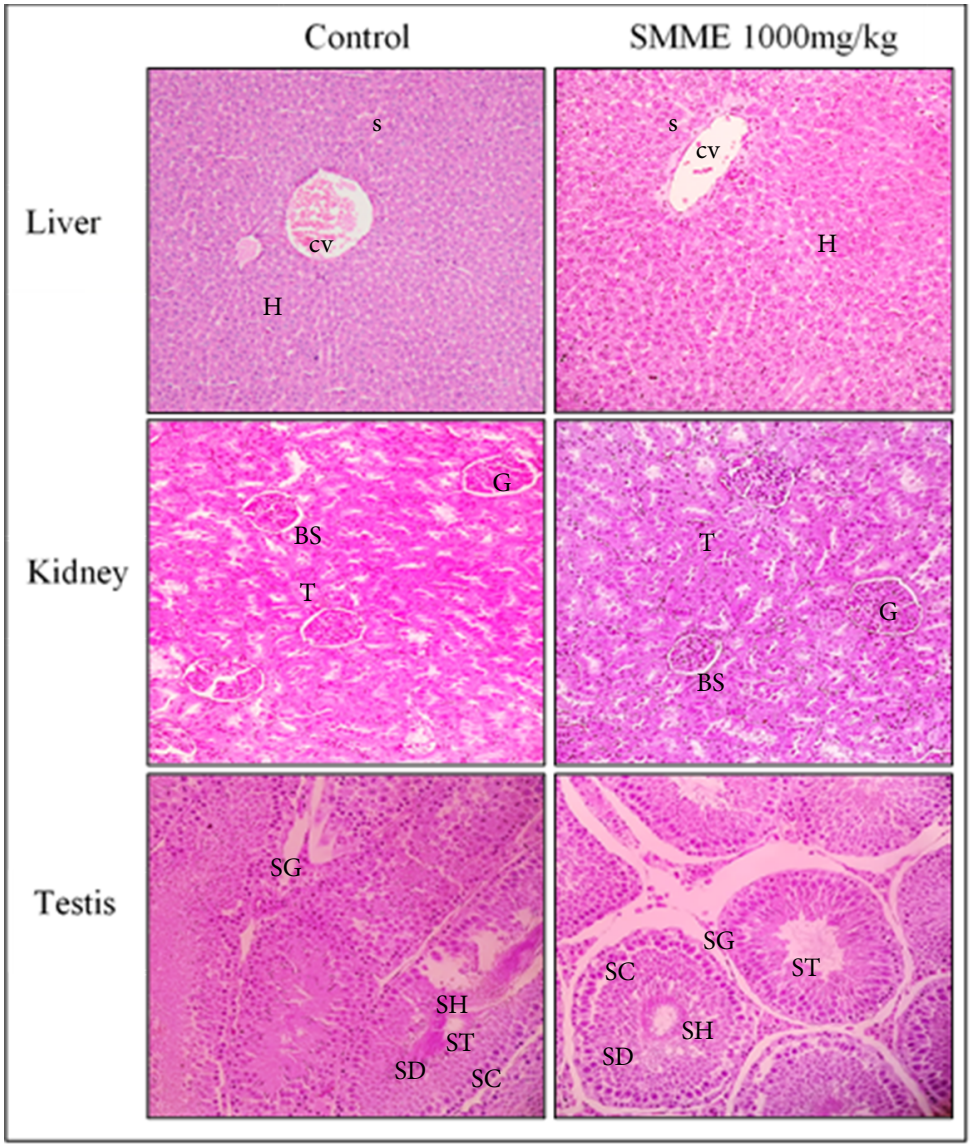

Figure 3. Histological result of liver, kidney, and testes under magnification 200x from subacute toxicity test where all tissues displayed normal architecture and morphologies. *Abbreviations*: S-Sinusoids, CV-Central vein, H-Hepatocytes, G-Glomerulus, BS-Bowman's space, T-Renal tubules, SC-Spermatocytes, SG-Spermatogonia, SD-Spermatids, SH-Sperm heads, ST-Sperm tails.

Aligned with the up-trend research and applications of medicinal plants to treat numerous medicinal issues and diseases, the toxicology studies are supposed to be along to ensure the safety use of the plant, especially when it relates to the reproductive organ system. This study has evaluated the toxic effects of horny little devil, a male aphrodisiac plant, in methanol extract through *in vitro* and *in vivo* studies. BSLT is regarded as an effective method to assess the cytotoxicity levels of substances due to its cost-effectiveness, time efficiency, and the no animal euthanasia needed. The determination of the LC_{50} through *in vitro* studies such as the BSLT will provide the first hint in the toxicology profile of the particular substance, including the *S. myosotiflora*. The value of LC_{50} from BSLT is defined as the concentration of a particular compound that can cause 50% mortality of the brine shrimps. For the SMME in this study, the LC_{50} was found to be 674.4ppm where this was classified as moderate toxic (LC_{50}: 500-1000ppm) according to Meyer's classification (Meyer et al., 1982). While based on the United State Environmental Protection Agency (US EPA) (United State Environmental Protection Agency, n.d.), SMME was considered as 'Practically nontoxic' since its LC_{50} was more than 100ppm; hence not harmful to the aquatic organism, which in this study was referred to the brine shrimps. Previously, the LC_{50} of SMME was reported to be greater than 1000mg/mL in a study by Wan et al. (2016).

Rodents including rats, were among the most common and primary predictive models of human effect in the toxicity assessments via acute and subacute tests through the OECD guidelines. In this study, both tests were performed using the Limit Test of acute no. 425 and subacute no. 407. The application of the Limit Test method can be applied efficiently for assessing particular substances which anticipated low toxicity risk based on the evidence derived from previous studies or experiments. Priorly, *S. myosotiflora* caused no changes in general behavior, sperm morphology, and pregnancy outcome after the SMME treatments in the paternal rats (Ahmad et al., 2014; Hilmi et al., 2015). Elsewhere, the LC_{50} of *S. myosotiflora* in petroleum ether, and ethyl acetate extracts also revealed high values ranging from 2900 to 4800 ppm (H. Osman et al., 2001). Hence, the Limit Test was executed to assay the acute and subacute toxicity profiles of *S. myosotiflora* in this study. As seen in the acute toxicity test, the SMME in 2000 and 5000mg/kg dosages did not exhibit any unusual behavior, mortality, morbidity, or adverse clinical signs in the treated rats. The AOW and ROW were also normal between the groups. According to the GHS, SMME could be classified under Category 5 (Safe) as its LD_{50} was higher than 5000mg/kg; thus, it considered as a nontoxic substance. Other aphrodisiac plants which reported to be in the same category were *Gardenia aqualla* (Mahmudul Hasan et al., 2018), *Pseudopanax arboreus* (Besong et al., 2018)we evaluated the effects of the leaf-aqueous extract of P. arboreus on the sexual behavior of normal male rats. The present study was designed to assess the effects of the leaf-methanolic extract of P. arboreus on amitriptyline-induced sexual dysfunction in male rats. Sexually impaired male rats were randomly divided into 4 groups of 8 rats each. Group 1 received 10 ml/kg distilled water, while group 2 was given 6 mg/kg Viagra. Groups 3 and 4 received 46.5 and 93 mg/kg of the leaf-methanolic extract, respectively. Female rats were made receptive by ovariectomy and subsequent hormonal treatment. Sexual behavior parameters were monitored on days 1, 7, 14, and 21 by pairing each male to a receptive female. The extract-treated rats registered significant decrease in mount latency (ML, *Nymphaea lotus* (Mahmudul Hasan et al., 2018), and *Cassia sieberiana* (Evenamede et al., 2019).

Meanwhile, all the elements measured in the subacute, including food intake, body weight gain, ROW, hematological and biochemicals analyses, and histopathology evaluation, were found to be normal, except there were significantly different in the AST and testosterone of the male group and significantly increased the sodium and triglycerides in the female treated group. AST is one of the biomarker enzymes

which monitor the structural integrity and damage in the liver (Mahmudul Hasan et al., 2018). The normal range of the AST in rats is 50-150 IU/L (Patrick & Villano, 1998). Thus, high AST enzyme in the blood of the male control group could be a sign of damaged in the liver caused by ischemic or toxic. Through this study, SMME has significantly helped to diminish the toxic compounds and restore the level of AST back to the normal range in the treated rats. For female rats, the level of AST was considerably normal and comparable between the control and treated groups, as its standard value is slightly higher. Meanwhile, the level of an essential male hormone, testosterone, was significantly increased in the SMME male-treated rats (P-value < 0.001) but not in female's. The remarkable increase of testosterone only in males is a great sign that the SMME may work selectively to increase the testosterone level through specific pathways, for example, the reproductive system while no detrimental effect to the liver and kidney occurred. This finding is aligned with the former studies (Dasuki et al., 2012; Wan Ghazali et al., 2016; Wan et al., 2013) and proved that *S. myosotiflora* is a potent male aphrodisiac and might be a promising agent to treat the MSD problem.

For female rats, the sodium was significantly increased in the SMME-treated group. However, the increment of the sodium was not considered as an adverse effect of the substance since the value was still within the normal range, 135-145 nmol/L. Simultaneously, the level of triglycerides exhibited a significant increase in the female rats treated with SMME. In response to excessive energy intake, the body stores surplus energy as triglycerides in adipose tissue. In the case of SMME treatment, the extract likely served as an energy source for the rats, resulting in elevated triglyceride levels in the treated group compared to the control group. Due to potential differences in energy requirements between gender, thus, female rats accumulated more triglycerides leading to a higher level in the treated female rats. Nevertheless, these stored triglycerides can be hydrolyzed into fatty acids to meet future energy demands. This situation explained the use of *S. myosotiflora* in traditional medicine as an energy booster where the plant might contain high-calorie content. Meanwhile, the treatment of *S. myosoriflora* in methanol extract form did not induce any abnormalities in the histopathology of liver, kidney, and testis rats. All examined organs appeared with the normal architecture of the respective organs. Some variations were spotted; however, they were very minimal and also detected in the control group. The histopathological results in the study have strengthened overall findings that *S. myosotiflora* is nontoxic and can be further investigated in human trials prior to the development of *S. myosotiflora*-based drug. It is recommended to perform other toxicity evaluations, for instance, genotoxicity, carcinogenicity, and teratogenicity studies in order to scrutinize and validate the safety profile of the *S. myosotiflora* plant.

CONCLUSION

The *in vitro* and *in vivo* toxicity evaluations of the SMME were carried out in the present study. Through the *in vitro* test of BSLT, the LC_{50} of SMME was 674.4ppm hence it was classified as 'Practically nontoxic' according to the US EPA. Meanwhile, the LD_{50} of SMME from the acute toxicity test *in vivo* was revealed to be more than 5000mg/kg and categorized as Category 5 (Safe). The SMME did not exhibit any treatment-related adverse effects in the subacute toxicity test after the behavior, food intake, weight gain, gross necropsy, hematological, biochemical, and histological analyses appeared no significant changes between the control and treated groups. SMME increased the testosterone level of males but not in female rats has shown that the plant was considerably safe after those *in vitro* and *in vivo* toxicity tests and can be further investigated for the application as a male aphrodisiac and a potent drug related to male testosterone deficiency provided more clinical research were carried out in the future.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Sains Malaysia for the financial support through the Research Universiti Initiative (RUI) with grant no. RUI/1001/8012209. Many appreciations also to the staff of the Science Lab Management Unit of School of Health Sciences and the Central Research Laboratory of School of Medical Sciences, USM, for all the help given during the experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

RAR: Experimenting, preparing the study text, reviewing the text, statistics analysis, interpretation of the data, literature research & etc. DSD: Developing hypothesis & reviewing the text. MDS: Developing hypothesis, literature research & reviewing the text. BNBB: Experimenting, interpretation of the data & technical supports. NFA: Experimenting & technical supports. WRWI: Developing hypothesis & reviewing the text.

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FABAD J. Pharm. Sci., 48, 2, 279-284, 2023
Doi: 10.55262/fabadeczacilik.1192521
RESEARCH ARTICLE

Phytochemical and Antimicrobial Study of *Glycosmis mauritiana* (Lam.) Tanaka

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Phytochemical and Antimicrobial Study of Glycosmis mauritiana (Lam.) Tanaka

SUMMARY

We have successfully extracted a novel geranyl flavanone from the root barks of Glycosmis mauritiana. The structure was elicudated as 6-geranyl-5-hydroxy-3'-methoxy-7, 8- (2", 2"-dimethyl pyrano) flavanone- 4'-O-D-glucopyranoside (GM-1), along with 3 known compounds;1-hydroxy-2,3-dimethoxy-10-methylacridin-9-one (GM-2), 1,3,5-trihydroxy-10-methyl-4-(3-methylbut-2-enyl) acridin-9-one (GM-3), 6,11-dihydroxy-3,3-dimethyl-12H-pyrano[2,3-c]acridin-7-one (GM-4), was separated and identified by a variety of spectroscopic techniques. The range of inhibition against four bacteria examined was shown to be 4-25 mm; the GM-1 revealed a significant inhibition zone.

Key Words: *Rutaceae, Glycosmis mauritiana, Flavanone*

Glycosmis mauritiana (Lam.) Tanaka'nın Fitokimyasal ve Antimikrobiyal Çalışması

ÖZ

Glycosmis mauritiana'nın kök kabuklarından başarılı bir şekilde yeni bir geranil flavanon elde ettik. Bu bileşiğin yapısı, 6-geranil-5-hidroksi-3'-metoksi-7,8-(2",2"-dimetil piran)flavanon-4'-O-D-glukopiranozit (GM-1) olarak aydınlatılmıştır. Ayrıca, çeşitli spektroskopik tekniklerle yapıları tanımlanmış olan 1-hidroksi-2,3-dimetoksi-10-metilakridin-9-on (GM-2), 1,3,5-trihidroksi-10-metil-4-(3-metilbut-2-enil)akridin-9-on (GM-3), 6,11-dihidroksi-3,3-dimetil-12H-pirano[2,3-c]akridin-7-on (GM-4) adlı üç bilinen bileşik bulunmuştur. Dört farklı bakteriye karşı inhibisyon aralığı 4-25 mm olarak gösterilmiş olup, GM-1 önemli bir inhibisyon bölgesi göstermiştir.

Anahtar Kelimeler: *Rutaceae, Glycosmis mauritiana, Flavanon*

Received: 21.10.2022
Revised: 09.07.2023
Accepted: 10.07.2023

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INTRODUCTION

In the Rutaceae family, *Glycosmis mauritiana* (syn. *Limonia pentaphylla* Auct., *Glycosmis pentaphylla* Auct.), often called Ash-sheora, Orange Berry, Rum Berry, and Gin Berry. The *Glycosmis maurtiana* found in its natural habitats in India, Pakistan, Malaysia, China, Sri Lanka, Myanmar, Thailand, Indonesia, and Malaya. Plants in this genus have a long history of usage as traditional medicine for treating a wide range of medical conditions (Khare, 2011). As previous research has shown, the *Glycosmis* genus contains quinazolines, furoquinolines (Yasir et al., 2019), carbazoles (Chakraborty, 2022), coumarins (Blanco Carcache et al., 2022), sulphur-containing amides (Hofer and Greger, 2000), quinolones, flavonoids and acridone-type alkaloids (Intekhab, et al., 2011), which have demonstrated to have broad-spectrum activity. The examination of pertinent scholarly works suggests that further investigation is required to explore the phytochemicals in *Glycosmis mauritiana*. As a result, our research endeavors were directed toward examining phytochemicals and their antimicrobial properties with the *Glycosmis mauritiana*.

MATERIAL AND METHODS

Instrumental

Ultraviolet (UV) spectrometer Perkin-Elmer Lambda Bio 20 (Perkin Elmer, USA) was used to record ultraviolet absorption spectrum. On Perkin-Elmer 1710 Fourier transform spectrometer (Perkin Elmer, USA), infrared (IR) spectroscopy was carried out utilising the KBr disc. As an internal standard, tetramethylsilane (TMS) is used to calculate δ values (ppm). The FEBMS were recorded using the JEOLSX 1021/DA-6000 mass spectrometer (JEOL Ltd. Japan). The Bruker AVANCE DRX-400 (German) was used to record NMR spectra (400, 100 MHz). Silica gel (60–120 mesh) was used for column chromatography. Chemicals and reagents used in this experiment were AR quality from E-Merck (Pakistan).

Plant material

In June 2021, root barks of *Glycosmis mauritiana* were picked throughout remote areas of the Faisalabad District. The herbarium specimens kept in the faculty of botany's herbarium at Agriculture University Faisalabad, Pakistan were compared to ensure validity. Secondary plant components can be extracted from both fresh and dried plant material. It is preferable to utilize newly collected and dried material, as dried substance that has been stored pro a longer duration may lose a significant amount of its original qualities.

Extraction

Air-dried roots of *Glycosmis mauritiana* were first defatted with petrol-ether (3L x 5 times) to obtain 80 g of petrol-extract on distillation under reduced pressure. The marc was then extracted with chloroform, methanol and ethyl acetate (3L x 5 times each). On standing at room temperature, the petrol-ether extract produced a yellow precipitate, which on re-crystallization with chloroform, produced yellow crystals of compound **GM-4**. Similarly, the chloroform extract was subjected to column chromatography. The column was uninterruptedly eluted with the solvents and solvent mixtures of increasing polarity. Elution with $CHCl_3$: EtOAc (8:2) yielded compound **GM-3**.

The alcoholic extract thus obtained was decanted in 500 ml distilled water to get water soluble and insoluble portions. After partitioning with benzene, the water-insoluble part (ppt.) was dissolved in methanol to provide 15 g of methanolic extract. The slurry was made to methanolic extract with 5 g of silica gel in pet-ether and was digested to well settle column. The column was uninterruptedly eluted with the solvents and solvent mixtures of increasing polarity. Elution with $CHCl_3$: MeOH (7:1) afforded **GM-2**.

The ethyl acetate extract was evaporated under a vacuum on a rotatory evaporator below 50 ^{O}C temperature to yield 35 g of ethyl acetate extract. A well-stirred silica gel suspension (100 -150 g in pet-ether 60-80^{0}) was poured into a column (150 cm long and

50 mm diameter). When the absorbent was well settled, the excess petrol was allowed to pass through the column. The slurry was made of 5 g of silica gel in ethyl acetate and was digested to well resolve column. The column was eluted with the solvents and solvent mixtures of increasing polarity. Elution with $CHCl_3$: MeOH (6:4) yielded compound **GM-1**.

RESULTS AND DISCUSSION

GM-1 was isolated as a yellow powder from the ethyl acetate extract by eluting the column with $CHCl_3$: MeOH (6: 4). The compound exhibits a purple spot on the thin layer chromatography plate when evaluated under UV light. The compound showed a positive chemical test for flavonoid glycoside (Mabry, 1970). This compound is a flavanone containing at least one hydroxyl group that was identified by UV spectral investigations, which revealed a dark blue color in Gibb's test, green with $FeCl_3$, and a pink color with magnesium-HCl (conc.) acid (Mabry, 1970). The UV spectra of the compound revealed bands with maximum wavelengths at 218 and 291 nm, typical of a flavanone skeleton (Mabry, 1970) (Figure 1).

In IR spectra, absorption bands appeared at 1645 cm^{-1} and 3327^{-1} of the molecule were ascribed to conjugated ketone and hydroxyl groups, respectively. Thus, the UV and IR of the compound show absorption bands, indicating that it belongs to the flavanone class (Mabry, 1970). When the ^{1}H NMR spectrum of the molecule was examined, it was evident that the signals of aromatic protons and signals from the pyran ring, the glucosyl moiety, and the geranyl group were present.

In the ^{1}H NMR spectra of the compound, signals were observed at 5.57 (1H, dd, J= 12.5, 3.2 Hz), 2.98 (1H, dd, J= 17.6, 3.2 Hz), and 2.81 (1H, dd, J= 17.6, 12.5 Hz). These protons were allocated to the flavanone C-ring protons. These protons are found in flavanones in the H-2, H-3_{ax}, and H-3_{eq} configurations. In ^{1}H NMR, a signal was found at δ_H 12.30, which corresponding to the C-5 hydroxyl group. Three protons signals were also seen at δ_H 7.14 (1H, d, J = 2.3 Hz, H-2′), 7.09 (1H, d, J= 8.3 Hz, H-5′), and 6.99 (1H, dd, J= 8.3, 2.3 Hz, H-6′) attributed to the aromatic protons of the 3′ and 4′ disubstituted B- ring (Andersen, 2006). Aside from that, two proton doublets of the AB pattern were observed at δ 5.39 (1H, d, J = 9.8 Hz) and 6.48 (1H, d, J = 9.8 Hz), which were attributed to the chromen protons H-3" and H-4", respectively (site C-7 /C-8) of the flavanone (Mabry, 1970). In order to account for the two methyl groups at C-2", the sharp singlets were observed and assigned to δ_H 1.43 and 1.45, each for three protons. Furthermore, the ^{1}H NMR singlets detected at δ_H 1.54, 1.63, and 1.67 were assigned to three vinylic methyl protons, consistent with the literature. Additionally, this flavanone allocated signals at δ_H 3.35, 2.09, and 1.98 for the three methylene protons. Additionally, signals were seen at δ_H 5.16 (H-6‴) and 5.27 (H-2‴) assigned for two methine protons, ascribed to the presence of a geranyl substituent in the compound (Andersen, 2006; Harborne, 1986; Phillips, 1996; Smejkal, 2007). According to the findings, the ^{1}H NMR also revealed a signal at δ_H 3.87 ascribed to a methoxyl group in ring B. Moreover, a one proton singlet detected at δ_H 5.43 (1H, d, J= 8.4 Hz) is assignable for an anomeric proton (H-1⁗), indicative that this sugar has β- arrangement. Additionally, signals found in the range δ_H 3.35–4.46 are assignable to different protons of sugar molecules (Mabry, 1970; Harborne, 1986). The compound was hydrolyzed with 2N HCl and treated with the standard workup to form glucose. A comparison of Co-PC's results with authentic samples revealed that the sugar is D-glucose. Ring A comprises the geranyl and 2, 2-dimethyl chromen groups. There is substantial evidence that the C-6 geranyl group can be attached to the C-6 in the compound as the chemical shift δ_C 113.12 (Agrawal, 1989; Markham, 1982). The geranyl moiety was proven to be at C-6 of ring A using HMBC correlations between H-1‴ at δ_H 3.35 and C-6 (108.23), C-5 (160.21) and C-7 (159.33). Moreover, connection at δ_H 5.43 of H-1⁗ with C-4′ (146.26) was used to determine the position of the β-D-glucose moiety at C-4′. The ^{13}C NMR spectrum

indicates that the molecule contains a 4'-O-D-glucopyranoside linkage, as evidenced by the presence of a signal at δc 102.57, which is attributed to the anomeric carbon. (Agrawal, 1989; Markham, 1982). These data indicated the presence of angular chromen ring with a geranyl group at C-6. Additionally, the protons at δ 3.87 correlated to C-3′ (149.31), designated that the methoxyl group positioned at C-3′ (Agrawal, 1989; Markham, 1982). Spectral studies ultimately identified the compound as 6-geranyl-5-hydroxy-3′ -methoxy- 7, 8- (2″, 2″ -dimethyl pyrano) flavanone-4'-*O*-β-D-glucopyranoside, a new flavanone glycoside from this plant.

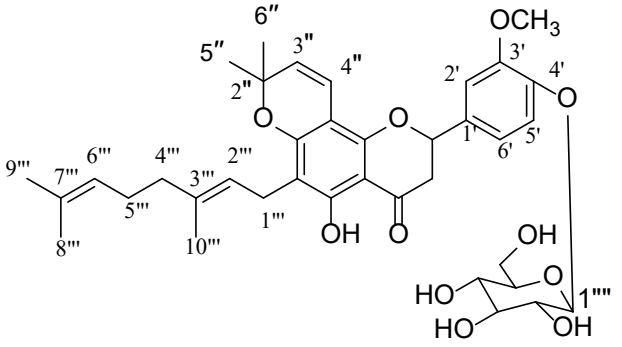
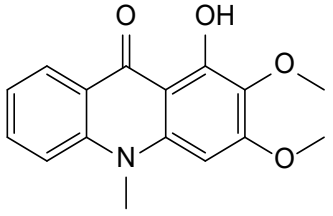
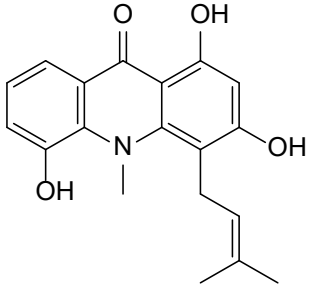


Figure 1. GM-1

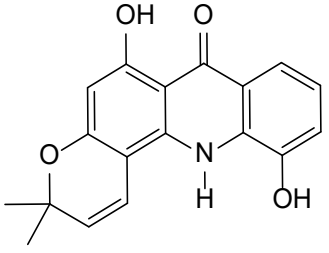
Three known compounds were isolated and identified as 1-hydroxy-2,3-dimethoxy-10-methylacridin-9-one (arborinine GM-2), 1,3,5-trihydroxy-10-methyl-4-(3-methylbut-2-enyl)acridin-9-one (GM-3), 6,11-dihydroxy-3,3-dimethyl-12*H*-pyrano[2,3-c]acridin-7-one (GM-4) through matching their NMR values to one previously published (Figure 2).



GM-2



GM-3



GM-4

Figure 2. The chemical formulas of GM-2, 3 and 4.

Antimicrobial activity

Nutritional agar media preparation

Nutrient agar, 28 g, mixed with 1 L of deionised water, autoclaved for 15 minutes at 121°C, then chilled to 47°C before being placed into Petri dishes.

Testing for antibacterial activity

The antibacterial activity of the compounds was evaluated using the cup-plate agar diffusion method. During the experiment, 100 ml of molten nutritional agar was mixed thoroughly with one milliliter of the standard bacterial stock solution (108-109 CFU/ml). In sterile Petri plates, 20 ml aliquots of the infected nutrient agar were delivered to each recipient. The agars were allowed to be set before 5 cups (10mm in diameter) were cut out of each plate using a sterile cork borer (No. 5), and the agar disc was carefully stood apart. On alternating days, a 0.1ml sample of each compound dilution in methanol was pipetted into alternate cups using an automated microliter pipette. The cups were permitted to diffuse for two hours at room temperature. The plates were then incubated for 18 hours in an upright position at 37°C to finish the procedure. Each compound was tested against each test organism three times, with three duplicates for each extract. When the growth inhibition zones formed during the incubation process were measured, the diameters were averaged, and the mean values were calculated (Table 1).

Table 1. Zones of inhibitions of different extracts of selected medicinal plant Concentration of extracts

Compound/ Bacterial organisms	Concentration in mg/ml	*Bacillus subtitles*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas arginosa*
GM-1	100	25	21	19	15
	50	24	17	18	10
	25	23	14	17	8
	12.5	22	12	12	6
GM-2	100	22	18	17	9
	50	20	17	16	7
	25	19	15	15	6
	12.5	18	14	11	4
GM-3	100	20	8	9	11
	50	19	7	8	10
	25	17	5	7	8
	12.5	15	2	4	7
GM-4	100	12	20	17	10
	50	11	19	15	9
	25	9	17	14	8
	12.5	7	16	11	5

The extract of *Glycosmis mauritiana* was tested for antibacterial activity against four bacterial strains and one fungus at different dosages using the cup plate agar diffusion technique, and the inhibition zone was determined in millimeters (mm). The inhibition range was 4-25 mm, against four bacteria tested; the GM-1 demonstrated a substantial inhibition zone.

CONCLUSION

A literature survey indicated that 6-geranyl-5-hydroxy-3′ -methoxy-7, 8- (2", 2"-dimethyl pyrano) flavanone- 4'-O-D-glucopyranoside is a novel flavanone.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

SK: Conceptualization, Analysis and/or Interpretation

AM: Conceptualization , Reviewing the article before submission, Materials, Supervision, Design

SMV, RJ: Literature Review, Data collection

ARS, RJ: Data Collection

SU: Taking responsibility in the construction of the whole manuscript

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FABAD J. Pharm. Sci., 48, 2, 285-294, 2023
Doi: 10.55262/fabadeczacilik.1259636
RESEARCH ARTICLE

Validation Of Rp-Hplc Uv Method for Determination Ketoconazole in Rabbit Plasma: an Application to The Pharmacokinetic Study

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Validation of RP-HPLC UV method for determination ketoconazole in rabbit plasma: An application to the pharmacokinetic study

SUMMARY

The validated method for determining ketoconazole in plasma rabbit is not yet reported. The HPLC-UV method is simple, rapid, cost-effective, sensitive, and only requires a small blood sampling. The chromatographic system used a mobile phase consist of NaH2PO4:Acetonitrile (30:70) and stationary phase as a reversed-phase C18 column (250 x 4.6 mm, 5 µm) at a flow rate of 1 ml/ min and detection wavelength of 240 nm, and the retention time of about 5 minutes for ketoconazole and 11 min for itraconazole as internal standard. The peak of ketoconazole can separate from other peaks and has no interference from the diluent, indicating this method was selective to detect ketoconazole. The calibration curve presented linearity in the 0.05-8 µg/ml with R2=0.9969, which showed good linearity. Precision and accuracy of the method were obtained. The result is 9.47 %diff and 10.13-12.08 RSD% for LLOQ and 0.59-3.94 %diff and 1.82-13.56 %RSD <20% for low, medium, and high levels. The LLOQ in this method is 0.05 µg/mL. Plasma stability under storage in a freezer (-200C) for three days was studied. The validated analytical method was successfully applied to determine the pharmacokinetics parameter of KTZ after a single oral administration.

Key Words: *Chromatography, imidazole, plasma, pharmacokinetic, validation method*

Tavşan plazmasındaki ketokonazol'un belirlenmesi için RP-HPLC UV yönteminin doğrulanması: Farmakokinetik çalışma için bir uygulama

ÖZ

Tavşan plazmasında ketokonazol tayini için doğrulanmış yöntem henüz bildirilmemiştir. HPLC-UV yöntemi basit, hızlı, uygun maliyetli, hassastır ve yalnızca küçük bir kan örneği gerektirir. Kromatografik sistem, NaH2PO4:asetonitril (30:70) içeren bir mobil faz ve sabit faz olarak ters faz bir C18 kolon (250 x 4.6 mm, 5 µm) kullanıldı. işlemde akış hızı 1 ml/dk, saptama dalga boyu 240 nm olarak ayarlandı ve dahili standart olarak alıkonma süresi yaklaşık 5 dakika olan ketokonazol ve 11 dakika olan itrakonazol kullanıldı. Ketokonazol piki diğer piklerden ayrılabilir olması ve dilüe ediciden etkilenmemesi bu yöntemin ketokonazolü saptamak için seçici olduğunu gösterir. Kalibrasyon eğrisi, iyi doğrusallığı gösteren R2=0.9969 ile 0.05-8 µg/ml'de doğrusallık gösterdi. Yöntemin kesinliği ve doğruluğu elde edildi. Sonuç, LLOQ için %9.47 fark ve %10.13-12.08 RSD ve düşük, orta ve yüksek seviyeler için %0.59-3.94 fark ve %1.82-13.56 RSD <%20'dir. Bu yöntemdeki LLOQ 0.05 µg/mL'dir. Üç gün boyunca bir dondurucuda (-20°C) depolama altında plazma stabilitesi incelenmiştir. Doğrulanmış analitik yöntem, tek bir oral uygulamadan sonra KTZ'nin farmakokinetik parametresini belirlemek için başarıyla uygulandı.

Anahtar Kelimeler: *Kromatografi, imidazol, plazma, farmakokinetik, validasyon yöntemi*

Received: 03.03.2023
Revised: 19.07.2023
Accepted: 20.07.2023

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INTRODUCTION

Ketoconazole (KTZ) is an imidazole broad-spectrum antifungal agent used for systemic and local infections (Hamdy & Brocks, 2009). KTZ is reported to have the ability to inhibit CYP3A4 and P-gp, which could inhibit several other tyrosine kinase inhibitors' substrates (Wang et al., 2022). It is potent inhibitor biosynthesis in *Candida albicans* of ergosterol that major sterol to synthesizing the fungal cell wall and other complexes (Oji, 1982). It is used as oral administration to control systemic mucocutaneous fungal infection. KTZ has poor water solubility (0.04 mg/ml), highly degraded *in vivo*, and eliminated through the bile-solubilized feces pathway (Aljurbui et al., 2022).

High-performance liquid chromatography (HPLC) is one of the most effective techniques for separating various mixtures and determining the number of compounds in many samples (Ban & Jinno, 2001). The HPLC method has been reported for determining ketoconazole in plasma (Bajad et al., 2002). There were HPLC-UV (Bajad et al., 2002; Hamdy & Brocks, 2010; Vertzoni et al., 2006), HPLC coupled with fluorescence detection (Alvinerie et al., 2008; Han et al., 2019), and HPLC tandem MS-MS (Chen et al., 2002). The HPLC with the fluorimetric detector and tandem MS-MS has a higher cost than UV (Ranjan et al., 2013). The HPLC-UV method is simple, rapid, cost-effective, sensitive, and only requires a small blood sampling (Bajad et al., 2002; Hamdy & Brocks, 2010).

In the HPLC-UV method, the internal standard (IS) is crucial for extraction efficiency and recovery (Chen et al., 2002). In the previous study, the IS that was used took a long elution time (>20 min). There was phenothiazine (Pascucci et al., 1983), R41300 (Swezey et al., 1982), terconazole (Turner et al., 1986), and clotrimazole (Riley & James, 1986). With a long retention time, the IS makes analysis inefficient, causing high costs and a wasteful mobile phase. An ideal method should have cost-effective by short total elution time of analyte and IS (Chen et al., 2002). In the early 2000s, the HPLC-UV methods for determining ketoconazole were still limited. Vertzoni et al. presented a technique that used a reversed-phase Hypersil BDS-C18 column with mobile phase methanol, water, and diethylamine (74:26:0.1), used 9-acetyl anthracene as IS with an elution time about 7-10 min use canine/dog plasma (Vertzoni et al., 2006). Hamdy et al. published the method to separate midazolam and ketoconazole, used a Symmetry C18 column with the mobile phase as Acetonitrile and KH_2PO_4 (45:55), and diazepam as IS (run time was 10 min for rat plasma and 19 min for human plasma) (Hamdy & Brocks, 2010). Bajad et al. described a method that used HPLC to determine ketoconazole and piperine in rat and human plasma simultaneously. The column used Water's Symmetry C18 with Acetonitrile: KH_2PO_4 (50:50) as mobile phase, without internal standard (Bajad et al., 2002).

The pharmacokinetic information for ketoconazole is relatively limited. The bioavailability data of ketoconazole is available only for rats, dogs, monkeys, and humans following oral doses was 35.8%, 50%, 22%, and 81.2%, respectively (CHMP, 2014). The data for rabbits have not been reported yet. Due to inter-subjects in several species, data have variability, so it is important to be researched. Before conducting a pharmacokinetic study, it is essential to validate the method for determining the analyte. In addition, no validation method of HPLC-UV for the determination of ketoconazole in rabbit plasma has been reported to date.

This study aims to validate a simple, sensitive, less time utilizing, and reliable RP-HPLC method with UV detection to determine ketoconazole in rabbit plasma according to the EMA guidelines. We present a rapid, selective, and sensitive HPLC-UV way with simple pretreatment procedures to determine KTZ in rabbit plasma with 12 min elution time and itraconazole as IS. To the best of our knowledge, this is the first method for the pharmacokinetic study of KTZ in

rabbits that has been thoroughly validated.

MATERIAL AND METHODS

Materials

Ketoconazole and itraconazole standard were bought from BPOM, Indonesia. Ketoconazole's active component was obtained from PT. Kimia Farma, Indonesia. The sterile water for injection was manufactured by Ikapharmindo, Indonesia. Deionized water was supplied from CV. Alfa Kimia. Acetonitrile gradient grades for HPLC (Merck, Germany), NaH_2PO_2 (Merck, Germany), and NaOH (Merck, Germany).

Animals

Three male New Zealand White rabbits, 3-4 months, weight 2,5-3 kg, were purchased from rabbit breeder Yogyakarta, Indonesia. Three rabbits were kept in a clean caught (Laboratory Animal, Pharmacology Department, Faculty of Pharmacy, Indonesia) with a 12-h light and dark cycle, with diet food of Vital Rabbit (Citrafeed, Indonesia) and water available ad libitum. Ethical clearance of the study was approved by the Faculty of Veterinary, Universitas Gadjah Mada, Indonesia (approval number 039/EC-FKH/Eks./2022).

Blood sampling and sample preparation

Rabbits fasted overnight before the experiment. The rabbits were administered a 14 mg/kg KTZ (equivalent to 400 mg KTZ human doses). The drug was put into the rabbit's mouth through the tube. An aliquot of approximately 2 ml blood samples was collected from the ear marginals vein at 0 (before administration), 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, and 420 after the oral dosing. The sample was collected into an EDTA tube containing 3 mL EDTA. Blood samples were centrifuged for 10 min at 3000 x g rpm to get plasma. The supernatant as a plasma sample was transferred to a 1.5 mL microtube. The samples were kept at -18^{0}C until assayed.

The IS (100 µL) was added to 150 µL plasma sample rabbit plasma in a 1.5 ml microtube. Then the sample was deproteinized with 300 µL acetonitrile. After vortexing for 1 min, the plasma was separated by centrifugation at 12,000 x g for 10 min, and 100 µL supernatant was transferred to a clean 1.5 mL microtube. Before analysis, the analyte was filtered by a 0.45 µm membrane filter. The concentration in the supernatant was determined by the RP-HPLC method.

Chromatographic condition

The HPLC system consisted of Elite LaChrom HPLC, Hitachi UV-Vis detector L-2420, and Hitachi pump L-2130. The utilized column was Phenomenex Luna (250 x 4.6 mm, 5 µm). The UV wavelength was set at 240 nm. The mobile phase consisted of a mixture of 0.02 sodium dihydrogen phosphate pH 7.0 adjusted with 1 M sodium hydroxide and acetonitrile (30:70) at a 1 ml/min flow rate. The injection volume was 20 µl. The mobile phase was filtered using the cellulose 0.45 µm membrane filter. The retention time for ketoconazole and itraconazole (as IS) took around 5 and 11 min, respectively.

System suitability test

Six replicates of the system suitability test were injected at 4 g/mL. The retention time, peak area, theoretical plates, and tailing factor were calculated. To be considered acceptable, the relative standard deviation (%RSD) of retention time should be less than 2%, the peak area should be less than 20%, the tailing factor should be 2, and the theoretical plates of the column (N) should be 2000.

Preparation of stock solution

The stock solution of ketoconazole was made by dissolving 10.0 mg of ketoconazole, then diluted with 100 mL methanol to get the final concentration of 500 µg/mL. The Acetonitrile diluted the stock solution to make 20 µg/mL.

Linearity

Linearity was performed by seven series of standard solution with concentrations of 0.05; 0.1; 0.4; 0.5; 2; 4, and 8 µg/mL as follows: in microtube containing 135 µL blank plasma, 15 µL ketoconazole working solutions (0.5-80 µg/mL) were transferred. After

vortexing for the 30s, add 100 µL IS working solution (25 µg/mL) and 300 µL Acetonitrile. After vortexing for the '30s, the samples were centrifuged for 10 min at 12.000 rpm, at 4^0C. The supernatant was filtered with a nylon filter of 0.22 µm and then injected into the HPLC system. The plotting of concentration (x) and peak area (y) was obtained to get the calibration curve. The linear regression results calculated the intercept, slope, and correlation coefficient.

Specificity

Specificity was determined with a sample of blank plasma 25 µm/ml IS and ketoconazole spiked plasma standard (4 µm/ml).

Accuracy and Precision

Accuracy and precision were determined in spiked plasma standards at four levels (LLOQ, low, medium, and high), which were 0.05, 0.4, 4, and 8 µg/mL, respectively, measured three replicates (n=3) for accuracy and intraday precision, and measured three days consecutively for interday precision.

Stability study

The stability of KTZ was evaluated using a calibration standard of KTZ 8 µg/ml. The condition used in stability testing is a freshly prepared sample and storage in a freezer (-20^0C) for three days.

Data and statistical analysis

Peak-area ratios of the analyte and IS were used for calculation. One compartmental analysis (PKSolver-an add of Microsoft Excel) was used to estimate the pharmacokinetic parameters: the total area under the curve from time zero to infinity ($AUC_{0-\infty}$), Kel, Kab, $t_{1/2}$, Vd, Cl, $C_{max,}$ T_{max}. The data of the parameter result was analyzed using the One Sample T-test. Differences with *p* value less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The chromatographic method was a reversed-phase mode that used a C-18 column (250x4.6 mm, 5 µm particle size) as a stationary phase. The mobile phase consisted of a mixture of 0.02 sodium dihydrogen phosphate pH 7.0 adjusted with 1 M sodium hydroxide and acetonitrile (30:70) at a 1 ml/min flow rate. The injection volume was 20 µl. Absorption was measured at 240 nm. The elution times for ketoconazole and itraconazole (as IS) were approximately 5 and 11 min, respectively.

HPLC with UV detection was chosen due to its simple, fast, and good separation method for determining KTZ. The optimal chromatographic conditions were achieved as described above. The mobile phase composition is essential for separating observed compounds from the other analyte. The best result mobile phase is Acetonitrile and NaH_2PO_4 pH 7 (70:30). Acetonitrile was utilized to obtain peaks with better resolution and symmetry. Alaa et al. observed that using methanol to detect the plasma samples resulted in the band broadening with more retardation (Khedr, 2008). Generally, increasing the concentration of organic solvent in the mobile phase impacts reducing the distance between the solute molecule and the terminal carbon atoms (C18) in the ODS ligand column, which causes a decrease in the retention time (Sankalia et al., 2007)

Itraconazole has been chosen for IS because of its similar properties and chemical structure. It can be obtained in the same condition as sample preparation, chromatographic system, and detected using a UV detector at the same wavelength with KTZ. The IS demonstrates stable ionization efficiency and adequate and reproducible extraction recovery using the sample pretreatment method in this study (Gu et al., 2016). The retention time of KTZ and IS were eluted at about 5 and 11 min, respectively. The resolution between KTZ and IS has been good.

Validation method

Acetonitrile was used for protein precipitation to obtain satisfactory values for recovery KTZ and IS. The increase in Acetonitrile led to an improvement in extraction recovery. However, diluted samples with

extensive acetonitrile will reduce sensitivity (You et al., 2005). In this study, the optimal ratio of plasma sample to acetonitrile is 1:2. The chromatography system condition shows the assay's ability to separate the KTZ and the IS from the plasma sample without interference from any endogenous compounds.

The HPLC system was optimized to demonstrate the system's suitability, including the retention time, tailing factor, number of plates (N), retention factor (k'), and peak area (%RSD) (Table 1). In the system suitability test, the %RSD result of retention time was less than 2%, and the peak area was less than 20%, indicating that the system is suitable for analysis. Ketoconazole separated well from the front solvent and formed a symmetrical peak with a tailing factor of 1.38 (good if 2). The number of theoretical plates (N) at 7813 indicates that the criteria of N (>2000) are acceptable. The retention factor (k') is found to be 514. It meets the requirements that should be > 2.

Table 1. System suitability test result

System parameters	Acceptance criteria	Result
Retention time (min)	RSD < 2%	5.15±0.02 RSD: 0.32%
The ratio of Peak area	RSD < 20%	0.24±0.009 RSD: 4.09%
Tailing factor	< 2	1.38±1.37
Theoretical plates	>2000	7813±116
Retention factor (k')	>2	514±1.37

Selectivity has been evaluated by blank plasma, standard internal (IS), and a spiked plasma standard (4 µm/ml) containing 25 µm/ml IS and standard internal (Figure 1). The ketoconazole retention time was found to be 5.15 minutes. The peak of ketoconazole can separate from other peaks and has no interference from the diluent, indicating this method was selective to detect ketoconazole.

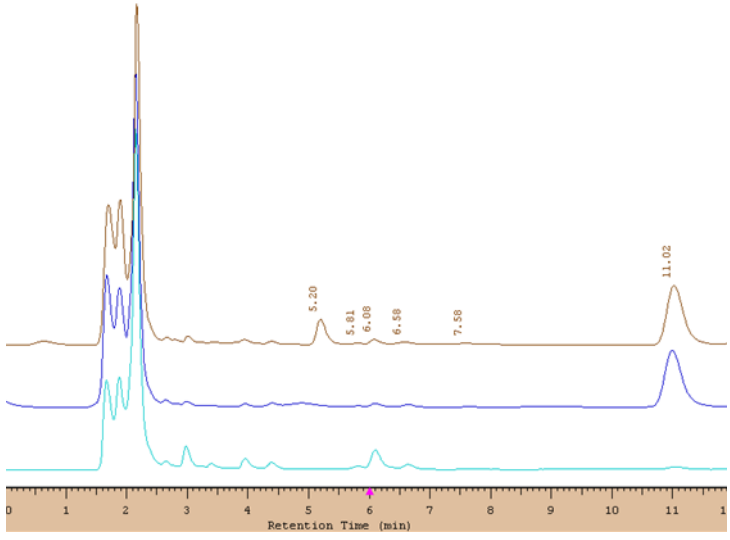

Figure 1. Specificity chromatogram of blank plasma (A), Internal Standard (IS) (B), and ketoconazole spiked plasma standard (4 µm/ml) containing 25 µm/ml IS (C), obtained for ketoconazole with mobile phase used NaH_2PO_4 pH 7:Acetonitrile (30:70)

C

Figure 2 depicts the standard calibration curve with concentration ranges of 0.05 to 8 µm. The intercept (a), slope (b), and coefficient (r2) were used to evaluate linearity. The method's capacity to proportionate correlation concentration in the sample is known as linearity. Sample concentration was linearly correlated with calibration curves. All chemicals' coefficients of determination (R2) were 0.9969, indicating that the approach has a solid linear relationship between peak area and concentration.

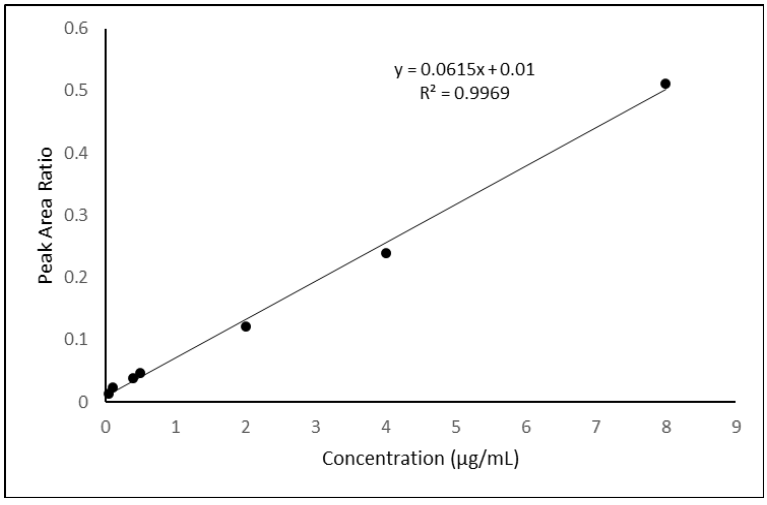

Figure 2. Calibration curve of with mobile phase used NaH_2PO_4 pH 7.0:Acetonitrile (30:70)

The evaluation of accuracy (Table 2) and precision (Table 3) used the standard addition method to calculate the %diff and %RSD for four concentration levels. There were LLOQ, Low, Medium, and High, with three replicates from each concentration. The result of LLOQ was 9.47 for %diff and 12.08 for %RSD. Other levels' results were <3.94 for %diff and 13.56 for %RSD. These values are within the acceptable range, indicating that the method is accurate and precise.

Table 2. Accuracy data of the proposed method

Level	Cons. standard (µg/mL)	Cons. detected (µg/mL)	%Diff
LLOQ	0.05	0.055±0.01	9.47
Low	0.4	0.402±0.05	0.59
Medium	4	3.842±0.12	3.94
High	8	8.208±0.15	2.59

Table 3. Precision data of the proposed method

Level	Cons. standard (µg/mL)	Intraday RSD(%)	Interday RSD (%)
LLOQ	0.05	12.08	10.13
Low	0.4	11.21	13.56
Medium	4	3.07	7.91
High	8	1.82	2.39

The stability of KTZ in different storage conditions was summarized in Table 4, which indicated that KTZ was stable for three days and kept in a freezer (-20^{0}C) without changes in the concentrations tested.

Table 4. Stability data of the proposed method

Condition	Cons. observed ± SD (n=3)	Diff (%)	RSD(%)
Freshly	8.177±0.64	2.21	7.83
Frezeer day 1	8.322±0.79	4.02	9.53
Frezeer day 3	8.240±0.84	3.00	10.26

Pharmacokinetic application

The procedure described above was used to measure the plasma concentration of KTZ following an oral dosage experiment on three rabbits. Chromatogram of ketoconazole in rabbit plasma after oral administration showed in Figure 3.

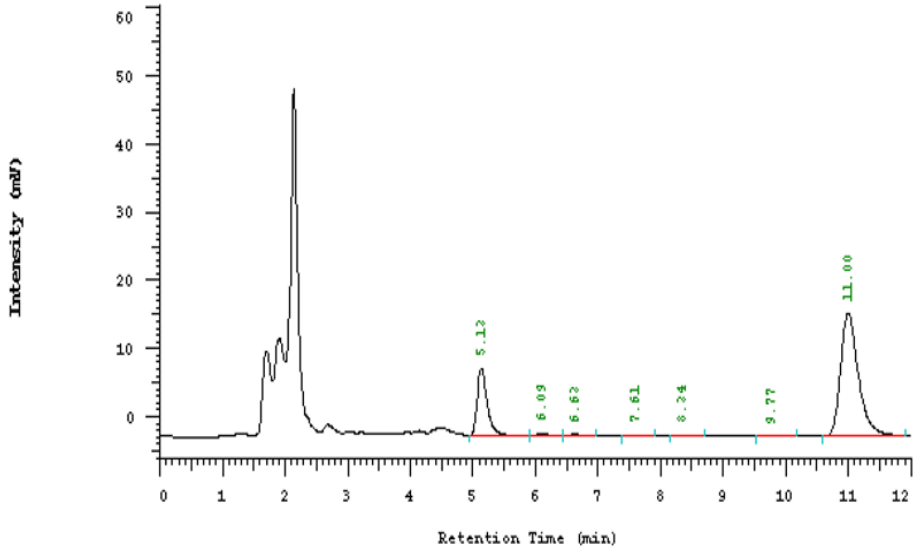

Figure 3. Chromatogram of ketoconazole in rabbit plasma after oral administration

Figure 4 shows three individual pharmacokinetic profiles of ketoconazole in rabbit plasma after oral administration of powder KTZ. Biological variations in the animal cause the difference in the data of the three replications.

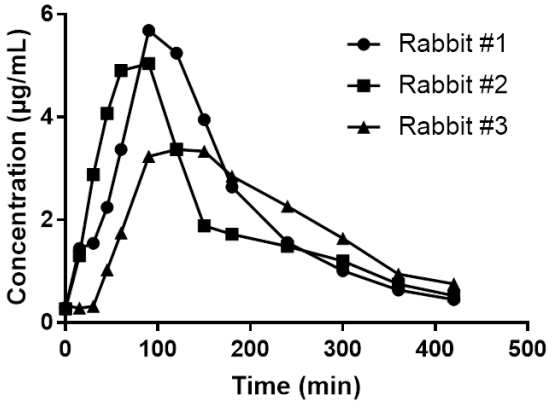

Figure 4. Pharmacokinetic profiles of ketoconazole in rabbit plasma after oral administration

Table 5 presents the corresponding individual pharmacokinetic parameters. The PK parameters were calculated using one-compartmental analysis by computing the particular value of the concentration at each time point. After formulation administration, a peak concentration of 3.74±0.67 μg/mL (C_{max}) was reached at 99.06±28.39 min (T_{max}). The peak concentration of the drug is defined as its highest concentration in blood plasma. It is one of the most important pharmacokinetic parameters for assessing how the medicine affects. The plasma levels may have different results based on dose, route of administration, ease of absorption, the distribution of the drug throughout the body, the bioavailability, and the effectiveness metabolized of the drug (Webb, 2011). The half-life was found to be 78.09±16.74. The half-life typically refers to eliminating the drug in plasma levels that fall to 50% of the initial dose. Different drugs have varying half-lives; the half-life value helps determine any particular drug's excretion rates and steady-state concentrations. The half-life of elimination can be used to predict the length of elimination of the drug. For the first order, the relationship between $t_{1/2}$ and elimination is after 3.3x half-lives, 90% of the given drug will be eliminated, and after 4-5x half-lives, elimination can be considered complete (Hallare & Gerriets, 2022). The area under plasma concentration ($AUC_{0-\infty}$) was 972±104. The AUC is commonly used to assess how much a drug reaches the bloodstream in each period following dose administration. The data can be used to evaluate pharmacodynamic responses (Scheff et al., 2011).

Table 5. Pharmacokinetic parameters of ketoconazole after oral administration

Pharmacokinetic parameter	Rabbit #1	Rabbit #2	Rabbit #3	Avarage±SD (n=3)	P value
C_{max} (μg/mL)	4.15	4.29	2.79	3.74±0.67	0.016
T_{max} (min)	93.73	67.26	136.19	99.06±28.39	0.039
$AUC_{0-\infty}$ (μg/mL*min)	1057	825	1034	972±104	0.006
$T_{1/2}$ (min)	66.98	65.54	101.76	78.09±16.74	0.022

The concentration of ketoconazole in plasma was obtained with a peak area ratio of ketoconazole to internal standard from calibration curves. The method had good linearity with R^2=0.9969, good selectivity, good precision within and interday (%RSD) for 4 level concentrations (LLOQ, low, medium, and high), good accuracy (%diff) for 4 level concentrations (LLOQ, low, medium, and high), and the plasma sample can be used for three days kept in the freezer (-20^{0}C) due to its stability. The LLOQ for this validated method is 0.05 μg/mL for plasma. It meets the criteria for detecting KTZ in rabbit plasma after oral dosing cause the lowest concentration obtained is 0.4 μg/mL. The *p*-value of the parameters is less than 0.05, indicating significant differences between the three replicates of rabbits. The differences in the results of pharmacokinetic data are primarily due to biological variations present in the animal.

CONCLUSION

The HPLC-UV method was successfully developed and comprehensively validated for application to the pharmacokinetic study of ketoconazole. Linearity, selectivity, sensitivity, accuracy, precision, recovery, and stability were measured using the HPLC-UV method. The calibration curve presented linearity in the 0.05-8 μg/ml with R^2=0.9969, which indicated good linearity. Intra-day and inter-day repeatability studies obtained the method's precision, and accuracy was examined by %diff. The result is <15% for LLOQ and <20% for low, medium, and high levels. Plasma stability under storage in a freezer (-20^{0}C) for three days was studied. As a result, the analysis method proposed in this work could prove to be a promising alternative for preclinical pharmacokinetic studies.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the State Ministry of Research and Technology under the Master Program of Education Leading to Doctoral Degree for Excellent Graduates (PMDSU) program, Indonesia.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept (VA, AEN), Design (VA), Supervision (TNSS, AKN, AEN), Resources (VA, TNSS, AKN), Materials (AEN), Data Collection and/or Processing (VA), Analysis and/ or Interpretation (VA, TNSS, AKN), Literature Search (VA), Writing (VA), Critical Reviews (TNSS, AKN, AEN)

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FABAD J. Pharm. Sci., 48, 2, 295-302, 2023
Doi: 10.55262/fabadeczacilik.1324184
RESEARCH ARTICLE

Parasite-Derived MicroRNAs: Potential Alternative Targets for Laboratory Diagnosis of Cystic Echinococcosis

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Parasite-Derived MicroRNAs: Potential Alternative Targets for Laboratory Diagnosis of Cystic Echinococcosis

SUMMARY

Cystic Echinococcosis (CE) is a type of zoonotic infection that can be caused by a specific form of a parasite called Echinococcus granulosus sensu lato. Mainly, imaging techniques are utilized to diagnose CE. Serological tests are only used when imaging findings are atypical. Additionally, laboratory assays including, direct microscopy and PCR are used to confirm of diagnosis after treatment though obtained negative results with these tests cannot be ruled out the diagnosis. Specific miRNAs produced by the parasite could be used as markers to diagnose and monitor CE. This research investigates the diagnostic potential of parasite-derived miRNAs compared to the presence of protoscolex in animal-derived hydatid cyst samples. Accordingly, egr-let-7-5p, egr-miR71-5p, and egr-miR-9-5p were positive in 26, 25, and 11 out of 30 samples (86.6%, 83.3%, and (36.6%), respectively. There was no relationship between protoscolex presence and detection of either egr-let-7-5p or egr-miR-9-5p (p>0.05). On the other hand, egr-miR71-5p positivity was found to be statistically significant compared with protoscolex presence (p=0.04). As a result, egr-miR-71 is a promising potential target for the diagnosis of CE. Additional research is necessary to assess the diagnostic value of miRNAs in CE using a larger group of samples.

Key Words: *Echinococcus granulosus, cystic echinococcosis, diagnostics, microRNA*

Parazit Kaynaklı MikroRNA'lar: Kistik Ekinokokkoz'un Laboratuvar Tanısında Potansiyel Alternatif Hedefler

ÖZ

Kistik Ekinokokkoz (KE), Echinococcus granulosus sensu lato adlı parazitin larva formunun neden olduğu bir tür zoonotik enfeksiyondur. Çoğunlukla, KE tanısı için görüntüleme teknikleri kullanılmaktadır. Serolojik testler yalnızca görüntüleme bulguları atipik olduğunda tanıya yardımcı olmakta, tedavi sonrası tanının doğrulanmasında direkt mikroskopi ve PCR gibi laboratuvar tetkikleri de kullanılmaktadır. Ancak bu testlerle elde edilen negatif sonuçlar tanıyı dışlamamaktadır. Parazit tarafından üretilen spesifik mikroRNA'lar (miRNA), KE'yi teşhis etmek ve hastaları tedavi etkinliği açısından takip için belirteçler olarak kullanım potansiyeli taşımaktadır. Bu araştırmanın amacı, hayvan kaynaklı hidatik kist örneklerinde protoskoleks varlığına kıyasla parazit kaynaklı miRNA'ların teşhis potansiyelini araştırmaktır. Buna göre egr-let-7-5p, egr-miR71-5p ve egr-miR-9-5p, 30 örneğin sırasıyla 26, 25 ve 11'inde (%86,6, %83,3 ve %36,6) pozitif bulunmuştur. Protoskoleks varlığı ile egr-let-7-5p veya egr-miR-9-5p'nin saptanması arasında anlamlı bir ilişki (p>0.05) olmamasına karşın egr-miR71-5p pozitifliği ise protoskoleks varlığına göre istatistiksel olarak anlamlı bulunmuştur (p=0,04). Sonuç olarak, egr-miR-71, KE tanısı için umut verici bir potansiyel hedef olarak görülmektedir. İleri çalışmalarda, daha büyük bir örneklem kullanarak KE tanısında parazit kaynaklı miRNA'ların tanısal değerinin değerlendirilmesi gereklidir.

Anahtar Kelimeler: *Echinococcus granulosus, kistik ekinokokkoz, tanı, mikroRNA*

Received: 07.07.2023
Revised: 20.07.2023
Accepted: 21.07.2023

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INTRODUCTION

Cystic Echinococcosis (CE) is a type of zoonotic infection that can be caused by a specific form of a parasite called *Echinococcus granulosus* sensu lato (McManus, 2001). *E.granulosus* s.l. is recognized as a group of concealed species, which comprises *Echinococcus granulosus sensu stricto* (*s.s.*) (G1 and G3 genotypes), *Echinococcus equinus* (G4 genotype), *Echinococcus ortleppi* (G5 genotype) and *Echinococcus canadensis* cluster (G6/7, G8, G10 genotypes) and *E.felidis* (Hüttner et al., 2008; Romig, Ebi, & Wassermann, 2015; Thompson, 2008; Vuitton et al., 2020). CE is a prevalent helminthic disease globally, especially where individuals are engaged in animal husbandry and have contact with roaming and, or herding canines (McManus, 2001). Transmission occurs with the fecal-oral route via eggs scattered around from definitive hosts' feces and ingested by intermediate hosts. The life cycle of parasites continues between canids, mainly dogs and livestock. Humans are accidentally involved in this cycle. Hence, early diagnosis and treatment in humans are affectless in parasite transmission but are essential for raising awareness and reducing the disease burden (Tamarozzi, Deplazes, & Casulli, 2020). The World Health Organization (WHO) has included CE in its roadmap for neglected diseases, aimed at preventing, controlling, eliminating, and eradicating them by 2030 (Organization., 2020).

The clinical manifestations of individuals with CE may range from having no noticeable symptoms to a severe illness. Most cases remain asymptomatic for years and may be diagnosed mainly by chance (Eckert, Gemmell, Meslin, Pawlowski, & World Health, 2001; Tamarozzi et al., 2016). The time of the beginning of infection is virtually impossible to determine due to the slow progression of hydatid cyst development and the absence of symptoms of acute infection (Tamarozzi et al., 2020). Although hydatid cysts may occur in any organ, 80% of the patients have a single cyst in the liver (4/5) or lungs (1/5) (Brunetti, Kern, & Vuitton, 2010; Brunetti et al., 2018).

The identification of CE primarily depends on imaging modalities like ultrasonography (US), computed tomography (CT), x-ray, and magnetic resonance (MR), and serological tests are mainly utilized when the imaging findings are not typical (Tamarozzi et al., 2016). To confirm the diagnosis, a protoscolex or hooklet examination is performed in the hydatid fluid obtained after treatment with light microscopy, but in the presence of a sterile cyst, the diagnosis cannot be confirmed microscopically (Schwarz et al., 2017). In addition, negative microscopy results cannot exclude the diagnosis of CE (Örsten S. , 2020; Schwarz et al., 2017). Today, molecular techniques are widely used to confirm the diagnosis of the obtained cyst material and distinguish between species and strains. However, PCR approaches cannot evaluate cyst viability and a negative PCR result cannot rule out the disease, creating confusion about the diagnosis (Schwarz et al., 2017).

A type of small non-coding RNAs called microRNAs (miRNAs) are naturally plentiful and have been conserved throughout evolution. These have been discovered in a wide array of organisms, ranging from viruses to more complex higher eukaryotes. Additionally, they are accepted as primary regulators of gene expressions (Ameres & Zamore, 2013; Faruq & Vecchione, 2015). miRNAs are stable and are found in tissues and several body fluids (Mitchell et al., 2008). Therefore, they have great potential as diagnostic and prognostic biomarkers with high specificity and sensitivity (Faruq & Vecchione, 2015). During various developmental phases of its life cycle, *E. granulosus* expresses numerous miRNAs (Cucher et al., 2011). This study aims to evaluate whether parasite-derived miRNAs known to be abundantly expressed in the metacestode form of the parasite, have the potential for laboratory diagnostic of CE compared with protoscolex presence.

MATERIALS and METHODS

Sample Selection, Direct Examination, and Molecular Characterization

Hydatid fluids were obtained from different abattoirs in the Central Anatolia region of Turkey. The ethics committee approval is not necessary since the used samples were obtained from the slaughterhouse. Collected cyst fluids were examined for protoscolex or hooks under a light microscope. For this purpose, fluid was centrifuged at 3000 xg for 3 minutes to obtain a residue. In case of hooks or protoscoleces in microscopy were detected, the cyst was accepted as fertile. A commercial DNA Purification Kit was used for DNA extraction from the hydatid cysts. (GeneAll Biotechnology, Korea) following the instructions provided by the manufacturer. PCR amplification of a segment of the mitochondrial gene cytochrome c oxidase subunit 1 (mtCO1) was carried out using a protocol that had been published previously (Nakao, Sako, Yokoyama, Fukunaga, & Ito, 2000). Amplicons were evaluated via electrophoresis and the products were considered positive when a band size of ~875 bp was observed. Subsequently, sequencing was performed to identify all positive amplicons. The chromatograms were analyzed via FinchTV 1.4.0 (Geospiza Inc., Seattle, Washington, USA). The Basic Local Alignment Search Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST/) was utilized to confirm the species. Thirty isolates from 28 cattle and two sheep confirmed to be E.granulosus were included in the study.

RNA Isolation, cDNA Synthesis, and miRNA Detection

RNA extraction from the hydatid cysts was performed using RNA Extracol (EURx) according to the manufacturer's recommendations. To measure the concentration and assessment of the purity of RNA, a FLUOstar Omega Microplate Reader (manufactured by BMG LABTECH) was used in conjunction with an LVis plate. The cDNA synthesis was performed using a commercial kit per the manufacturer's guidelines (cDNA Synthesis Kit with RNase Inh. (High Capacity) by A.B.T.™, Turkey).

To determine *E. granulosus*-specific miRNAs in the hydatid cyst material, miR-71a-5p, miR-9-5p, and let-7-5p were chosen for assay. MiRNA primers were used in conjunction with SYBR Green Master Mix (manufactured by A.B.T.™, Turkey) for conducting real-time polymerase chain reaction (RT-PCR) with ViiA™ 7 RT PCR System (Thermo Fisher Scientific) (Örsten et al., 2022) (Table 1). Data were collected with the previously published protocol (Örsten et al., 2022). As a negative control, nuclease-free water was used.

Table 1. Primer Sequences

miRNA	Primer Sequence
egr-let-7- 5p	TGAGGTAGTGTTTCGAATGTCT
egr-miR-9- 5p	TCTTTGGTTATCTAGCTGTGTGT
egr-miR-71a-5p	TGAAAGACGATGGTAGTGAGA

Statistical Analysis

The data collected were analyzed using the Chi-Square test with SPSS 23 (SPSS Inc., Chicago, IL, USA) program. A significance level was accepted as $p \leq 0.05$.

RESULTS AND DISCUSSION

The study investigated 30 hydatid cysts obtained from sheep and cattle, with 60% (18/30) of the cysts found in the liver and the remaining 40% (12/30) in the lungs. Upon microscopic examination, 60% (18/30) of the cysts were classified as fertile, while the remaining cysts were categorized as sterile. Most fertile cysts (13/18, 72.2%) were located in the liver. The BLAST algorithm confirmed that all samples were *E. granulosus* s.s. as per the PCR results.

RT PCR was used to assess the existence of parasite-specific miRNAs, namely egr-let-7-5p, egr-miR71-5p, and egr-miR-9-5p. Accordingly, egr-let-7-5p and egr-miR71-5p were positive in 26 and 25 out of 30 samples (86.6% and 83.3%), respectively. On the other hand, egr-miR-9-5p (11/30, 36.6%) was found to be positive in 11 out of 30 samples (36.6%). The positivity rates of egr-let-7-5p, egr-miR71-5p, and

egr-miR-9-5p in liver cysts were 89.9%, 83.3%, and 38.9%, respectively. In addition, the positivity rates of egr-let-7-5p, egr-miR71-5p, and egr-miR-9-5p in lung cysts were 83.3%, 91.7%, and 33.3%, respectively. On a sample basis, specific parameters such as cyst location, results of protoscolex examination, and miRNA detection status are given in Table 2.

Table 2. The parameters evaluated in this study

Isolate number	Location	Protoscolex	Let-7	miR-71	miR-9
1	Lung	-	+	+	-
2	Lung	-	+	+	-
3	Lung	-	+	+	-
4	Lung	-	-	+	+
5	Lung	-	-	+	-
6	Lung	+	+	-	+
7	Lung	+	+	+	+
8	Lung	+	+	+	-
9	Lung	-	+	+	+
10	Lung	-	+	+	-
11	Lung	+	+	+	-
12	Lung	+	+	-	-
13	Liver	+	+	+	+
14	Liver	+	+	+	+
15	Liver	+	+	+	-
16	Liver	-	-	+	+
17	Liver	-	+	+	-
18	Liver	+	+	+	-
19	Liver	+	+	-	-
20	Liver	-	+	+	-
21	Liver	-	+	+	-
22	Liver	+	-	+	-
23	Liver	+	+	+	+
24	Liver	+	+	+	-
25	Liver	+	+	+	+
26	Liver	+	+	-	-
27	Liver	-	+	+	-
28	Liver	+	+	-	+
29	Liver	+	+	+	-
30	Liver	+	+	+	+

(+): Positive, (-): Negative

There was no relationship between protoscolex presence and detection of either egr-let-7-5p or egr-miR-9-5p ($p>0.05$). On the other hand, egr-miR71-5p positivity was found statistically significant compared to protoscolex presence in molecularly confirmed samples ($p=0.04$) (Figure 1).

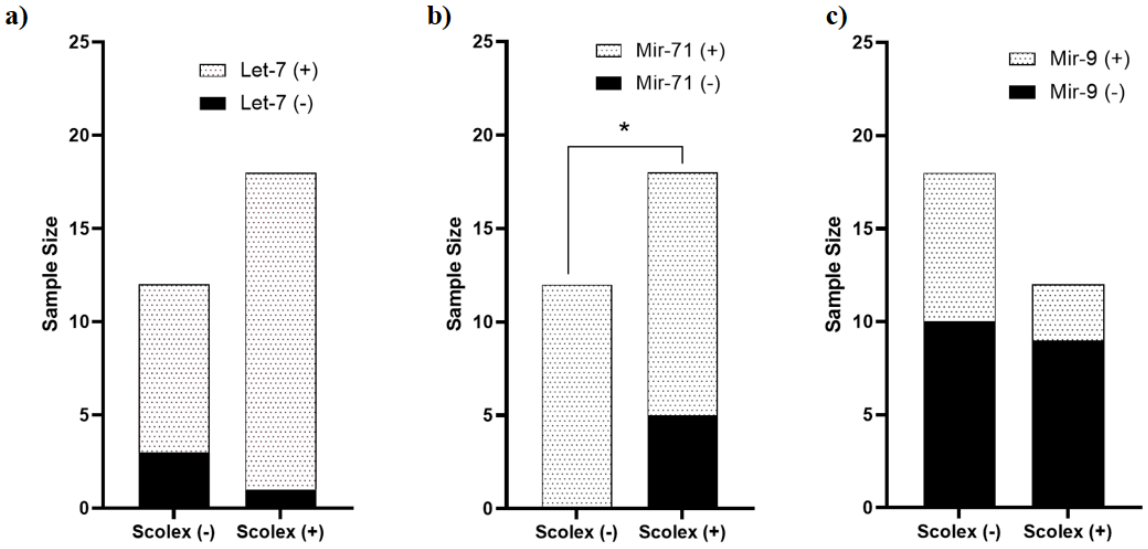

Figure 1. Presence of scolex and miRNA detection rates

Currently, imaging techniques serve as the primary diagnostic tool for CE in human patients, while laboratory methods are employed solely for confirmation purposes albeit their potential inadequacy. For instance, serological tests used for confirmation in humans are not standardized (World Health Organization, 2020). Characteristic protoscolex examination in the hydatid cyst fluid is performed with light microscopy after the treatment to confirm the diagnosis. However, it cannot be confirmed microbiologically in sterile cysts (Brunetti et al., 2010). Molecular techniques, such as PCR, are regarded as specific and sensitive methods that can be used in the species identification of parasites and confirmation of CE diagnosis (Grimm et al., 2021). However, it is known that PCR negativity targeting the DNA of *E.granulosus* does not rule out the diagnosis (Schwarz et al., 2017). Consequently, there is a need for alternative targets that can be used in the diagnosis of CE. In this study, the diagnostic potential of miRNAs was evaluated by comparison with protoscolex examination in hydatid cyst material derived from intermediate hosts, including cattle and sheep. For this purpose, specific parasite miRNAs, namely egr-let-7, egr-miR71a, and egr-miR-9, were chosen. The outcomes showed that 86.6% of all samples were positive for egr-let-7, while egr-miR71a and egr-miR-9 were detected in 83.3% and 36.6% of the samples, respectively.

Several studies have consistently found that cysts derived from sheep display notable fertility and viability, while cysts from cattle tend to exhibit a significant level of sterility (Fikire, Tolosa, Nigussie, Macías, & Kebede, 2012; Kebede, Mitiku, & Tilahun, 2009). According to studies from Turkey, cyst fertility rates have changed between 5.42-93.3% in hydatid cysts derived from cattle (Macin et al., 2021; Yildiz & Gurcan, 2003; Yildiz & Tunçer, 2005). Consistent with the literature, in this study fertility rate of hydatid cysts from cattle was found to be 57.1% (16/28). In addition, according to the organ location of the hydatid cysts, protoscolex positivity was found to be 72.2% in the liver and 41.6% in the lung.

Investigation of *E. granulosus*-specific antigens in the hydatid fluid and showing the parasite's DNA (eg PCR) are considered more advanced techniques that can be used in the diagnosis, especially in sterile cysts (Eckert et al., 2001). A recent study showed that 25 out of 39 samples accepted sterile by light microscopy were PCR positive. This result reveals the neces-

sity of examining the negative samples by light microscopy with molecular methods as well (Örsten S. , 2020). The study revealed that protoscolex-negative samples exhibited a positivity of 75% for egr-let-7-5p and 100% for egr-miR71a-5p, respectively. However, only the egr-miR71a-5p positivity showed statistical significance (p=0.04). Surprisingly, the egr-miR-9-5p positivity rate was observed as 25% and 44.4% in protoscolex-negative and protoscolex-positive samples, respectively. Prior research found that specific miRNAs derived from the parasite, namely let-7, miR71a, and miR-9, were significantly more abundant in the serum of patients with CE compared to the healthy subjects (with fold changes of >200, 107.90, and 126.56, respectively), with a statistical significance level of $p<0.05$. Furthermore, based on its expression levels in active and inactive CE patients, egr-miR-9-5p was identified as the most effective discriminatory miRNA (Örsten et al., 2022). In this study, it was surprising to find that egr-miR-9-5p exhibited the lowest miRNA positivity rate in the cyst sample. This might be due to its abundant presence as the highest circulating miRNA in the host serum. It is important to interpret the empirical findings presented in this study while acknowledging certain limitations. One such limitation is the restriction of the hydatid materials used to those of animal origin. Therefore, conducting extensive research involving hydatid material derived from humans is imperative to ascertain the diagnostic capabilities of the findings.

CONCLUSION

In conclusion, the diagnostic potential of certain miRNAs was evaluated using hydatid isolates originating from cattle and sheep. This study has indicated that parasite-derived miRNAs can be used as an alternative target molecule in the laboratory diagnosis of CE. Especially, egr-miR71a-5p has shown promising diagnostic potential compared to the other miRNAs. Additional research is necessary to assess the diagnostic value of miRNAs in CE using a larger group of samples.

ACKNOWLEDGMENTS

We are grateful to veterinarians for their help collecting samples in slaughterhouses. The author declares that no funds, grants, or other support were received during the preparation of this manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

AUTHOR CONTRIBUTION STATEMENT

S.Ö: Developing hypothesis, experimenting, preparing the study text, literature research. İ.B: Statistics, analysis, and interpretation of the data, reviewing the text. S.M: Sample collection, literature research, reviewing the text.

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FABAD J. Pharm. Sci., 48, 2, 303-318, 2023
Doi: 10.55262/fabadeczacilik.1144926
REVIEW ARTICLE

Thirdhand Smoke Exposure and Its Toxicological Impacts: A Review on Target Organ-Based Studies

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Thirdhand Smoke Exposure and Its Toxicological Impacts: A Review on Target Organ-Based Studies

SUMMARY

Thirdhand smoke (THS) is a newly described environmental health hazard that might be defined as residual cigarette smoke that occurs due to the accumulation of toxins of second-hand smoke (SHS) in the smoking environment. In addition, the chemicodynamic of THS may alter due to the interaction with other gases and chemicals in the environment. THS may cause serious health outcomes in the lungs, liver, skin, heart, and nervous system as well. Hence, it is thought to represent a major health hazard for people, particularly children, who are exposed to THS, where they interact more frequently with these surfaces exposed to THS via hand-to-mouth transfer. In the present study, it was aimed to summarize the proposed toxicity mechanisms based on in vitro and in vivo studies based on target organ toxicity. In this study, it is aimed to review the toxicity mechanisms of THS based on in vitro and in vivo studies on target organ toxicity. Recent studies reported that THS might induce unwanted effects in the respiratory, cardiovascular, nervous, hematopoietic, and skeletal systems and skin. Literature data indicated that THS-mediated oxidative damage and an increase in inflammatory response may play an important role in the pathogenesis of cardiovascular and neurobehavioral diseases, especially the target organ lung. In the future, THS might be defined as a preventable environmental risk factor. Therefore, further studies on THS are needed to define its toxicity mechanism as well as increase social awareness and legal regulations.

Key Words: *Thirdhand smoke, cigarette, oxidative stress, lung, nicotine*

Üçüncü El Dumanı Maruziyeti ve Toksikolojik Etkileri: Hedef Organa Yönelik Çalışmalara İlişkin Bir Derleme

ÖZ

Üçüncü el duman (THS), sigara içilen ortamda ikinci el dumanının (SHS) toksinlerinin birikmesi nedeniyle oluşan "artık sigara dumanı" olarak tanımlanabilecek kısmen yeni bir çevresel sağlık tehlikesidir. Ayrıca, THS'nin kimyasal dinamiği, çevrede bulunan diğer gazlar ve kimyasallarla etkileşime bağlı olarak değişebilmektedir. THS akciğer, karaciğer, cilt, kalp ve sinir sisteminde de ciddi sağlık sorunlarına neden olabilmektedir. Bu nedenle, el-ağız transferi yoluyla THS'ye maruz kalan, bu yüzeylerle daha sık etkileşime girerek THS'ye maruz kalan insanlar ve özellikle çocuklar için büyük bir sağlık tehlikesi oluşturduğu düşünülmektedir. Bu çalışmada, hedef organ toksisitesine dayalı in vitro ve in vivo çalışmalara dayalı olarak söz konusu toksisite mekanizmalarının özetlenmesi amaçlanmıştır. Son zamanlarda yapılan çalışmalar THS'nin solunum, kardiyovasküler, sinir, hematopoietik ve iskelet sistemi ve cilt üzerinde olumsuz etkileri olduğuna yönelik bulgular rapor etmektedir. Literatür verileri, özellikle THS aracılı oksidatif hasarın tetiklenmesinin ve inflamatuvar yanıtın artışının gelecekte başta hedef organ akciğer olmak üzere, kardiyovasküler ve nörodavranışsal hastalıkların patojenezinde önemli rol oynayabileceği ve THS'nin önlenebilir bir çevresel risk faktörü olarak tanımlanabileceğine işaret etmektedir. Bu nedenle THS'nin toksisite mekanizmasının belirlenmesi, toplumsal farkındalığın ve yasal düzenlemelerin artırılması için daha kapsamlı ileri çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: *Üçüncü el duman, sigara, oksidatif stres, akciğer, nikotin*

Received: 18.07.2022
Revised: 21.02.2023
Accepted: 22.02.2023

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INTRODUCTION

Cigarette smoke (CS) is one of the most common and preventable health hazard for public health; that causes more than 8 million deaths annually. More than 1 million of those are passive smokers exposed to second-hand smoke (SHS) (WHO, 2021). Even if it has not resulted in death, severe health outcomes such as cancer, heart diseases, diabetes, chronic obstructive pulmonary disease (COPD), eye disorders, and tuberculosis might be seen in people who smoke. Thus, it contributes to a decrease in life quality gradually. The toxicity of CS in numerous target organs has been well-studied previously both *in vivo* and *in vitro* (Guan et al., 2012; Zong et al., 2019; Reis et al., 2021.) In addition to active smoke and SHS, a new toxicological concept, thirdhand smoke (THS) has arisen due to cigarette smoke's environmental residue. Basically, THS can be described as the 'residual tobacco smoke' or 'aged tobacco smoke', which is formed due to the accumulation of SHS toxins on surfaces of environments where smoking has occurred, such as carpets, tabletops, utensils, foods, household dust and cloths of smokers after the smoking was finished. The pollution of THS may remain in the environment for more than a month, depending on the degree of absorption or adhesion rates (Moon et al., 2019). Even though the public awareness of the health impacts of direct exposure to CS and SHS is well-acknowledged, people still do not have consciousness of the negative health impacts of THS as well as its concept. According to several studies on people from different age groups, education levels, and health professions, people were asked what THS is and which health problems it may cause. The results implicated that more than half of them did not know about it (Roberts et al., 2017; Moon et al., 2019; Lidón-Moyano et al., 2021). Today, smoking is prohibited in public places such as restaurants, cafes, and playgrounds due to various health outcomes, particularly lung diseases; however, according to the most recent World Health Organization (WHO) report, smoking in outdoor places is still not prohibited in many countries, including Turkey (WHO, 2020). According to the latest published information, public awareness of the concept of THS was highest in the United States (U.S.) and several prohibitions have been applied based on the published articles in the U.S. (Delgado-Rendon, et al., 2017). The majority of the studies on THS have shown that THS exposure might represent a public health concern, especially for children, due to the different exposure routes and children's developing organ-body system (Martins-Green et al., 2014; Mahabee-Gittens, et al., 2018; Myers, et al., 2018). Therefore, the toxicological profile of THS in the aspect of both public and environmental health should be elucidated to define its possible effects on human health. Although THS is a great risk to human health, very few people have awareness of its potential danger (Winickoff et al., 2009). In most countries, smoke-free laws are becoming widespread recently. In 2006, half of the states of the U.S. restricted smoking in workplaces and public areas (Delgado-Rendon et al., 2017). By keeping the value of smoke-free homes and cars higher, it is planned to prevent indoor smoking and involuntary exposure to THS. Studies show that this sanction implemented in the U.S. is successful in reducing exposure (Bundy et al., 2018). Based on the content of THS, nicotine is the main constituent of THS. It might be accumulated on surfaces for weeks to months, which can also react with oxidant gases (e.g., ozone and nitrous acid) to form carcinogenic nitrosamines such as 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA), 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). These carcinogens may persist for a long time due to their low volatility property, and people can be exposed to these carcinogens for a while by involuntary ingestion, inhalation, and dermal contact (Ferrante et al., 2013). THS, which contains many different chemicals such as nicotine, phenol, cresols, acetaldehyde, formaldehyde, polycyclic aromatic hydrocarbons, isocyanic acid (HNCO), and TSNAs, has the potential to ad-

here and accumulate on many surfaces (Matt et al., 2011). People are chronically exposed to this because it can remain on indoor surfaces for weeks to months and can be re-emitted in the air (Acuff et al., 2016). According to several literature data, lung diseases, cardiovascular diseases, chronic depression, and poor wound healing were seen in people exposed to THS (Martins-Green et al., 2014; Karim et al., 2015; Dhall et al., 2016a; Adhami et al., 2018). In addition, abnormal melatonin catabolism through hypo-methylation of CYP1A2-promoter is another outcome of exposure to THS (Jiang et al., 2021). Besides its harmful effects on human health, THS might be a leading cause of an economic burden with estimated hundreds of billions of dollars in costs annually (Martins-Green et al., 2014). Hence, as a new toxicological concept, the present study is aimed to review the recent findings on THS exposure and possible health impacts on public health, primarily on children and people who are suffering from chronic diseases.

THE CONCEPT OF THS

CS is a serious health risk for both smokers and non-smokers, with almost 7000 toxin content (Centers for Disease Control and Prevention (U.S.); National Center for Chronic Disease Prevention and Health Promotion (U.S.); Office on Smoking and Health (U.S.), 2010). Therefore, THS might be classified as an involuntary exposure route to these toxins due to its accumulation and aging capacity (Acuff, et al., 2016). It was first described in 2006; however, thoroughly investigated in 2009 by pediatrician Winickoff (Ganjre et al., 2016). In the study of Winickoff et al. (2009), it was mentioned that was no safe exposure level to THS and, the children were uniquely susceptible to exposure. In addition, the SHS can be removed from the indoor environment by ventilation. However, the THS may persist for several days, weeks, or even months after cigarettes have been smoked (Ferrante et al., 2013; Chen, et al., 2018). The differences between SHS and THS exposure and chemical content are described in Table 1.

Table 1. Main differences between SHS and THS based on their chemical content, exposure frequency and accumulation capacity.

	SHS	THS
Route of exposure	Inhalation of both side-stream and main-stream smoke (Hang et al., 2013)	Inhalation, ingestion, and dermal absorption of pollution (Acuff et al., 2016)
Exposure frequency	High levels over a short time (Ferrante et al., 2013)	Low levels over a long time (Ferrante et al., 2013)
Physical characteristics	Removal through ventilation (Acuff et al., 2016)	Persistence on indoor surfaces and above humans (Acuff et al., 2016)
Accumulation	—	Walls, doors, carpets, pillows, curtains, furniture, clothes, skin, and hair (Acuff et al., 2016; Dhall et al., 2016a)
Chemical content	Carbon dioxide, carbon monoxide, nicotine, carbonyls (acetaldehyde, formaldehyde, acrolein), hydrocarbons (benzene, toluene, PAHs), nitrogen oxides, pyridine, ammonia, nitrosamines, and hydrogen cyanide (CalEPA, 2005)	Nicotine, 3-ethenylpyridine, phenol, cresols, naphthalene, formaldehyde, and TSNAs (NNN, NNK, NNA) (Northrup et al., 2016)

The pollution of SHS that accumulates on surfaces reacts with dust, other chemicals, or gases in the environment, and this reaction produces THS, as depicted in Figure 1. By this conversion, aged THS becomes more toxic and harmful than SHS (Dhall et al., 2016a).

Figure 1. Illustration of THS and its exposure in the environmental system

People might be exposed to THS via involuntary inhalation, contact with the surfaces, or ingestion, as shown in Figure 2 (Ferrante et al., 2013). Especially children tend to be exposed to dust ingestion because of close interaction with household surfaces and frequent hand-to-mouth transmission. Therefore, it has been seen that the effects of THS are mostly seen in infants and children, and negative health impacts have been reported by several studies (Ferrante et al., 2013; Mahabee-Gittens et al., 2018). In addition, exposure to THS by dermal intake and inhalation may occur when non-smokers are present in a previously smoked environment (Acuff et al., 2016; Chen et al., 2018).

There are three common pathways for exposure to THS: inhalation of gaseous chemicals, dermal absorption, and oral ingestion by hand-to-mouth transfer of chemicals (Li et al., 2021). Since THS is an involuntary source of exposure by individuals, it is an issue with public health implications (Matt et al., 2011). The most commonly investigated target organs were the lungs, skin, heart, and brain (Escoffery et al., 2013; Ferrante et al., 2013; Martins-Green et al., 2014; Acuff et al., 2016). According to the results of *in vitro* and *in vivo* studies (Bahl et al., 2016; Hang, et al., 2018), it was shown that THS has negative impacts on the primary target, the respiratory system, by increasing oxidative damage (Hang et al., 2013), inflammatory response (Jacob et al., 2017), and fibrosis (Martins-Green et al., 2014). On the other hand, dermal and systemic exposure to THS has exerted its toxicity via several mechanisms. In the skin, THS deteriorates the wound-healing process which, involves hemostasis, inflammation, cell proliferation, and tissue remodeling. While carbon monoxide (CO) affects wound healing by binding to hemoglobin to form carboxyhemoglobin and preventing oxygen supply to tissues, hydrogen cyanide (HCN) disrupts oxidative metabolism and oxygen transport, creating a hypoxic effect (Silverstein, 1992; Dhall et al., 2016b). Since the inflammatory response, collagen deposition, and angiogenesis are decreased; clot formation and blood vessel leakage are increased (Dhall et al., 2016a). The wound healing delays, because of the decrease in the level of fibrillar collagen, resulted in a reduction in the strength of tissue (Martins-Green et al., 2014). In the cardiovascular system, THS has exerted its toxicity via stimulation of high levels of inflammatory cytokines and increased lipid levels (Martins-Green et al., 2014). Tail bleeding time assay revealed that bleeding times had been significantly shortened in THS-exposed mice, which poses a

great risk for thrombosis (Karim et al., 2015). Besides thrombosis, atherosclerosis, hyperlipidemia, and hypertension are the other health consequences of THS exposure (Deivanayagi et al., 2021). Some of the neurotoxic effects of THS are anxiety, memory loss, attention deficit hyperactivity disorder, and learning disorders, and these are the consequences of elevated nitro tyrosine levels causing oxidative stress and decreased ATP levels with THS exposure (Martins-Green et al., 2014; Chen et al., 2018; He et al., 2021).

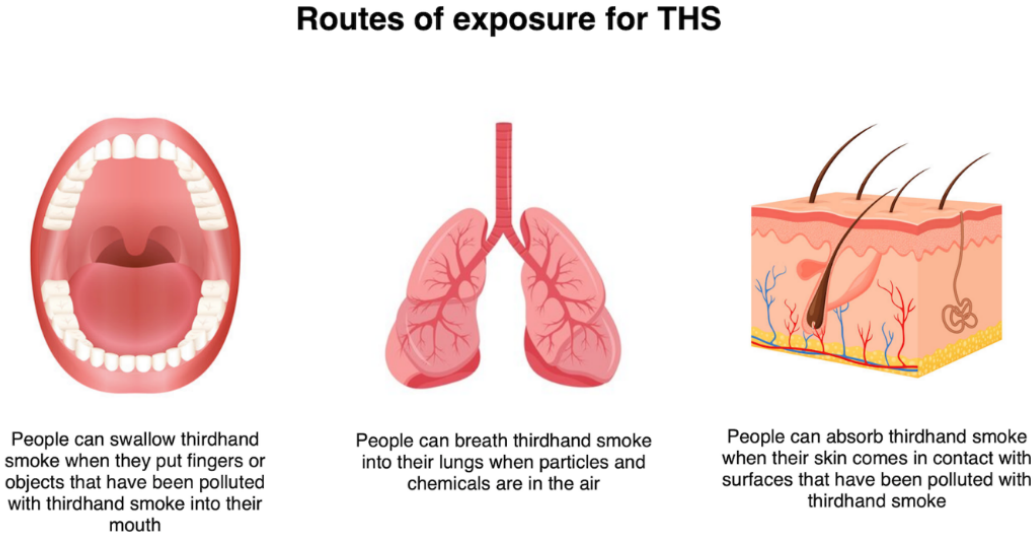

Figure 2. Illustration of the route of exposure to THS

Apart from the exposure differences compared to the SHS, the THS consists of mainly nicotine, tobacco-specific nitrosamines (TSNAs), phenol, cresols, naphthalene, formaldehyde, and 3-ethenylpyridine. After the release of smoke from a smoker several physical, and chemical transformations occur in the environment and it can continue at different levels just after the smoke is released. The most abundant disseminated constituent, nicotine, has the majority of accumulation potential among the other components, which persists for weeks to months (Matt et al., 2011). It might easily react with ozone and nitrous acid that, are oxidant gases present in indoor environments, and form TSNAs, such as NNK, NNN, and NNA, which are classified as group 1 lung carcinogens by the International Agency for Research on Cancer (IARC) (Burton, 2011; Stepanov, et al., 2013). These TSNAs, even in low doses, might lead to long-term health problem for infants since they are in close contact with surfaces, and have a higher respiration rate, and a lower body weight (Ferrante et al., 2013). Moreover, nicotine can form other volatile compounds such as formaldehydes, phenols, carbon monoxide, benzene, etc. are available to be exposed via inhalation due to their lower volatility and classified as human carcinogens with no safe level of exposure (Bogdanovica, et al., 2011). In addition, heavy metals such as chromium (Cr), lead (Pb), cadmium (Cd), copper (Cu), aluminum (Al), and nickel (Ni) are released during CS release and accumulate on surfaces (Yaprak et al., 2017). In a study conducted in an area exposed to THS, it has been proven that Pb and Cd, in particular, are highly mixed with house dust and accumulate for a long time (Matt et al., 2021). These heavy metals have lifelong health effects on humans. According to the studies, adverse cognitive and behavioral outcomes, increase in the risk of cardiovascular diseases, cancer and COPD thought to be related to the THS may be due to the accumulation of these heavy metals (Richter, et al., 2008; Obeng-Gyasi, et al., 2018; Zeng et al., 2018).

GENERAL HEALTH IMPACTS OF THS

Respiratory system

CS poses a great risk to the respiratory system due to its potential to induce target organ toxicity. Nicotine, the main content of CS, is also might be oxidized and produces several nitrosamines, the TSNAs, in the presence of ozone and nitrous acid (Rehan, et al., 2011). In addition to active smoke, the harmful effects of passive smoke on the lungs and several other organs are well acknowledged according to previous studies (Wannamethee et al., 2001; Fujii et al., 2012; Vani et al., 2015; Akhavan Rezayat et al., 2018; Reis et al., 2021). Cigarette smoke serves hazards especially for the human being exposed to passive smoke. Over the last decades, the effects of natural compounds on smoking-mediated respiratory diseases such as COPD, asthma, and lung cancer have been under investigation, as well as the mechanistic aspects of disease progression. In the present study, the protective mechanism of eucalyptol SHS causes a significant increase in hospitalizations and health expenditures of children each year due to pneumonia, bronchiolitis, or severe asthma (Cook et al., 1999; Li, et al., 1999). Although cough and nasal symptoms are more severe in SHS groups, THS also have a great impact on children's respiratory system (Ferrante et al., 2013).

In an *in vitro* study conducted by Bahl et al. (2015), the effects of acrolein, a toxic residue of THS, on human health revealed that acrolein killed lung fibroblasts. In another study conducted by Yu et al. (2018), even low levels of exposure to residual THS nitric oxide cause severe airway inflammation. In this study, the relationship between epicutaneous exposure to NNK, an important component of THS, and asthma was demonstrated by using a cockroach antigen (CRA) induced model of asthma in mice. As an adverse effect of this exposure, exacerbations in airway hyperreactivity, airflow inflammation, and airway remodeling were observed in mice (Yu, et al., 2018) which are the features of asthma (Busse et al., 2001; Umetsu, et al., 2002). Disruptions and thickening in the walls of the alveoli of mice were observed under THS exposure (Martins-Green et al., 2014). Besides, in the respiratory bronchioles, leukocyte infiltration, especially macrophages which indicate inflammation and highly disorganized collagen fibers, were observed in the interstitial tissue. All these observations and the pro-inflammatory environment in the lungs suggested an increased risk for the development of lung fibrosis in people who have been chronically exposed to THS (Martins-Green et al., 2014; Jacob et al., 2017). It is known that children might be exposed to THS easier because of both physiological, and behavioral reasons (Jacob et al., 2017). Hang et al. (2018) investigated how early exposure affects lung cancer by using 4 to 7 weeks age mice. In this study, the mice were exposed to THS at measured concentrations that a small child would be exposed to, and their lungs were observed after forty weeks. According to this study, a significant increase in the incidence of lung cancer was observed in mice exposed to THS compared to the control group. Also, *in vitro* studies conducted by Hang et al. (2018) using human cancer cell lines suggested an increase in lung cancer risk. It was concluded that THS might induce tumorigenic phenotypes, including cell proliferation and colony formation, in cells exposed to THS. The data confirmed that THS increases the cancer risk due to the induction of DNA double-strand breaks (Hang et al., 2013). As a result, the risk of lung cancer in children most exposed to THS for physiological and behavioral reasons increases continuously depending on the exposure time (Hang, et al., 2019). Our previous *in vitro* study (Reis et al. 2022) has also shown that THS might be led to an increase in oxidative stress, IL-6 level, and intracellular ROS level in A549 human lung adenocarcinoma cells. Based on the studies conducted with THS and its impact on the respiratory system, it might be concluded that repeated exposure to THS might induce fibrosis, oxidative and pro-inflammatory responses in the airway epithelia and lung fibroblast thus, THS might contribute to the symptoms of people with chronic pulmonary diseases.

Skin

Skin is one of the most important barriers to environmental exposures and to chemical substances that are embedded on the surfaces where people contact physically. Since THS differs from SHS and direct CS exposure, one of the main important exposure routes for THS is dermal exposure (Dhall et al., 2016b). Limited studies have shown the effects of active and passive smoking and their possible effects on the dermal system such as aging, wrinkle forming, atopic dermatitis, skin darkening, and thickening of the skin on the cheek (Knuutinen et al., 2002; Bernhard et al., 2007; Norman et al., 2010; Ishiwata et al., 2013; Yazdanparast et al., 2019). However, dermal side effects of THS are not elucidated clearly in the literature. According to the limited data on dermal exposure to THS, it was shown that the greatest known impact of THS on the skin was a delay in wound healing (Martins-Green et al., 2014; Acuff et al., 2016; Jacob et al., 2017). Wound healing is a dynamic process that involves four stages as hemostasis (blood clotting), inflammation, cell proliferation (tissue growth), and tissue remodeling. Delays, imbalances, and external factors occurring in these stages may disrupt the normal recovery period and cause adverse effects on human health (Sen et al., 2009). According to studies and tests carried out on mice, THS is one of the factors that cause a delay in wound healing. Mainly, nicotine, CO, and HCN in THS are suggested to be responsible for the aforementioned healing delay (Martins-Green et al., 2014). Since the affinity of CO to hemoglobin is greater than that of oxygen, it binds to hemoglobin to form carboxyhemoglobin and prevents adequate oxygen supply to the tissue. On the other hand, HCN disrupts oxidative metabolism, and oxygen transport, creates a hypoxic effect in the tissue, and delays healing (Nolan et al., 1985; Silverstein, 1992). The main active ingredient of THS, nicotine, is also known to cause tissue ischemia as a result of vasoconstriction in the peripheral vascular system, increases the tendency to thrombus and causes deterioration of the wound healing process due to its negative effects on blood cells (Mosely et al., 1978). Another study by Dhall et al. (2016), mice were placed in cages containing materials that had been smoked for several days and exposed to THS for one week. Full-thickness excision wounds were performed on the shaved skins of mice 24 h after exposure. The same procedure was performed on a group of mice not exposed to THS for comparison at the end of the study. Then wound tissues were collected from each group at various times to measure superoxide dismutase, hydrogen peroxide (H_2O_2), catalase, glutathione peroxidase (GPx), and lipid peroxidase activities, nitrotyrosine, cytokine and chemokine levels. According to the results, while the wounds of the mice were healed in 12 days, the wounds of the mice exposed to THS were healed on the 14th day, which showed that THS-exposed wounds took approximately 25% more time to heal compared to the control. Although the cellular and molecular mechanism underlying the delay of wound healing due to THS is unknown, it has been observed that increased oxidative stress, and related tissue damage cause an imbalance in chemokines and cytokines. As evidenced by the tests performed as a result of this imbalance, the inflammatory response, collagen deposition and angiogenesis are decreased; clot formation and blood vessel leakage are increased (Dhall et al., 2016). The decrease in the level of fibrillar collagen in THS-exposed mice is the main reason of delaying wound closure since it results in a notable reduction of the strength of wound tissue (Martins-Green et al., 2014). Another critical factor in impaired wound healing associated with THS is the imbalance of reactive oxygen species (ROS) levels (Nolan et al., 1985) and elevated superoxide dismutase activity, which causes an increase in H_2O_2. When H_2O_2 cannot be broken down to H_2O and O_2 by the oxidant enzymes, GPx, and catalase, it leads to an increase in oxidative stress. As a consequence of the increment of oxidative stress, DNA damage in tissues might be seen and negatively affects wound healing (Buettner, 2011). Hence, the overall literature data points that THS-induced ROS production and imbalances in the collagen deposition

of skin might be the leading causes of delay in wound healing, but further studies are needed, though.

Cardiovascular system

As it is known, although CS is one of the most obvious causes of coronary heart disease (CHD), it is a preventable risk factor compared to other factors such as age, gender, family history of CHD, diabetes mellitus (DM), hypertension, dyslipidemia, and ethnicity (Kitamura et al., 2013).

There are limited studies are elucidating the effects of THS in the cardiovascular system (Martins-Green et al., 2014; Karim et al., 2015). Although it is known that both active and passive smoking is a major risk factor for the cardiovascular system, especially acute coronary thrombosis, the risk of THS on this system has been newly started to be investigated in recent years (Karim et al., 2015). According to a study conducted by Martins-Green et al. (2014), THS has a negative effect on the cardiovascular system by causing stimulation of high levels of inflammatory cytokines and increased lipid levels. In another study, Karim et al. (2015) suggested that enhanced platelet aggregation, glycoprotein IIb-IIIa activation and platelet secretion were observed in platelets of mice that were exposed to THS. Based on the performed tail bleeding time assay to evaluate hemostasis, it was revealed that bleeding times had been significantly shortened in THS-exposed mice. In the same study, platelet aggregation and secretion were also evaluated in $FeCl_3$-induced carotid artery injury in mice exposed to THS. According to their findings, the occlusion time was observed to be almost 10-fold shorter in mice exposed to THS (Karim et al., 2015). All these data showed that THS may pose a preventable risk factor for the progression of thrombosis, particularly for people at the risk of CVD.

Besides the aforementioned studies, several findings conducted the effects of chemical constituents of CS. Among them, nicotine, the major content of THS, is a sympathomimetic chemical, that acts peripherally and centrally by increasing the release of catecholamine and other neurotransmitters. Apart from its cardiovascular effects, such as elevation in the heart rate, high cardiac output, and increased arterial blood pressure (Ambrose et al., 2004), it also has metabolic effects, such as increased lipolysis. Lipolysis increases the amount of free fatty acids and glycerol in the blood and increased fat metabolism increases the oxygen demand of the whole system. Thus, nicotine-induced sympathetic activity increases the myocardium's oxygen demand without an increase in blood flow to the myocardium. On the contrary, vasoconstriction occurs in the coronary arteries, and all these effects cause symptoms of ischemia (Pur Özyiğit, 2019). On the other hand, the hemodynamic effects of nicotine are thought to increase atherosclerosis by causing endothelial damage (Beere et al., 1984). In addition, nicotine acts on nicotinic cholinergic receptors on endothelial cells, causing increased blood pressure, which causes the highest death rate (Arima et al., 2011) from cardiovascular diseases. These receptors lead to hypertension by arrangement in a barrel-like configuration forming a channel in the cell membrane, and increasing cation permeability from this channel (Lee et al., 2011). Another important mechanism is lipid peroxidation, which defines the oxidation of lipids, proteins, and DNA, which leads to cell damage and forms the basis of atherogenesis. CS is rich in oxidant chemicals such as hydrogen peroxide, peroxynitrite and superoxide. It also contributes to oxidant production *in vivo* (Burke et al., 2003). It is well acknowledged that reduced nitric oxide levels in smokers may cause acute cardiovascular events, and accelerated atherogenesis (Kiowski et al., 1994). Therefore, further studies are needed to conduct the possible health impacts of THS in the cardiovascular system based on exposure frequency, exposure level, age and cardiovascular health status.

Nervous system

Neurotoxicity can be defined as the alteration of the normal activity of the nervous system by disruption or death of neurons due to exposure to toxic

substances. Since the children might be exposed to THS via hand-to-mouth transfer as well, one of the expected target organs might be defined as the central nervous system (CNS). According to the data of the National Survey on Children's Health in 2007, among approximately fifty-five thousand children under the age of twelve that were exposed to SHS and relatively THS, 6% of them had attention-deficit/hyperactivity disorder, 9% had learning disabilities, and 4% had other behavioral disorders (Yolton et al., 2005; Kabir et al., 2011). Some studies have shown that CS causes many behavioral health problems, especially increasing depressive symptoms (Prokhorov et al., 2016). On the other hand, there are many studies reveal the effects of THS on human behavioral health (Martins-Green et al., 2014; Adhami et al., 2018; He et al., 2021).

In the tests conducted by Martins-Green et al. (2014) on mice, it was concluded that mice exposed to THS were hyperactive and anxious compared to non-exposed. It was observed that THS exposure caused elevated nitrotyrosine level. Consequently, oxidative stress increased in the brains of mice which caused penetration of the blood-brain barrier damaging many molecular structures in brain tissue. At the same time, ATP levels decreased in the brain tissues of mice exposed to THS (Chen et al., 2018). In another study, anxiety-like behavior in six strains exposed to THS was measured in the light/dark box, and one strain, the anxiety level was detected to be increased. They also measured the memory potential using the passive avoidance assay, and again, one strain displayed significant memory loss in mice that were exposed to THS from 4 to 9 weeks. This data showed that THS exposure may negatively affect anxiety-like behavior and memory in a strain-dependent manner (He et al., 2021). According to the aforementioned studies, THS might be important environmental pollutant, particularly for crawling babies and children exposed to accumulated THS on surfaces via hand-to-mouth transfer. However, to define the exact neurotoxicity mechanisms, its capacity to pass the blood-brain barrier, and the physicochemical dynamics of THS content have a major role and need to be elucidated with further studies.

Hematopoietic system

The hematopoietic system is the other suggested target for THS exposure. According to the limited data, an *in vivo* study showed that complete blood count values of neonatal and adult mice exposed to THS were elevated. Moreover, the number of neutrophils in female mice and the amount of basophils in male mice were higher than that of the non-exposed control (Hang et al., 2017). In the same study, it was also reported that THS exposure permanently affected the presence of B cells, and myeloid cells. Although there was no significant difference in blood lymphocyte counts between control mice and THS-exposed mice, significant differences were reported in the lymphocyte subpopulations and a significant decrease was observed in the myeloid/NK cell fraction, while the B cell fraction of neonatal and adult mice was increased. In another recent study in pregnant Cdkn2a/ dams exposed to THS, THS-exposed and control Cdkn2a/ mice bone marrow samples were transplanted into wild-type recipient mice with bone marrow ablation (irradiated) and then observed for one year to determine the effect of THS exposure in leukemia/ lymphoma risk. According to the measured cytokines above thirty types, twenty of them in THS-exposed pups were found to be less than that of control mice. Moreover, the β-subunit of basic fibroblast growth factor and platelet-derived growth factor was found to be higher in mice exposed to THS, and plasma cytokine differences were elevated in both genders exposed to THS (Snijders et al. 2021). Based on the literature findings, it might be suggested that repeated exposure to THS might affect complete blood counts as well as cytokine levels.

CONCLUSION

THS might be defined as important and preventable environmental pollution. Thus, exposure to THS might contribute to adverse health outcomes in many

systems, particularly in the respiratory and cardiovascular systems, by inducing oxidative stress and inflammatory responses. Hence, it might be suggested that repeated exposure to THS may cause serious health problems, especially in children, due to their involuntary contact with the surfaces. Therefore, further experimental, and clinical studies should conduct to determine the risk level of THS exposure and to raise public awareness. In addition, the legal sanctions that restrict smoking, especially indoors, should be brought to the agenda, and it should be ensured that these negative health effects related to THS could be minimized. As a result, it might be suggested that repeated exposure to THS may constitute a risk factor not only for public health but also for the environment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Literature research(KK, NG, RR), preparing the study text (KK, NG, RR), reviewing the text (KK, RR); developing hypothesis (RR)

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FABAD J. Pharm. Sci., 48, 2, 319-336, 2023
Doi: 10.55262/fabadeczacilik.1191048
REVIEW ARTICLE

The Role of Radiopharmaceuticals in the Bone Metastases Therapy

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The Role of Radiopharmaceuticals in the Bone Metastases Therapy

SUMMARY

Cancer, having high morbidity and mortality rates, has become a significant public health problem in recent years, and it is the second leading cause of death after heart disease in the world. Metastases are one of the most serious complications of cancer and bone metastases are detected in 2/3 of metastatic cancer cases. Therapy approaches in bone metastases can be classified as surgery, bisphosphonates, radiotherapy, and radionuclide therapy. Radionuclide therapy using alpha and beta-emitting radionuclides is more selective and effective than other local and systemic treatment methods, and this feature provides superiority over other therapeutic methods. Radionuclide therapy is used in bone metastasis to reduce pain, to kill tumor cells, to prolong life span, and to improve quality of life. In recent years, alpha-emitting radiopharmaceuticals [such as Radium-223 (Ra-223) chloride] and beta-emitting radiopharmaceuticals [such as Strontium-89 (Sr-89) chloride, Lutetium-177 (Lu-177) labeled Ethylenediamine Tetra Methylene Phosphonic Acid (EDTMP), Samarium-153 (Sm-153) labeled EDTMP] are introduced in the clinic for especially the treatment of painful bone metastases and on the other hand new radiopharmaceutical development studies also continue intensively, like Actinium-225 labeled prostate-specific membrane antigen-617 (Ac-225-PSMA). Number of studies are proven that using radionuclide therapy in bone metastases improves the patient's general health, reduces pain and the risk of pathological fractures, and increases survival. This review presents an overview of radionuclide therapy used in bone metastases. In this context, following the general information about radiopharmaceuticals, the importance of radiopharmaceuticals used in bone metastases therapy is explained with experimental and clinical studies examples.

Key Words: *Cancer, Bone Metastases, Radionuclide Therapy, Radiopharmaceutical*

Kemik Metastazlarının Tedavisinde Radyofarmasötiklerin Rolü

ÖZ

Kanser, morbidite ve mortalite oranları yüksek olan ve son yıllarda büyük bir halk sağlığı sorunu haline gelen bir hastalıktır ve dünyada kalp hastalıklarından sonra ölüm nedenleri arasında 2. sırada yer almaktadır. Metastazlar kanserin en ciddi komplikasyonlarından biri olup kemik metastazları, metastatik kanser olgularının 2/3'sinde saptanmaktadır. Kemik metastazlarında genel tedavi yaklaşımları, cerrahi, bifosfanatlarla tedavi, radyoterapi ve radyonüklid tedavi olarak sınıflandırılabilir. Radyonüklid tedavinin diğer lokal ve sistemik tedavi yöntemleriyle karşılaştırıldığında daha seçici ve etkili olması yöntemi avantajlı hale getirmektedir. Alfa ve beta partikülü yayan radyonüklidlerin kullanıldığı bu tedavi yöntemi, kemik metastazı olan hastalarda sıklıkla görülen ağrının azaltılmasında, tümör hücrelerinin öldürülmesinde, yaşam süresinin uzatılmasında ve yaşam kalitesinin artırılmasında etkili bir yöntemdir. Son yıllarda alfa partikülü yayan; Radyum-223 (Ra-223) klorür, beta partikülü yayan; Stronsiyum-89 (Sr-89) klorür, Lutesyum-177 (Lu-177) işaretli Etilendiamin Tetra Metilen Fosfonik Asit (EDTMP) ve Samaryum-153 (Sm-153) işaretli EDTMP başta olmak üzere çok sayıda radyofarmasötik kemik metastazlarının tedavisinde klinik kullanıma girmişken Aktinyum-225 işaretli prostat spesifik membran antijeni-617 (PSMA-617) gibi yeni radyofarmasötiklerin geliştirilmesi üzerine de çalışmalar yoğun şekilde devam etmektedir. Yapılan birçok çalışma kemik metastazı tedavisinde radyofarmasötiklerin kullanımının hastanın genel sağlık durumunu iyileştirdiğini, ağrıları ve patolojik kırık riskini azalttığını ve sağkalımı artırdığını kanıtlamıştır. Bu derlemede kemik metastazı tedavisinde kullanılan radyonüklid tedaviye genel bir bakış açısı sunulmuştur. Bu kapsamda klinikte kullanılan ve araştırmaları devam eden radyofarmasötiklerle ilgili genel bilgiler verilmiş, deneysel ve klinik çalışma örnekleriyle kemik metastazı tedavisinde radyofarmasötiklerin kullanımının önemi açıklanmıştır.

Anahtar Kelimeler: *Kanser, Kemik metastazı, Radyonüklid Tedavi, Radyofarmasötik*

Received: 18.10.2022
Revised: 25.04.2023
Accepted: 02.05.2023

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INTRODUCTION

Cancer is a disease that occurs when some of the body's cells grow and multiply uncontrollably and spread to other areas of the body (National Cancer Institute, 2021). Cancer has become a major public health problem in recent years and has high morbidity and mortality rates. According to the World Health Organization (WHO) and Global Cancer Observatory (GLOBACON) data, approximately 19.3 million new cancer cases were encountered worldwide in 2020, and the number of cancer-related deaths was reported as approximately 10 million. In other words, one out of every eight deaths worldwide are caused by cancer. In our country, while the number of new cancer cases for 2020 is about 230.000, cancer-related deaths have been reported at about 130.000 (Global Cancer Observatory, 2020).

Metastases are one of the most critical complications of cancer (Ell & Gambhir, 2006), and 2/3 of metastatic cancer cases are bone metastases which are a widespread consequence of the spread of numerous solid cancers to distant sites (Arıkan, 2014). The primary tumors with the highest rate of bone metastases are prostate, breast, kidney, lung, and thyroid cancer (Maccauro et al., 2011; Çetin & Büyükberber, 2012). The incidence of bone metastasis of primary tumors calculated by postmortem examination is shown in Figure 1 (Coleman, 2006; Galasko, 1981).

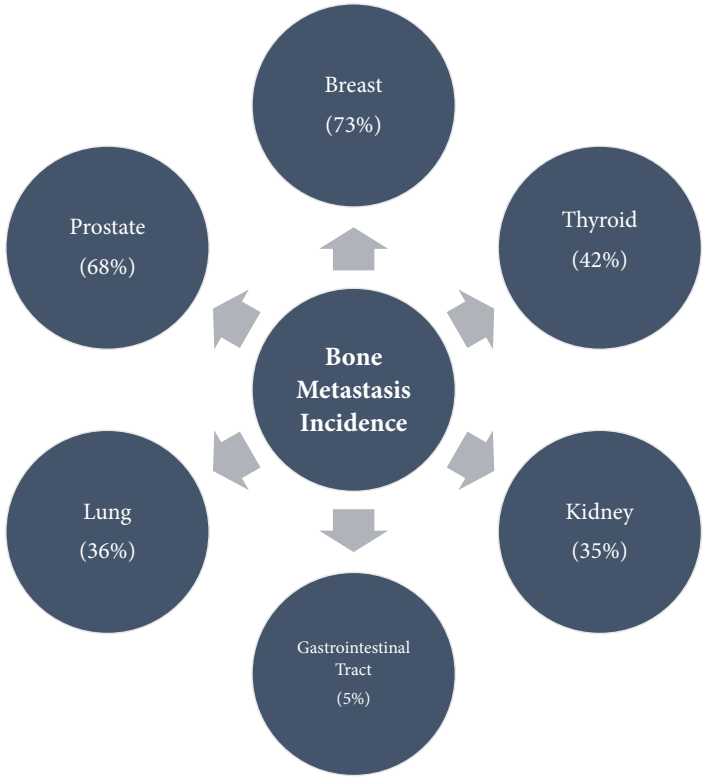

Figure 1. Incidence of bone metastases at postmortem examination in different cancers

(Data are adapted from Coleman, 2006 and Galasko, 1981 and presented as a figure)

The most common symptoms in bone metastases are severe pain, movement disorder, hypercalcemia, pathological bone fracture, spinal cord or nerve root compression, and bone marrow infiltration (Maisano et al., 2001; Macedo et al., 2017). Pain is the most widespread symptom in bone metastases. This symptom, which negatively affects the patient's quality of life, is increasingly severe at night and does not go away with sleep or lying down (Clohisy & Mantyh, 2003). In the therapy of bone metastases, it is crucial to increase the patient's survival and improve the quality of life by reducing the symptoms. For this reason, fac-

tors such as localized or widespread bone disease, the type of tumor, previous treatments, and the patient's response to previous treatments should be evaluated, and appropriate treatment should be decided. Therapy methods such as radiotherapy, endocrine therapy, chemotherapy, therapy with bisphosphonates, and radionuclide therapy come to the fore in bone metastases therapy (Maisano et al., 2001).

In this review, therapy methods for bone metastases, where primary tumors frequently metastasize and constitute a significant percentage of metastatic cancer cases, are briefly mentioned. In addition, the radiopharmaceuticals used in this therapy are summarized by emphasizing the importance of radionuclide therapy, and examples from studies on the subject are presented.

THERAPY METHODS FOR BONE METASTASES

Bone metastasis therapy aims to reduce tumor burden, to prevent further progression of tumor and metastasis, and to prevent tumor-related bone pathologies such as pathological fracture, pain, and hypercalcemia (Suva et al., 2011). It is also essential to increase survival by improving the patient's quality of life (Mayadağlı et al., 2011). Local bone metastasis treatment strategies are palliative in nature; individual lesions are surgically excised, and the tumor 'bed' irradiated, either before or after surgery. The decision for or against surgery and/or radiation, alone or in combination with select bone-targeted agents, is profoundly influenced by the extent of systemic disease at the time of treatment.

Surgery; it is difficult to treat bone metastases surgically, multidisciplinary approach that must be applied to each patient depending on the specifics of their case is necessary. In general, surgery can be applied to treat existing or potential pathological fractures, decompress spinal cord and nerve roots, relieve pain and reestablish bone continuity (Güney et al., 2008; Macedo et al., 2017).

Radiotherapy; it is the most commonly used treatment for bone metastases. Primary purpose is to reduce bone pain and prevent pathological fractures and spinal cord compression while maintaining the patient's quality of life (De Felice et al., 2017).

Therapy with bisphosphonates; bisphosphonates are analogs of pyrophosphate, a natural inhibitor of bone demineralization. It has been shown that bisphosphonates is reduced skeletal complications and vertebral fractures in bone metastases and they cause osteoclasts' apoptosis by inhibiting their development and function (Janjan, 2001; Gralow & Tripathy, 2007). Therefore, bisphosphonates, alone or in combination with cytotoxic agents, have recently started to be used, especially in treatment of tumor-induced hypercalcemia (Maisano et al., 2001).

Radionuclide therapy; it is a therapy method using alpha (α) and beta (β-) emitting radionuclides. It can be more effective when combined with other therapy methods, such as chemotherapy, bisphosphonates, and radiotherapy (Macedo et al., 2017). The aim of the radionuclide therapy is to decelerate the development of the disease by preventing the development of new metastases. Thus, it can reduce morbidity and increase survival (Hillegonds et al., 2007).

RADIONUCLIDE THERAPY

In radionuclide therapy, the destructive effects of the radiation emitted by radioactive substances on target cells are utilized. Unlike the diagnostic radiopharmaceuticals, therapeutic radiopharmaceuticals' purposes are to kill cells. Therefore, it is desired that the radionuclide used for therapy should have high energy (E_{max} > 1 MeV). In addition, the localization of the radiopharmaceutical in the target organ is significant (Önsel, 2009). Therefore, the success of radionuclide therapy depends on adequate uptake and long-term retention of the radiopharmaceutical in the target tissue (Chatal & Hoefnagel, 1999).

The radioactive material can be delivered locally into an organ or tissue or targeted to the desired tissue

with the help of metabolic carriers (Naki Sivri & Özer, 2004). Contrary to systemic treatment approaches, in radionuclidic treatment cell death selectively occurs in cancer cells while damage to healthy tissues is minimal. In this way, the rest of the body is protected from the harmful effects of radiation (Ersahin et al., 2011). However, as well as the advantages of radionuclide therapy, there are some limitations. In Table 1, the pros and cons of radionuclide therapy were summarized (Chatal & Hoefnagel, 1999; Naki Sivri & Özer, 2004; Sgouros et al., 2020).

Table 1. Advantages and disadvantages of radionuclide therapy

Advantages
It can be applied systemically or locally.
It is effective in all areas with disease involvement.
Selective.
Non-invasive method.
Palliation ability.
Low normal tissue toxicity.
Minimal side effects.
The uptake and retention of the radioactive material in the tumor can be predetermined.
Multiple therapies are possible.
Disadvantages
Patient isolation may be required (for some therapies).
Special rules are required for the disposal and storage of radioactive waste.
Difficulties may be experienced in the supply of radiopharmaceuticals.
Some new therapies are costly.
A multidisciplinary approach is required.
Dosimetry calculations is required.
Patients may be prejudiced toward radioactivity.

Radionuclide therapy in bone metastases

The failure of cancer treatments and the fact that metastases are the leading cause of cancer-related deaths have increased the attention on the development of more effective treatment methods for palliative treatment and the treatment of metastases, especially bone metastases, in recent years (Ferreira et al., 2012). Radionuclide therapy is one of these methods and mainly used for pain relief associated with bone metastases. It is applied in cases where pain due to bone metastasis, recurrent pain in the radiotherapy area, no response to opiate analgesics, pain circulating in more than one region, and more than one abnormality from a bone scan are observed (Ell & Gambhir, 2006). Although the early use of radionuclide therapy in osteoblastic metastases has proven to be a reliable method in the treatment of pain (Paes & Serafini, 2010), it is currently used as the last preferred method in cases where other treatments have failed.

In the clinic, α and β emitting radionuclides are used in the therapy of bone metastases and pain relief associated with bone metastases. These radionuclides can be selectively delivered to bone sites with enhanced osteoblastic activity (Tsukamoto et al., 2021). An ideal radiopharmaceutical to be used for the therapy of bone metastasis should have the following characteristics (Ferreira et al., 2012; Choi, 2018). It should;

- show selective, high, and long-term uptake in bone lesions,

- has limited uptake by other organs,
- excrete rapidly from non-skeletal sites,
- show low toxicity in the bone marrow and other healthy tissues,
- has a low risk of side effects,
- has easy and simple preparation methods,
- be produced in high radiochemical purity,
- be suitable for clinic use,
- be safe for patients and staff,
- provide bone pain palliation and improve survival.

Although β-emitting radionuclides, such as P-32, Sr-89, Re-186, Re-188, Lu-177 and Sm-153 have been used more frequently in the therapy of bone metastases and pain palliation associated with bone metastasis, in recent years, α-emitting radionuclides, such as Ra-223 and Ac-225 have been received increasing attention (Sathekge et al., 2019; Satapathy et al., 2020; Nava-Cabrera et al., 2021). The properties of β and α radionuclides used in radionuclide therapy are summarized in Table 2 (Zustovich & Barsanti, 2017; Choi, 2018; Marcu et al., 2018).

Table 2. Properties of β and α radionuclides used in the bone metastases.

β emitting radionuclides	**α emitting radionuclides**
Low energy	High energy
Low LET (~0.3 keV/μm)	High LET (~100 keV/μm)
Less tumoricidal activity	More tumoricidal activity
Long distance (2000–11500 μm)	Short distance (20-80 μm)
Delivers more radiation to neighboring tissue	Delivers less radiation to neighboring tissue
High risk of bone marrow toxicity	Low risk of bone marrow toxicity
Use in pain palliation	Use in pain palliation
Required patient isolation	Outpatient therapy for radium-223

In radionuclide therapy of bone metastasis, the radiation dose absorbed in the metastatic focus is a function of the activity uptake in the tumor and this function is determined by parameters such as the concentration and retention time of the radiopharmaceutical as well as the particle energy and half-life of the radionuclides. Mechanism of pain relief is not yet fully understood; however, it is thought that radiopharmaceuticals used in treating pain palliation work by adsorption or fixation in areas with increased osteoblastic activity. Tumor size is reduced by the effect of radiation, thus reducing the periosteum pressure, and as a result, the patient is relieved. It is also reported that the destruction of cells that secrete pain mediators may play a role in reducing pain, especially in the early period of metastases (Şahin et al., 1998; Ferreira et al., 2012). Bone marrow toxicity is a parameter that limits the use of radionuclides. High doses of radiation may lead to the risk of bone marrow suppression (Zustovich & Barsanti, 2017).

α-particles have particular advantageous in targeted therapy due to their high potential and specificity in addition, the distance that α particles go to in human tissue is very short (2-10 tumor cell diameter) which is especially important for dosimetry of surrounding tissue. α-particles have a high linear energy transfer (LET) (about 100 keV/μm), and thus they cause breaks in the double-stranded structure of DNA. When the double-stranded DNA structure is disrupted, it becomes difficult to repair, the unrepaired structure undergoes apoptosis (Zustovich & Barsanti, 2017).

Radiopharmaceuticals used for the therapy of bone metastases

Phosphorus-32 (P-32) Orthophosphate (Phosphotope)

P-32, the radioisotope of phosphor, is produced directly by the reactor and has a physical half-life of 14.3 days. It is a pure β emitter with high specific radioactivity (Ferreira et al., 2012). The maximum and average β particle energies are 1.71 and 0.695 MeV, respectively. The maximum and mean penetration ranges in tissues are 8 and 3 mm, respectively (Pandit-Taskar et al., 2004).

Phosphate is widely distributed throughout the body, contributing to energy metabolism, neuromuscular function, hematopoiesis, and bone metabolism. The skeletal system is the greatest pool of phosphate in the body, and phosphor, found in the bone as inorganic phosphate, binds to the hydroxyapatite matrix (Ell & Gambhir, 2006). Phosphate is eliminated mainly by the renal route, with minimal fecal excretion (Şahin et al., 1998).

P-32 was one of the first radionuclides used to decrease pain from bone metastases and was in widespread use until the 1980s. It is thought that the mechanism of action of P-32 is to cause damage to the DNA of the tumor cell as well as to reduce pain due to damage to cells that produce pain modulators (such as lymphokines). However, various toxicities, including myelosuppression and pancytopenia in patients, limited the its clinical use (Pandit-Taskar et al., 2004).

Strontium-89 (Sr-89) Chloride (Metastron™)

Sr-89 is a pure β emitter with a half-life of 50.5 days. It has a maximum energy of 1.46 MeV, an average soft tissue penetration of 2.4 mm, and high specific radioactivity (Paes & Serafini, 2010; Kuroda, 2014).

Strontium, like calcium, is a divalent cation and is incorporated into the hydroxyapatite structure in bone in rate to osteoblastic activity. In osteoblastic lesions in the bone, it is taken five times more than in normal bone areas, and long-term uptake occurs (Paes & Serafini, 2010). About 80% of Sr-89, which is not concentrated in the bone, is excreted through the kidneys and 20% through the gastrointestinal tract (Pandit-Taskar et al., 2004). It has been reported that Sr-89 chloride exerts its effect by effectively killing tumor cells and inducing tumor cell apoptosis (Ye et al., 2018).

Sr-89 labeled strontium chloride $^{89}[Sr]SrCl_2$ was the first radiopharmaceutical approved by the FDA in 1993 for bone pain therapy and is currently licensed in many countries (Paes & Serafini, 2010; Guerra Liberal et al., 2016). Pecher first reported using Sr-89 chloride to treat painful bone metastases in 1942. Studies have shown that Sr-89 is an alternative treatment method for patients with bone pain caused by metastatic prostate cancer, provides benefits in addition to External Beam Radiotherapy (EBRT), and is cost-effective (Furubayashi et al., 2015). In addition, it has been emphasized that Sr-89 can reduce the need for both EBRT and narcotic analgesics, helping reduce lifelong health costs without hospitalization (Pandit-Taskar et al., 2004).

Robinson et al. stated that in 622 patients, a response rate of 81% occurred in pain palliation therapy with Sr-89, and no myelotoxicity occurred in the patients (Robinson et al., 1995). In a study by Kuroda et al., the lethal effect of Sr-89 on the tumor, pain therapy efficacy, and survival were investigated in prostate cancer patients with bone metastases. As a result of the study, Sr-89; has been stated that it controls prostate-specific antigen (PSA) and increases survival time. However, it has been emphasized that large-scale studies are needed to examine its tumor-killing effect (Kuroda, 2014).

Ye et al. evaluated the efficacy of Sr-89 chloride in pain relief associated with bone metastasis in lung, breast, or prostate cancer patients. The study included one hundred twenty-six patients with lung cancer, 71 with breast cancer, and 49 with prostate cancer. The study's results indicated that Sr-89 chloride could safely and efficiently alleviate bone pain caused by

bone metastasis in lung cancer. However, its efficacy is lower in patients with lung cancer than in breast or prostate cancer (Ye et al., 2018).

Samarium-153 (Sm-153) Ethylenediamine Tetra Methylene Phosphonic Acid (EDTMP) (Quadramet®)

Samarium-153 is a radionuclide with a physical half-life of 1.9 days. It emits beta particles with a maximum energy of 810 keV. It emits β particle with a maximum energy of 810 keV and gamma rays with maximum energy of 103 keV. The average penetration range of Sm-153 is 0.5 mm, and Sm-152 oxide is produced with high radionuclidic purity by neutron bombardment (Rubini et al., 2014).

Goeckeler et al. (1987) reported for the first time the synthesis of a series of samarium complexes has been produced using multidentate acetate and phosphonate ligands. Of the complexes studied, Sm-153 labeled EDTMP provides the optimum combination as a result of its high bone uptake, rapid blood clearance, and low soft tissue uptake. Sm-153-labeled EDTMP, concentrated in proportion to osteoblastic activity in the skeleton (Pandit-Taskar et al., 2004), is used in the clinic for the effective palliative therapy of bone metastasis. After injection, Sm-153 labeled EDTMP is quickly excreted from the blood into the urine. Only 1% of the injected activity remains in the blood 4 hours after application, while retained in bone for a long time (Ferreira et al., 2012). Excretion from the body is carried out through the kidneys and is completed in approximately 6 hours (Paes & Serafini, 2010).

The first study, conducted in 1989, included 35 patients with bone metastases that spread from different tumor types. In 65% of patients treated with Sm-153 labeled EDTMP, pain relief was reported between 4 and 35 weeks. In addition, dose-limiting toxicity appeared to be myelosuppression, and platelet counts were reported to return to treatment baseline levels within ten weeks of therapy (Turner et al., 1989; Rubini et al., 2014).

In a study carried out to determine the clinical efficacy of Sm-153-labeled EDTMP, the following four parameters were evaluated by asking patients at each visit (before and after therapy) of all patients treated with ^{153}Sm-EDTMP in the clinic:

(i) Pain assessment according to the visual analog scale (VAS),

(ii) Pain-related sleep disorder,

(iii) Analgesic drug dose,

(iv) Answer the question, 'Do you think you have benefited from the treatment?'

In conclusion, it has been reported that Sm-153 labeled EDTMP therapy is an effective supportive treatment, especially in patients with bone metastases originating from breast or prostate cancer (Kolesnikov-Gauthier et al., 2018).

Barai et al. investigated whether combining capecitabine in radio-sensitizing dose with Sm-153-EDTMP produces superior analgesia compared to Sm alone. Capecitabine is a chemotherapeutic drug and used with external beam radiation to make the target more radiosensitive. For eight days, patients with skeletal metastases from various primaries received either Sm-153 labeled EDTMP plus capecitabine or Sm-153 labeled EDTMP plus placebo (control group) and all patients were followed up for 12 weeks to evaluate the degree and duration of pain palliation and hematologic toxicity. As a result, it was stated that the combination of radiosensitive capecitabine and ^{153}Sm-EDTMP radiopharmaceutical increased the analgesic effect without increasing bone marrow toxicity (Barai et al., 2015).

Sm-153 labeled EDTMP has been reported to be safe and effective and has been reported to cause only mild reversible bone marrow suppression in patients, while it has been reported to be safe and effective (Paes & Serafini, 2010). However, although ease of use, ability to display its distribution, and clinical results make Sm-153 labeled EDTMP attractive, the risk of developing myelosuppression limits its wider use (Pandit-Taskar et al., 2004).

Lutetium-177 (Lu-177) EDTMP

Lu-177 is a radioisotope used for both therapeutic and diagnostic purposes, with a physical half-life of 6.73 days, a maximum β energy of 497 keV, γ Energy of 113 keV (6.4%), and 208 keV (11%). Lutetium-177 is produced in nuclear reactors either directly by the ^{176}Lu (n, γ) ^{177}Lu reaction or indirectly by the ^{176}Yb (n, γ) ^{177}Yb $\xrightarrow{\beta-}$ ^{177}Lu reaction.

Lu-177 labeled EDTMP has appropriate biological and physical properties for the palliative therapy of patients with painful bone metastases (Ando et al., 1998). It was shown that the uptake of the ^{177}Lu-EDTMP compound in soft tissues is very low and high in bones. In addition, Lu-177 nuclide has an appropriate physical half-life, and its low-energy gamma-rays allow the imaging of bone lesions by scintigraphy. Furthermore, the potential for side effects is lower than the other beta-particle emitting radionuclides due to their moderate beta energy. Thus, Lu-177 is considered an alternative nuclide to clinically used radionuclides such as Sr-89 and Sm-153 (Chopra, 2004), and its clinical use is increasing rapidly.

In the phase II study, the therapeutic efficacy and safety of 177Lu-EDTMP were examined in breast cancer and hormone-resistant prostate cancer patients with bone metastases. In addition, patients were observed for pain scores, Karnofsky indices, mobility scores, and analgesic needs. The results have been reported to show that using 177Lu-EDTMP is effective and safe (Yuan et al., 2013).

In a randomized, double-blind clinical study, 153Sm-EDTMP and 177Lu-EDTMP were compared to the effectiveness of pain palliation in patients with bone metastases. Fifty patients with painful bone metastases were included in the study. It was stated that similar results were obtained in the patient groups treated with these two radiopharmaceuticals and that 153Sm-EDTMP and 177Lu-EDTMP could be used in the pain therapy of multiple bone metastases, and were effective and safe (Taheri et al., 2018).

Rhenium-186 (Re-186) Hydroxyethylidene Diphosphonate (HEDP)

Re-186 is a β^- emitting radioisotope of rhenium with a physical half-life of 3.7 days, a maximum energy of 1.07 MeV, an average energy of 0.349 MeV, and a mean penetration range of 1.1 mm. This isotope also emits a low level of 137 keV gamma radiation (Bodei et al., 2008). Re-186 is produced directly in the reactor and has moderate specific radioactivity (Ferreira et al., 2012).

Re-186 is used as a HEDP compound for palliative therapy. Mathieu et al. first reported the possible use of ^{186}Re-HEDP in the treatment of bone metastases in 1979 (Mathieu et al., 1979). Re-186-labeled HEDP binds to hydroxyapatite crystals by building hydroxide bridges in a hydrolysis reaction and is significantly concentrated in primary and metastatic bone lesions (Finlay et al., 2005; Lam et al., 2007). This process is thought to mediate the metabolic activity of osteoclastic cells. Studies on the biokinetics of ^{186}Re-HEDP have shown that it binds to plasma proteins in a time-dependent manner and is a compound with rapid blood clearance. Approximately 70% of the dose is excreted in the urine within 24 hours (Finlay et al., 2005).

In the phase II study, to evaluate the effect of the compound, 60 persons with painful bone metastases from different tumors were treated with ^{186}Re-HEDP, and 80% of the patients stated that they experienced pain relief. Of these, 31% reported that the pain was utterly relieved, 34% partially relieved, and 15% relieved at a low level. The duration of pain relief has been noted to range from 2 to 52 weeks, with moderate and transient hematological toxicity (Sciuto et al., 2000).

Kucuk et al. investigated the palliative and side effects of Re-186 HEDP in patients with different cancer types with bone metastases. Thirty-one patients were included in the study (10 prostate, ten breast, four rectum, five lung, and two nasopharyngeal cancer patients). In conclusion, it was reported that the overall response rate was 67.5%; the average response

rate was 87.5% in patients with breast and prostate cancer, 75% in patients with rectal cancer, and 20% in patients with lung cancer. Furthermore, when the side effects were evaluated, it was stated that no serious side effects were observed except for mild hematological toxicity (Küçük et al., 2000).

The Phase III study, entitled PLACORHEN, was a randomized controlled trial in which 111 patients with metastatic castration-resistant prostate cancer (mCRPC) with bone metastases received ^{186}Re-HEDP or placebo. It was reported that a higher rate of pain relief response (65% vs. 36%) was observed in the group receiving ^{186}Re-HEDP compared to the group receiving placebo, and the pain response with ^{186}Re-HEDP was longer than those associated with placebo (Han et al., 2002).

Because of the delivery of a substantial dose to bone marrow, marrow toxicity side effects such as thrombocytopenia or, most rarely, leucopenia may be observed. The baseline white blood cells and platelet counts are essential parameters for therapy. Platelets and white blood cells count decrease during therapy; however, it is reported that they return to average levels within eight weeks after administration (Argyrou et al., 2013; Sciuto et al., 2000).

Although Re-186 labeled HEDP, has been evaluated in various studies for the therapy of painful bone metastases, its use is still experimental and has not yet entered routine clinical use.

Rhenium-188 (Re-188) HEDP

Re-188, an isotope of rhenium, has a physical half-life of 16.9 hours and a maximum beta energy of 2.1 MeV, with a mean penetration range into the soft tissue of 3 mm (Li et al., 2001). Re-188 also emits 155 keV gamma radiation. This isotope's high β energy can potentially kill tumor cells. Re-188 is synthesized from the Tungsten-188/Rhenium-188 generator, so it is cheap, and a kit combined with HEDP is also available (Pandit-Taskar et al., 2004).

Re-188 is evaluated by complexing with HEDP, just like the Re-186 compound, for bone pain relief but ^{188}Re-HEDP, like ^{186}Re-HEDP, has not yet entered routine clinical use. Average biological half-life of ^{188}Re-HEDP in bone is about 16 hours, almost 40% of the radiopharmaceutical is excreted in the urine within 8 hours (Finlay et al., 2005). ^{188}Re-HEDP has high bone uptake and shows similar results to ^{186}Re-HEDP. The hematological toxicity of ^{188}Re-HEDP is a decrease in platelet and leukocyte counts, and it has been stated that these changes are reversible (Pandit-Taskar et al., 2004; Cheng et al., 2011; Rubini et al., 2014).

Although there are some studies to show its effectiveness, the information on the use of the Re-188 labeled HEDP compound is limited. In the study by Cheng et al., various doses of ^{188}Re-HEDP were administered in 64 patients, and pain reduction, blood counts, biochemical parameters, and side effects were reviewed. As a result of the study, it was reported that there were no clinically significant changes in biochemical parameters, and no vital adverse effects were observed. It has been reported that thrombopenia and leukopenia are rarely seen in patients, and thrombocyte and leukocyte levels return to baseline at the end of the therapy. In addition, it was stated that pain relief was achieved by 84.62% in patients with prostate cancer, 78.57% in patients with breast cancer, 62.50% in patients with lung cancer, and 55.56% in patients with liver cancer from specific tumor types. As a result of the study, it was pointed out that ^{188}Re-HEDP is a beneficial radiopharmaceutical to improve bone pain in patients with progressed cancer with painful bone metastases (Cheng et al., 2011).

Li et al. evaluated the therapeutic efficacy of ^{188}Re-HEDP for the palliation of painful bone metastases in patients with dissimilar types of advanced cancer. Sixty-one patients were included in the study and were treated with various doses of Re-188 HEDP. As a result, it was reported that most patients had an essential reduction in bone pain, and no serious side effects or hematopoietic toxicity were observed (Li et al., 2001).

Radium-223 (Ra-223) Dichloride (Xofigo®)

Ra-223, a radioactive isotope of radium, supplied as a radium chloride salt solution and given intrave-

nously, has a half-life of 11.4 days. It has an average path length of less than 0.1 mm in soft tissue and an alpha particle energy of 5850 keV.

Radium-223 is chemically like calcium and replaces calcium by participating in the hydroxyapatite structure ((), a bone mineral ingredient. In addition to the bone surface and skeletal metastases, it is concentrated in growth zones, including sites of bone turnover (Rubini et al., 2014; Zustovich & Barsanti, 2017). It shows its radiobiological effects mainly by the forming double helix breaks in tumor cell DNA by α particles. A cytotoxic effect occurs due to the high LET of α particles. At the same time, since the distance that α-particles can travel in the tissue is very short (2-10 cells), the suppressive effect of Ra-223 on the bone marrow is significantly less than that of β-particles (Alan Selçuk & Yencilek, 2018). Approximately 25% of the injected Ra-223 at therapeutic doses is uptake by the bones, and its elimination is primarily accomplished by the gastrointestinal tract (Pandit-Taskar et al., 2014; Alan Selçuk & Yencilek, 2018).

Studies conducted on mCRPC patients with only bone metastases and no internal organ metastases showed that radium-223 dichloride increased overall survival versus placebo, and Ra-223 had a palliative effect of 50-60%. In addition, it was emphasized that it was superior in pain relief (Alan Selçuk & Yencilek, 2018).

The first human study to examine the safety and tolerability of Ra-223 was performed by Nilsson et al. As a result of the study, it was emphasized that Ra-223 was tolerable at therapeutic doses. It could be an effective radiopharmaceutical in cancer therapy due to its pain-reducing properties and positive effects on serum markers (Nilsson et al., 2005). A randomized, double-blind, placebo-controlled multicenter phase II study conducted in 2002 indicated that adverse events, including serious ones, were more common when patients were not treated with Ra-223 (Gupta et al., 2017).

In a multicenter study with Ra-223 titled Alpha-Alphadine (Alpharadin in Symptomatic Prostate Cancer=ALSYMPCA) in Phase III Symptomatic Prostate Cancer, Ra-223 was reported to improve overall survival. In this study, the efficacy and safety of Ra-223 in mCRPC patients were compared with the placebo group. The study's results emphasized that the overall survival was prolonged, the risk of death was reduced by 30%, and the development time of symptomatic skeletal-related events was delayed in patients treated with Ra-223 compared to the placebo group. Ra-223 has also been shown to have a palliative effect on pain associated with bone metastases. It was stated that the most common side effects were anemia, thrombocytopenia, and diarrhea (Parker et al., 2013).

Ra-223 dichloride has been approved for use in CRPC patients with bone metastases and no visceral metastases. This radiopharmaceutical is commercially available under the trade name Xofigo®. Many studies are ongoing regarding extending other indications of radium-223 dichloride in patients with prostate cancer and its use in bone metastases caused by different types of cancer (Pandit-Taskar et al., 2014; Alan Selçuk & Yencilek, 2018).

Actinium-225 labeled prostate-specific membrane antigen-617 (Ac-225-PSMA-617)

Ac-225 is an α-emitting radioisotope with a half-life of 10 days. It emits four α, two β particles and two γ photons during decay. Alpha particles have 5.8 to 8.4 MeV energies, and tissue ranges from 47 to 85 μm (Hooijman et al., 2021). The emitted γ photons can be used for post-therapy monitoring and dosimetric experiments. Its favorable half-life and decay properties make Ac-225 a promising compound for targeted alpha therapy (TAT). Preclinical or early clinical trials of many Ac-225-labeled molecules (peptides, antibodies, nanobodies) are ongoing, and some of them (^{225}Ac-PSMA-617 and ^{225}Ac-DOTATATE) have reached clinical practice (Dhiman et al., 2022).

Ac-225-labeled PSMA-617, developed and characterized in 2013, has shown remarkable therapeutic efficacy in previously intensively treated mCRPC patients (Sathekge et al., 2019). Compared with the Lu-177 labeled PSMA used in the clinic, Ac-225 has higher energy, a shorter distance, and a higher tumoricidal effect. It was reported that the therapeutic re-

sponse of ^{177}Lu labeled PSMA in bone metastases is weaker than in soft tissue lesions. Nava-Cabrera et al. calculated the absorbed dose of ^{177}Lu-PSMA and ^{225}Ac-PSMA by comparing experimental data from the bone metastasis model and cellular fractionation in animals and performed a comparison study. The study's results reported that ^{225}Ac-PSMA accumulates in bone lesions and efficiently kills tumoral cells in the bone lumen (Nava-Cabrera et al., 2021). Moreover, ^{225}Ac-PSMA-617 can target any metastatic tissue and has good application potential for small tumors, diffuse cancers, and micro-metastasis. Clinical trials are being gradually conducted at multiple centers to evaluate the efficacy and safety of ^{225}Ac-PSMA-617 radiopharmaceutical in mCRPC patients (Ma et al., 2022).

^{225}Ac-PSMA-17 therapy was administered to patients with advanced prostate cancer who did not receive chemotherapy. Bone metastasis was reported in 80% of the 17 patients included in the study. Therapy efficacy was evaluated by ^{68}Ga-PSMA-11 PET scans and measuring prostate-specific antigen (PSA) values. As a result of the study, it was reported that an excellent antitumor effect was observed based on PSA levels and PET results. In addition, ^{68}Ga-PSMA-11 PET scans showeda decrease in tracer avidity in metastatic nodal and skeletal lesions and relief in bone pain after the first therapy cycle (Sathekge et al., 2019).

Satapathy et al. use the "National Comprehensive Cancer Network Functional Assessment of Cancer Therapy-Prostate Symptom Index 17 (NCCN-FACT-FPSI-17)" questionnaire to evaluate the health status of mCRPC patients (previously heavily pretreated) after ^{225}Ac-PSMA-17 therapy. As a result of the study, it was stated that PSA decreased in 5 out of 11 patients, while PSA remained stable or progressed in 3 of them. In addition, index scores before and after treatment were compared. It has been reported that the patients recorded significant improvement in physical symptoms such as pain, difficulty in urination, bone pain, fatigue, and limitation in physical activity (Satapathy et al., 2020).

The information obtained in the clinical studies with Ac-225 radiopharmaceuticals is encouraging, thus leading to the development and use of different α-emitters such as Terbium-149 (Tb-149), Astatine-211 (At-211), Lead-212 (Pb-212), Bismuth-213 (Bi-213), and Thorium-227 (Th-227) (Dhiman et al., 2022).

In addition, there are studies on different radiopharmaceuticals that can be used in the therapy of bone metastases, which are still in the research phase. These radiopharmaceuticals and their properties are summarized in Table 3 (International Atomic Energy Agency, 2021).

Table 3. Examples of radiopharmaceuticals under investigation for use in bone metastases therapy and their properties (International Atomic Energy Agency, 2021)

Radionuclide	Labeled Compound	Half-life	Radiation Type	Maximum Energy of Beta Particles (keV)	References
Sn-117m	DTPA	14 days	β^-,γ	130	(Krishnamurthy et al., 1997) (Srivastava et al., 1998)
Lu-177	DOTMP	6.734 days	β^-, γ	498	(Zakaly et al., 2020) (Bollampally et al., 2021) (Chakraborty et al., 2008)
Sm-153	DOTMP	46.27 hours	β^-	808	(Simón et al., 2012) (Chakraborty et al., 2004)
Tm-170	EDTMP	128.6 days	β^-, γ	968	(Das et al., 2009) (Das et al., 2017)
Ho-166	EDTMP	26.83 hours	β^-, γ	1854	(Louw et al., 1996) (Pedraza- López et al., 2004)

Sn: Tin; **Lu:** Lutetium; **Sm:** Samarium; **Tm:** Thulium; **Ho:** Holmium; **DTPA:** Diethylene triaminepentaacetic acid; **DOTMP:** 1,4,7,10-tetraaxacyclododecane-1,4,7,10-tetramethylene phosphonic acid

CONCLUSION

Metastases are still one of the most serious complications of cancer, and their treatment is vital for patient health. Bone metastases are seen in many primary cancer types and cause significant problems in patient survival and living standards. Radionuclide therapy is becoming increasingly important in the treatment of bone metastases. Using a targeted systemic therapy approach in radionuclide therapy and radiopharmaceuticals is more selective and effective in bone metastases therapy than other local and systemic treatments, making this therapy method advantageous. For this reason, studies on radionuclide therapy and the use of new radiopharmaceuticals in bone metastases therapy continue intensively. It can be said that promising results have been obtained with radiopharmaceuticals that have entered clinical use.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Determining the subject of study (SE), literature research (HB), preparing the study text (HB, SE), reviewing the text (SE).

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FABAD J. Pharm. Sci., 48, 2, 337-358, 2023
Doi: 10.55262/fabadeczacilik.1187441
REVIEW ARTICLE

Interaction of Statins with Grapefruit Juice

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Interaction of Statins with Grapefruit Juice

SUMMARY

Grapefruit juice, which discovered to interact with felodipine for the first time, is now known to interact with more than 80 drugs. Statins are among the drugs that interact with grapefruit juice. Grapefruit juice-statin interactions were first investigated in 1998 in human pharmacokinetic studies with lovastatin and simvastatin. The pharmacokinetic and pharmacodynamic basis of the interaction has been extensively investigated in studies. Flavonoids and furanocoumarins, the main components of grapefruit juice, have been reported to cause drug interactions. Furthermore, statin-grapefruit juice interactions occur mostly through inhibition of cytochrome-3A4 (CYP3A4), to a lesser extent through inhibition of P-glycoprotein (P-gp) and organic anion transporting polypeptides (OATPs). Changes in plasma drug levels as a result of interaction may increase the side-effect of statins or reduce their therapeutic efficacy. Therefore, patients using statins are generally advised to avoid grapefruit juice consumption.

Key Words: *Grapefruit juice, drug interaction, statins, CYP3A4, P-gp, OATP*

Statinlerin Greyfurt Suyu ile Etkileşimi

ÖZ

İlk kez felodipin ile etkileştiği keşfedilen greyfurt suyunun günümüzde 80'den fazla ilaçla etkileştiği bilinmektedir. Statinler de greyfurt suyuyla etkileşen ilaçlar arasında yer almaktadır. Greyfurt suyu-statin etkileşimleri ilk olarak 1998 yılında lovastatin ve simvastatin ile insanlarda yapılan farmakokinetik çalışmalarla araştırılmıştır. Yapılan çalışmalarda etkileşimin farmakokinetik ve farmakodinamik temeli kapsamlı bir şekilde araştırılmıştır. Greyfurt suyunun ana bileşenleri olan flavonoidler ve furanokumarinlerin ilaç etkileşimlerine neden olduğu belirtilmiştir. Ayrıca, statin-greyfurt suyu etkileşimleri çoğunlukla sitokrom-3A4'ün (CYP3A4) inhibisyonu yoluyla, daha az oranda P-glikoprotein (P-gp) ve organik anyon taşıyıcı polipeptitlerinin (OATPler) inhibisyonu yoluyla meydana gelmektedir. Etkileşim sonucu plazma ilaç seviyelerindeki değişiklikler, statinlerin yan etkilerini artırabilir veya terapötik etkinliklerini azaltabilir. Bu nedenle statin kullanan hastaların genellikle greyfurt suyu tüketiminden uzak durmaları önerilmektedir.

Anahtar Kelimeler: *Greyfurt suyu, ilaç etkileşimi, statinler, CYP3A4, P-gp, OATP*

Received: 11.10.2022
Revised: 16.05.2023
Accepted: 17.05.2023

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INTRODUCTION

Drug-food interactions significantly affect the success of drug therapy. Food-drug interactions can be divided into: pharmacokinetic and pharmacodynamic interactions depending on the content, amount, and consumption time of nutrients (Schmidt, 2002). The pharmacodynamic interactions in which dietary components affect pharmacological activity at the receptor level are limited. More frequent pharmacokinetic interactions may change the effectiveness of therapy or increase toxicity. This may adversely affect patient care, increase morbidity, and extend treatment or hospital stay (Shirasaka, 2011). In a study examining the interaction between ethanol and felodipine, grapefruit juice was used to mask the taste of ethanol and found that plasma concentrations of felodipine were several times higher when grapefruit juice was used (Bailey, 1989; Bailey, 1998). Because of this accidental discovery, the idea that grapefruit juice and drug interaction has emerged, and numerous studies have been conducted on the subject (Dahan, 2004). In 2016, it was reported that the number of drugs interacting with grapefruit juice was more than 85 (Lee, 2016). When therapeutic agents are co-administered with grapefruit juice, the drug exposure is significantly increased. The drugs that are best known to interact with grapefruit juice are statins, also called 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, used to treat hypercholesterolemia (Kafle, 2018). The main substances that cause grapefruit juice to interact with statins are the components in grapefruit juice (Mouly, 2017). As a result of thisinteraction, serious side effects such as rhabdomyolysis, ischemic heart disease, and changes in low-density lipoprotein (LDL) cholesterol values have been reported (Shirasaka, 2013). In this review, grapefruit juice components, their role in drug interactions, statins interacting with grapefruit juice and their properties are summarized.

GRAPEFRUIT JUICE COMPOSITION

Grapefruit, one of the world's most popular citrus fruits, is rich in vitamins, dietary fiber, sugar, and minerals. In addition, antioxidant, anti-inflammatory, anticancer, and neuroprotective effects of secondary metabolites in grapefruit juice are known (Hung, 2017). Antioxidant activity is caused by phenolic compounds such as anthocyanins, flavonoids, and ascorbic acid. These compounds are the largest group of secondary metabolites that attract attention due to their physiological effects (Sicari, 2018). Furanocoumarins and flavonoids are components in grapefruit juice that inhibit intestinal metabolism and/or transport of many drugs. The amounts of these components in grapefruit juice may vary depending on production procedure, storage conditions, source, and maturity of the fruit. The amount of active ingredients is important in terms of the grapefruit juice-drug interaction mechanism, reversibility, and comparison with clinical data (Castro, 2006).

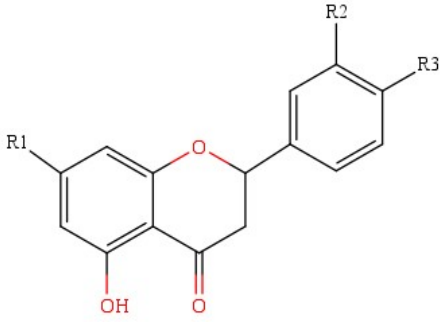
Flavonoids

There are six groups of flavonoids: Anthocyanins, flavan, flavanones, flavones, flavonols and isoflavones (Figure 1). Flavonones are abundant in citrus fruits and give their typical flavor. They exist in glycoside or aglycone forms (Igual, 2011). If sugar molecules are attached to the flavonoid core, which contains three-ring structure, they are called glycosides; and if there is no sugar, they are called aglycons (Zhang, 2007). Flavonoids are structurally similar to adenosine triphosphate (ATP). Therefore, they may be responsible for some biological effects by competing with ATP for binding to different enzymatic sites (Vanamala, 2006). Naringin is the most abundant flavonoid in grapefruit juice (200-2000 μmol/L). It is also the main component that causes the bitter taste in grapefruit juice and is an inhibitor of the cytochrome (CYP) enzymes. Naringenin is the aglycone form produced by the intestinal hydrolysis of naringin (Fukuda, 2000; Hanley, 2011). Many flavonoids such as narirutin, hesperidin, neohesperidin, quercetin, tangeretin, nobiletin, kemferol were detected in grapefruit juice (Ross, 2000).

Tangeretin and nobiletin increased the activity of benzopyrene hydroxylase and some CYP enzymes after oral administration, while naringin (capsule formulation) and quercetin did not show a significant inhibitory effect on CYP3A4 (Ho, 2001).

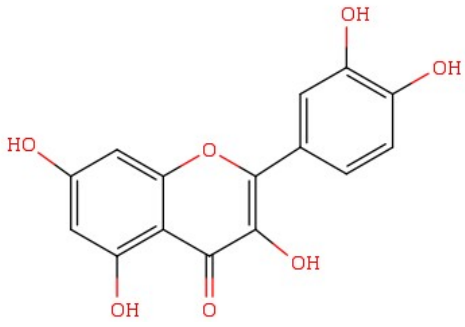
Furanocoumarins

Furanocoumarins have a three-ring head and an aliphatic tail. The furan ring is crucial for the production of a reactive precursor that binds irreversibly to the CYP apoprotein and inhibits enzymatic activity. Reported concentrations in grapefruit juice for bergamottin and dihydroxybergamottin are 1-37 μM and 0.2-52.5 μM, respectively (Hanley, 2011). Dihydroxybergamottin, one of the furanocoumarin derivatives found in the highest amount in grapefruit juice, has 1000 times the water solubility of bergamottin due to its hydroxyl groups. The CYP3A4 inhibitory effect of both components has been shown to be mechanism-based and reversible *in vitro* (Paine, 2004; Paine, 2006).



Flavanone

Flavonoid	R1	R2	R3
Naringenin	OH	H	OH
Narirutine	O-Ru	H	OH
Naringin	O-Nh	H	OH
Hesperidin	O-Ru	OH	OCH_3
Neohesperidin	O-Nh	OH	OCH_3
Didymin	O-Ru	H	OCH_3
Poncirin	O-Nh	H	OCH_3



Flavonol

Quercetin

Figure 1. Chemical structures of some flavonoids in grapefruit juice (Igual, 2011).

Furanocoumarin derivatives are divided into three groups: monomer, dimer, and trimer. The monomers can be angular with a furan ring attachment at 7,8-position or linear with a furan ring attachment at 6,7-position of coumarin. This structure is substituted at the 5 and/or 8 position with side chains of methoxy, prenyloxy or geranyloxy. Dimers are formed by an ether bond between the side chains of two linear furanocoumarin monomers or by attaching the side chain of one monomer to the pyrone ring of the other monomer (Guo, 2004). In 2006, chemical analogues of furanocoumarins were synthesized for the structural evaluation of their inhibitory effects on the CYP3A4 enzyme. According to the results of this study, the binding of geranyloxy chains with hydrophilic groups at the 6,7-positions to the structure increases the in-

hibitory effect. In this way, furanocoumarins interact with the CYP3A4 enzyme from both lipophilic and hydrophilic sites (Row, 2006). The chemical structure of some furanocoumarins in grapefruit juice is given in Figure 2 (Ohta, 2002). *In vitro* and *in vivo* studies have shown that furanocoumarins improve bone health as well as anti-inflammatory, antioxidative, and anticancer effects (Hung, 2017). It has also been used clinically in the treatment of skin diseases in some countries since the 2000s (Melough, 2018).

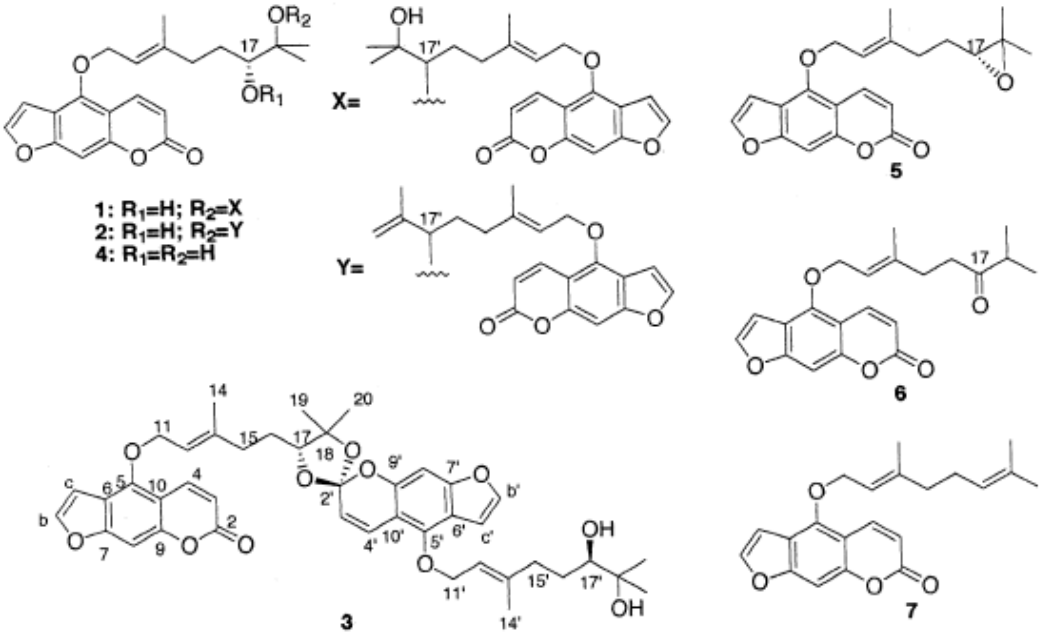


Figure 2. Some furanocoumarins in grapefruit juice (1: Paradisin A, 2: Paradisin B, 3: Paradisin C, 4: 17,18-dihydroxybergamottin, 5: 17-epoxybergamottin, 6: 17-ketobergamottin, 7: Bergamottin) (Ohta, 2002).

GRAPEFRUIT JUICE-DRUG INTERACTION MECHANISMS

Natural components in grapefruit juice cause drug interactions through different mechanisms. The most accepted interaction mechanism is the inhibition of CYP3A isoforms (Greenblatt, 2001). It is well known that grapefruit juice interacts with drugs that are substrates of intestinal CYP3A4, which controls the first-pass metabolism of many pharmaceuticals (Kiani, 2007). Recent research indicates that grapefruit-drug interactions may also occur by altering intestinal absorptive and efflux transporter proteins. These transporters are involved in the absorption of many drugs (De Castro, 2007). According to the literature, there are three mechanisms for grapefruit juice-drug interaction.

CYP3A4 Inhibition

CYP450 is a family of enzymes responsible for drug metabolism and more than 50 members have been elucidated. CYP3A4 is responsible for oxidative metabolization of many drugs in humans and is expressed at the apical surface of enterocytes and hepatocytes (Bailey, 2004; Bailey, 2013). The components in grapefruit juice cause competitive and non-competitive inhibition of this enzyme. To observe this inhibition, the drug should be taken orally, and grapefruit juice should be consumed as a glass (200 mL) of frozen concentrate, diluted from concentrate or freshly frozen (Bailey, 2004). Grapefruit

juice significantly increases the plasma concentration of many drugs that are CYP3A4 substrates, such as dihydropyridine, cyclosporine, midazolam, and terfenadine (Lilja, 1998). To examine the effect of grapefruit juice on the pharmacokinetics of midazolam, volunteers were administered midazolam with different amounts of grapefruit juice. When the pharmacokinetic parameters of midazolam, such as the area under the plasma drug concentration-time curve (AUC), maximum plasma concentration (C_{max}), elimination half-life ($t_{1/2}$) were evaluated, side effects of CYP3A4 substrates increased due to consumption of large amounts of grapefruit juice (Veronese, 2003). In another pharmacokinetic study, concomitant use of grapefruit juice and simvastatin in healthy volunteers increased the AUC_{0-24} by 3.6-fold and C_{max} by 3.9-fold. These results were attributed to the inhibition of CYP3A4-mediated first-pass metabolism of simvastatin in the intestinal wall (Lilja, 2004). Components in grapefruit juice (especially furanocoumarins) are converted into reactive intermediates that cause inactivation by covalently binding to the CYP3A4 enzyme system. In this condition, known as mechanism-based inhibition, defective CYP3A4 undergoes proteolysis. In this process, there is no change in the content of messenger RNA (mRNA) in enterocytes or a decrease in CYP3A4 production (Bailey, 2004). In another study, it was reported that furanocoumarins (such as 6,7-dihydroxybergamottin) cause irreversible inhibition of CYP3A4 (Pirmohamed, 2013). Evaluation of CYP3A4 and P-gp interaction with five grapefruit juice components (quercetin, naringin, naringenin, 6,7-dihydroxybergamottin and bergamottin) showed that 6,7-dihydroxybergamottin and bergamottin inhibited the CYP3A4-mediated metabolism of model drug saquinavir. However, none of these grapefruit juice components had a significant effect on P-gp activity *in vitro* (Eagling, 1999). In another study, the inhibitory effect of four furanocoumarin derivatives isolated from grapefruit juice on CYP3A4 was evaluated and the inhibitory effects of these components were found to be equivalent or greater than the specific CYP3A4 inhibitor ketoconazole (Fukuda, 1997). In addition, evaluation the effects of five grapefruit juice components (bergamottin, 6,7-dihydroxybergamottin, GF-I-1, GF-I-4 and nootkatone) on CYP450 isoforms showed that four furanocoumarin derivatives inhibited CYP3A4-mediated oxidation of nifedipine in a concentration- and time-dependent manner. On the other hand, bergamottin inhibited CYP1A2, CYP2C9, CYP2C19, CYP2D6, while dihydroxybergamottin inhibited only CYP1A2. Nootkatone, a sesquiterpene, had no significant effect on CYP450 activity, except for CYP2A6 and CYP2C19 (Tassaneeyakul, 2000).

P-gp Inhibition

P-gp is an ATP-dependent efflux pump that affects the disposition and clinical response of its substrates. It is localized in the blood-brain barrier, testes, proximal tubule of the kidneys, canalicular membrane of the liver, and luminal surfaces of small intestinal epithelial cells. It has been reported that grapefruit juice inhibits P-gp-mediated pitavastatin transport in rats and humans (Shirasaka, 2011). Although the mechanism of inhibition is not fully elucidated, flavonoids (naringin, naringenin) in grapefruit juice are known to inhibit P-gp-mediated transport (Chen, 2018). The oral bioavailability of digoxin, a good P-gp substrate, is increased when co-administered with grapefruit juice (Bailey, 2004). Naringin had no direct inhibitory effect on the P-gp substrate talinolol. However, naringin is converted to aglycone naringin, which regulates P-gp activity in the intestinal microflora, and exerts its inhibitory effect. In the same study, it was reported that bergamottin did not significantly affect P-gp activity, but 6,7-dihydroxybergamottin was a potential P-gp inhibitor (De Castro, 2007). In another study, the effect of grapefruit juice and its components on intestinal absorption of colchicine was investigated using the human colorectal adenocarcinoma (Caco-2) cell line and an intestinal rat perfusion method. The decreased mucosal secretion in the presence of known P-gp inhibitors verapamil and quinidine indicates that basolateral-to-apical (B→A) per-

meability is greater than apical-to-basolateral (A→B) permeability. In the presence of grapefruit juice and its components, A→B permeability increased, whereas B→A permeability decreased due to P-gp inhibition. In addition, in rats, grapefruit juice increased the ileal and jejunal permeability of colchicine 2 and 1.5 times, respectively (Dahan, 2009).

Organic anion transporting polypeptides Inhibition

Organic anion transporting polypeptides (OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1, OATP4A1) are localized on the luminal surface of epithelial cells in the small intestine and facilitate the uptake of their substrates from the gastrointestinal tract into the portal circulation. OATPs localized on the basolateral membrane in the liver facilitate uptake of their substrates from the portal circulation to the hepatocytes. Grapefruit juice and its components (e.g. furanocoumarins and flavonoids) are potential inhibitors of OATPs. The oral bioavailability of fexofenadine (an OATP substrate) has been shown to be increased in humans when co-administered with grapefruit juice (Bailey, 2004). Grapefruit juice components, especially flavonoids (such as naringin, hesperidin) inhibit OATP. This directly affects the dose-response relationship by reducing substrate drug concentrations in the systemic circulation and tissues (Pirmohamed, 2013). In a pharmacokinetic study, co-administration of grapefruit juice with aliskiren dramatically decreased the plasma levels of aliskiren. OATP2B1 and OATP1A2 transfected cell lines were used to elucidate the mechanism of this effect. The accumulation of OATP1A2 substrates aliskiren and fexofenadine was significantly reduced in the presence of naringin (Rebello, 2012). Two views have been proposed regarding the OATP inhibitory effect of grapefruit juice. First, water absorbed faster than grapefruit juice, resulting in increased drug concentrations in the intestinal fluid and increased drug absorption due to higher drug exposure of OATP transporters. The second view is that non-specific osmotic effects of solutes increase the volume of intestinal fluid, and thus indirectly affect OATP function (Dresser, 2003).

STATINS

Statins are a class of drugs used to treat hypercholesterolemia. They are also known as HMG-CoA reductase inhibitors. In clinical studies with statins, it has been shown that morbidity and mortality rates due to cardiovascular dieseases are reduced (Bellosta, 2004). There are seven statins used in the clinic: Atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin, rosuvastatin, pitavastatin. With the exception of cerivastatin, which was withdrawn from the market in 2001, all are safe and well-tolerated drugs. Although their chemical structures are different, they mainly consist of three parts. These moieties are the binding site to the HMG-CoA enzyme, the complex hydrophobic ring and the side groups responsible for the solubility of the drug attached to this ring (Figure 3) (Schachter, 2004).

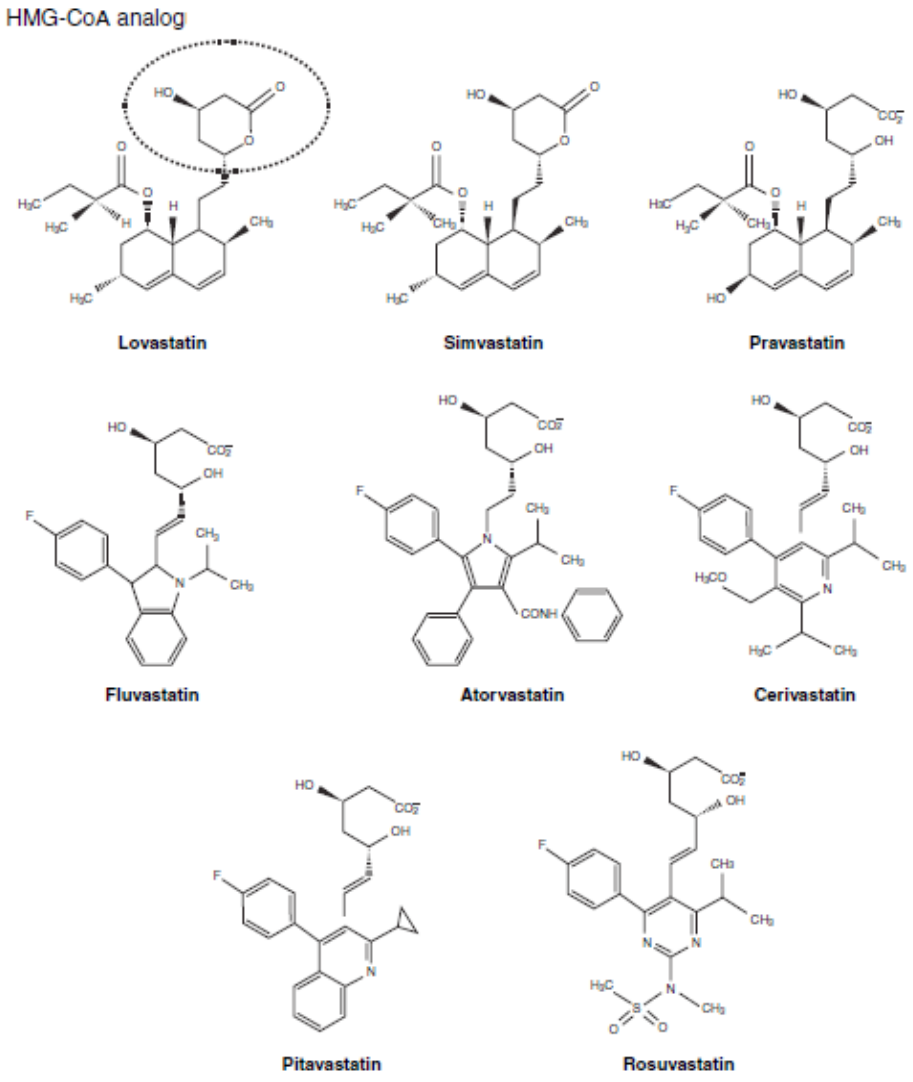


Figure 3. Chemical structures of statins (Schachter, 2004).

Statins competitively inhibit the HMG-CoA reductase enzyme, which is the rate-limiting step in cholesterol synthesis (Figure 4). Statins have 3 times higher affinity for the enzyme binding site than the natural substrate HMG-CoA, and reduce cholesterol synthesis by 10-60%, depending on dose and individual factors (Williams, 2002). The reduction of cholesterol in hepatocytes leads to an increase in hepatic LDL receptors, which reduces circulating LDL and its precursors (intermediate density - IDL and very low density - VLDL lipoproteins). After a single daily dose, all statins decrease LDL cholesterol in a non-linear and dose-dependent manner (Stancu, 2001). Table 1 shows statins-induced decreases in serum LDL cholesterol concentrations at different doses (Law, 2003). Additionally, HMG-CoA inhibition affects smooth muscle proliferation and platelet aggregation, and anti-inflammatory and antithrombotic effects can also be observed (Williams, 2002). The most known side effects of statins are on the musculoskeletal system. Possible side effects are muscle pain, fatigue, weakness and, in severe cases, rhabdomyolysis. In addition, a number of side effects such as gastrointestinal, neurological, psychiatric symptoms, sleep problems, and high blood sugar levels have also been reported (Golomb, 2008).

Table 1. Reductions in serum LDL cholesterol levels (%) based on daily dose of statins (Law, 2003).

	Daily dose (mg)				
	5	**10**	**20**	**40**	**80**
Atorvastatin	31%	37%	43%	49%	55%
Fluvastatin	10%	15%	21%	27%	33%
Lovastatin	-	21%	29%	37%	45%
Pravastatin	15%	20%	24%	29%	33%
Rosuvastatin	38%	43%	48%	53%	58%
Simvastatin	23%	27%	32%	37%	42%

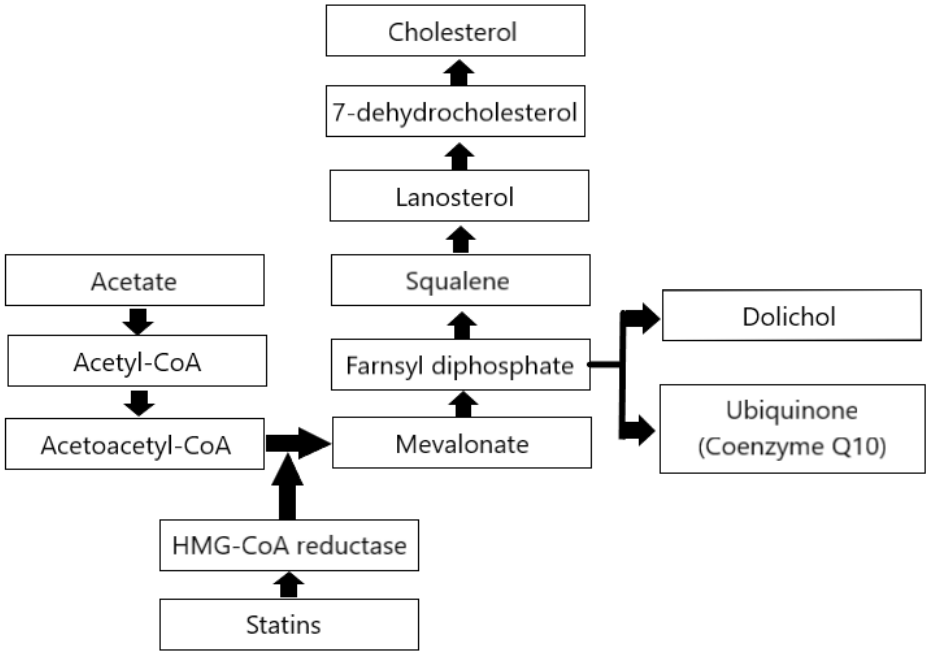


Figure 4. Cholesterol synthesis in the liver and the mechanism of action of statins (Williams, 2002).

Although statins have the same mechanism of action, their pharmacokinetic properties differ due to changes in their chemical structures (Amly, 2015). Their chemical structures also affect their binding potency to the HMG-CoA reductase enzyme, their lipophilicity, and their ability to enter hepatocytes. The pharmacokinetic parameters of statins are summarized in Table 2. Statins with 15-30% oral bioavailability have a short elimination half-life. Most statins are metabolized by CYP enzymes. Atorvastatin, cerivastatin, lovastatin, and simvastatin are metabolized by CYP3A4, while fluvastatin and rosuvastatin are metabolized by CYP2C9 (McKenney, 2003). CYP450 enzymes have no significant effect on the metabolism of pravastatin and pitavastatin (Schachter, 2004).

Table 2. Physicochemical and pharmacokinetic properties of statins.

	Lovastatin	Simvastatin	Pravastatin	Fluvastatin	Atorvastatin	Cerivastatin	Rosuvastatin	Pitavastatin
[1]IC_{50} (nM)	-	11.2	44.1	27.6	8.2	10.0	5.4	-
[1]Absorption (%)	30	60-85	35	98	30	>98	50	80
[1]Bioavailability (%)	5	<5	18	24-30	12	60	20	60-80
[1]Hepatic extraction (%)	≥70	≥80	45	≥70	70	50-60	63	-
[1]Renal extraction (%)	10	13	20	6	<5	30	10	-
[1]Protein binding (%)	>98	>95	50	>98	>98	>99	90	96
[1]Half-life (h)	2-5	2-5	1-3	1-3	7-20	1-3	20	10-13
[1]Metabolism	+++	+++	+	+++	+++	+++	+	++
[1]Active metabolites	3	3	2	No	2	2	Minor	Minor
[1]CYP enzyme metabolism	3A4/5, 2C8	3A4/5, 2C8	3A4	2C9	3A4, 2C8	3A4, 2C8	2C9, 2C19	2C9
[1]Uptake transporters	SLCO1B1, MCT4	SLCO1B1	SLCO1B1/2B1 OAT3, MCT1	SLCO1B1	SLCO1B1	SLCO1B1	SLCO1B1/1B3 /2B1/1A2, SLC10A1	SLCO1B1/1B3
[1]Efflux transporters	ABCB1	ABCB1	ABCB1/B11 /C2/G2	ABCG2	ABCB1/G2	ABCB1/C2/G2	ABCB1/C2/G2	ABCB1/C2/G2
[2,3]BCS class	II	II	III	I	II	II	II	II
[4]Hydrophilicity/ Lipophilicity	Lipophilic	Lipophilic	Hydrophilic	Lipophilic	Lipophilic	Lipophilic	Hydrophilic	Lipophilic
[5]log P (pH 7.0)	1.7	2.06	-0.23	1.67	1.61	2.05	0.13	-
[5]log D (pH 7.0)	3.91	4.4	-0.47	1.75	1.53	2.32	-	1.5
[6]Standard daily dose (mg)	10-40	10-40	10-40	80	10-80	-	5-40	1-4

r[1]Hu, 2009; [2]Lada, 2018; [3]Varma, 2012; [4]Bonsu, 2013; [5]Murphy, 2020, [6]Sirtori, 2014

IC_{50}: Half maximal inhibitory concentration, CYP: Cytochrome P450, BCS: Biopharmaceutics Classification System, log P: Partition coefficient, log D: Distribution coefficient,

SLC: Solute carrier organic anion transporters, MCT: Monocarboxylate transporters, ABC: ATP-binding cassette transporters

Atorvastatin

Atorvastatin is a second-generation statin and a reversible HMG-CoA reductase enzyme inhibitor. It is administered orally as the calcium salt of the active hydroxy acid form. It is used at doses of 10-80 mg/day in the clinic. Atorvastatin is converted to the lactone form in the body. The acid and lactone forms of the drug differ in solubility, lipophilicity, and octanol/water partition coefficient (Lennernas, 2003). Atorvastatin calcium salt is insoluble in aqueous solutions at pH ≤ 4.0 aqueous solutions, and slightly soluble in pH 7.4 phosphate buffer and water (Kim, 2008). According to the Biopharmaceutics Classification System (BCS), atorvastatin calcium is a Class II drug (low solubility, high permeability) and its oral bioavailability is approximately 12% (Shayanfar, 2013). Due to its high membrane permeability, it is rapidly absorbed and reaches its maximum plasma concentration within 1-3 hours after oral administration (Khan, 2017). Low solubility and first-pass elimination have been reported to be the causes of low bioavailability (Khan, 2011). The CYP3A4 enzyme in the gastrointestinal tract and liver, is responsible for the first-pass metabolism of atorvastatin (Kumar, 2017). The plasma protein binding of atorvastatin is greater than 98%, and the mean volume of distribution after 5 mg iv infusion is 381 L indicating high binding of atorvastatin to peripheral tissues (Khan, 2017).

There are many studies in the literature evaluating the interaction of atorvastatin with grapefruit juice. Co-administration with grapefruit juice increased the AUC of atorvastatin acid and pitavastatin acid (their active forms) in humans by 83% and 13%, respectively; this indicates that grapefruit juice inhibits the CYP3A4-mediated metabolism of atorvastatin, whereas the metabolism of pitavastatin does not extensively depend on CYP3A4. The time to maximum concentration (t_{max}) of 2-hydroxy atorvastatin acid (major active metabolite) was prolonged from 3.2 to 9.4 hours. $t_{1/2}$ and AUC of pitavastatin lactone (inactive form) were significantly increased (Ando, 2005). In another study, in the presence of grapefruit juice, the AUCs of atorvastatin acid and atorvastatin lactone increased by 1.4- and 1.56-fold, respectively, while there was no significant increase in the pharmacokinetic parameters of pravastatin (Fukazawa, 2003). Daily consumption of 240 mL grapefruit juice increased the AUC of atorvastatin by 37% after oral administration of 40 mg, but this increase was not clinically significant (Kellick, 2014). Concomitant use of atorvastatin (10, 20, or 40 mg/day) and grapefruit juice resulted in a 19-26% increase in serum atorvastatin concentrations in patients. When the daily dose was halved, serum concentration values increased by 12-25%. Additionally, myalgia and memory loss scoring were used to assess the quality of life of the patients. Serum creatine kinase (CPK) levels and liver function tests were evaluated. According to results, changes in serum atorvastatin levels did not cause liver and/or muscle toxicity (Reddy, 2011). Similarly, grapefruit juice consumption with 10 mg atorvastatin increased the serum levels by 1.8 times. Daily use of 10 mg of atorvastatin reduces LDL cholesterol level by 37%, reducing the risk of ischemic heart disease by 61%. When atorvastatin is taken with a glass of grapefruit juice, its blood level increases by approximately 80%. In this case, the reductions in LDL cholesterol level and ischemic heart disease risk are 42% and 66%, respectively (Lee et al., 2016). The intestinal concentration of CYP3A4 enzyme decreases by 50% within 4 h of drinking juice of a whole grapefruit. Inactivation of intestinal CYP3A4 increases the systemic bioavailability by affecting the presystemic degradation of statins (atorvastatin, lovastatin, simvastatin) metabolized by this enzyme. Therefore, it is not recommended to consume grapefruit juice while taking these statins. The half-life of drug, the time of consumption and the amount grapefruit juice consumed are important for interaction. It has been reported that with statins, this grapefruit juice effect is reduced to 10% of the maximum 24 hours after grapefruit juice ingestion, or using about a liter or more grapefruit juice poses a potential hazard as it increases the amount of

statin entering the circulation (Azemawah, 2019).

Simvastatin

Simvastatin is produced synthetically from the fermentation product of Aspergillus terreus. There is only one stable crystal form and no hydrate form. It is Class II drug (low solubility, high permeability) according to BCS (solubility at 25 ^{0}C 6.3x10^{-3} g/L, pH 1.0-7.0) (Graeser, 2008; Kong, 2017). After simvastatin lactone is administered as a prodrug, it is enzymatically hydrolyzed in the body and converted to its active acid form (Schachter, 2004). Although oral absorption is about 60-85%, the bioavailability of simvastatin is less than 5% due to pre-systemic elimination in the gastrointestinal tract via both CYP3A4 and P-gp. The CYP2C8 is also involved in metabolism of simvastatin. Lipophilic statins such as simvastatin tend to bind to peripheral tissues much more readily (Neuvonen, 2008). The major elimination organ of simvastatin is the kidneys, and its half-life is approximately 3 hours in adults with normal renal function (Srinivas, 2012). The t_{max} of simvastatin is between 1.3 and 2.4 hours after 40 mg oral dose (Bellosta, 2004). Total body clearance is 31.8 L/h, and protein bindings for simvastatin and simvastatin acid are 98% and 94%, respectively (Mauro, 1993). In nine healthy Malaysian male subjects, the mean volume of distribution was 232.57±132.54 L following a single oral dosage of 40 mg (Alakhali, 2013).

In a study investigating the effects of regular grapefruit juice consumption on the pharmacokinetics of simvastatin, 200 mL of grapefruit juice was given to 10 healthy volunteers every day for three days. Simvastatin (40 mg, single dose) was administered with 200 mL of grapefruit juice on the third day. The AUC values of simvastatin and simvastatin acid were increased by 3.6 and 3.3 times, respectively, following grapefruit juice administration (Lilja, 2004). In another study by the same researcher, the C_{max} of simvastatin increased 12-fold and the AUC increased 13.5-fold after consuming 200 mL grapefruit juice three times each day for three days, compared to the control group (consumed water only) (Lilja, 2000). Similarly, administration of 40 mg simvastatin daily with one grapefruit or one glass of grapefruit juice (approximately 240 mL) increased AUC by 3.6-fold, while excessive grapefruit juice consumption (equal to six grapefruits) increased AUC by 13.5-fold (Lee, 2016). In another study, 200 mL of double-strength (diluted 1/1) grapefruit juice was given 3 times a day for 2 days. On the 3rd day, 60 mg of simvastatin was administered with 200 mL of grapefruit juice. The increase in AUC values for simvastatin and simvastatin acid was 16 and 7-fold, respectively. Additionally, 200 mL of single-strength (diluted 1/3) grapefruit juice was consumed at breakfast for 3 days, and then 20 mg of simvastatin was administered in the evening on the 3rd day. The AUC of simvastatin and simvastatin acid increased 1.9 and 1.3 times, respectively. Based on the results, it was reported that the amount of grapefruit juice taken with simvastatin should not exceed 1 liter daily (Kellick, 2014). Bergamottin, a component of grapefruit juice, raises the plasma levels of simvastatin and simvastatin acid (the active metabolite) by preventing the CYP3A4-mediated first-pass metabolism of simvastatin in the intestines (Kiani, 2007). In a study using human and rat liver microsomes, bergamottin extracted from grapefruit juice was shown to inhibit the CYP450-dependent hepatic metabolism of simvastatin (Goff-Klein, 2004). Grapefruit juice and other CYP3A4 inhibitors have the potential to increase blood levels 20-fold. According to a case report published in Germany, a woman taking 80 mg of simvastatin developed rhabdomyolysis four days after she started consuming one grapefruit per day. Therefore, grapefruit juice consumption requires dose adjustment (Spence, 2016). Excessive consumption of grapefruit juice (400 mL 3 times daily, 3 days) increased the AUC of simvastatin by 700%, while this increase was 330% at low amounts (200 mL once daily, 3 days). Additionally, rhabdomyolysis has also been reported after consumption of fresh grapefruit for 10 days (Bailey, 2013).

Lovastatin

Lovastatin is a white crystalline powder and its

water solubility is 0.4 μg/mL at room temperature (Sharannavar, 2018). It is given as a prodrug in inactive lactone form and transformed into the active β-hydroxy acid form by carboxyesterases in the liver (Donovan, 2002). After oral administration, lovastatin is metabolized by CYP3A4 and reaches its maximum plasma concentration within 4 hours. The elimination half-life is 3 hours, and lovastatin is 95% protein bound. Renal and fecal excretion are approximately 10% and 83%, respectively. Lovastatin is a BCS Class II drug (low solubility, high permeability) and it should be taken twice daily (Zolkiflee, 2017). Due to its low water solubility and short half-life, its oral bioavailability is only 5%. It also undergoes extensive first-pass metabolism (Zhou, 2015).

Ten healthy volunteers were given 200 mL of grapefruit juice 3 times a day for 2 days, and 80 mg of lovastatin was co-administered with 200 mL of grapefruit juice on the 3rd day. In this pharmacokinetic study, AUC increased 15 times for lovastatin and 5 times for lovastatin acid. C_{max} of lovastatin and lovastatin acid increased approximately 12-fold and 4-fold, respectively. $t_{1/2}$ values remained unchanged (Kantola, 1998). In another study, AUC and C_{max} values of lovastatin increased by 2-fold, while for lovastatin acid these values increased by 1.6-fold with grapefruit juice (Rogers, 1999). A high daily intake of grapefruit juice (equivalent to six grapefruits) increases the systemic bioavailability of lovastatin by inhibiting its presystemic biotransformation. The time of consumption is as important as the amount of grapefruit juice taken. The pharmacokinetic properties of statins with short half-life, such as simvastatin and lovastatin are more affected when taken with grapefruit juice in the morning. This is because the effect of grapefruit juice is seen within 7-8 hours (Costache, 2019). In another study, a single dose of 40 mg lovastatin was administered after using 250 mL of single-strength grapefruit juice for 4 days. AUC values for lovastatin and lovastatin acid increased 1.94 and 1.57-fold, respectively (Kellick, 2014). When concomitant administration of 40 mg of lovastatin or simvastatin daily with grapefruit juice, the estimated reduction in LDL cholesterol level and heart disease risk is 48% and 70%, respectively. If grapefruit juice is consumed 12 hours before these statins, reductions are predicted to be 43% and 66%, respectively (Lee, 2016).

Pravastatin

Pravastatin is a hygroscopic, crystalline powder, readily soluble in water and methanol. Unlike other statins, it is a hydrophilic compound. The pH-dependent octanol-water partition coefficient is 0.59 at pH 7.0. It is an acidic drug with a pKa of 4.5. Although it is very rapidly absorbed after oral administration, its bioavailability is low (about 18%) due to low membrane permeability (Hatanaka, 2000; Quion, 1994; Bang, 2003). The protein binding of pravastatin is approximately 50% and the volume of distribution is 0.46 L/kg. Elimination of pravastatin occurs by renal (47%) or non-renal (53%) routes. Approximately 70% of the oral dose is excreted in the feces and 20% in the urine. Despite its high dissolution rate and solubility in water, pravastatin is unstable in acidic conditions. It is converted to an isomer (3α-isopravastatin) by chemical transformation in the stomach (Hatanaka, 2000; Quion, 1994; Bang, 2003).

Evaluation of the interaction of atorvastatin and pravastatin with grapefruit juice in healthy volunteers revealed that grapefruit juice raised the AUC of atorvastatin acid and atorvastatin lactone, while pharmacokinetic parameters of pravastatin remained unchanged. This observation was attributed to the insignificant role of CYP3A4 pravastatin metabolism (Lilja, 1999). It has been reported that grapefruit juice has no effect on statins that are not metabolized by CYP3A4, such as pravastatin, rosuvastatin, fluvastatin, and pitavastatin. On the other hand, taking CPY3A4 metabolized statins at least four hours after drinking grapefruit juice reduces the risk of interactions by more than 60%, as the effect of grapefruit juice on this enzyme system disappears within a few hours (Mouly, 2017). In a study with pravastatin and pitavastatin, drug interactions were investigated in rats using the *in*

situ intestinal closed-loop technique in the presence of grapefruit juice or naringin. Although both statins are OATP1A5 and OATP2B1 substrates, only pitavastatin is a P-gp substrate. Grapefruit juice and naringin decreased the plasma concentration of pravastatin, while increasing the plasma concentration of pitavastatin. Based on the results, it was stated that the inhibitory effect of naringin on OATP caused a decrease in the absorption of pravastatin, while P-gp inhibition caused an increase in the absorption of pitavastatin (Shirasaka, 2011). In another study by the same researchers using the same technique, pravastatin was administered together with elacridar (P-gp inhibitor) and naringin (OATP inhibitor). In the presence of naringin, rat intestinal permeability of pravastatin was significantly reduced, whereas there was no significant change with elacridar (Shirasaka, 2010). According to the results of these studies, although there is a cellular interaction between naringin and the OATP substrate pravastatin, there is no possible clinical interaction between grapefruit juice and pravastatin.

Pitavastatin

Clinically used pitavastatin calcium is white to light yellow and odorless powder. It is soluble in organic solvents such as pyridine and tetrahydrofuran, but slightly soluble in ethanol and water. Partition coefficient of pitavastatin is 31.7 (Hayashi, 2007). Pitavastatin is a synthetic lipophilic statin that was first used in the treatment of hyperlipidemia in Japan in 2003. Unlike other statins, the cyclopropyl group in the structure of pitavastatin binds to the hydrophobic regions of the HMG-CoA reductase enzyme with high affinity, leading to more effective inhibition. Pitavastatin is administered as the calcium salt in doses of 1 mg, 2 mg, and 4 mg. Its bioavailability is between 51-60% and C_{max} is reached within one hour after oral dosing. Plasma protein binding is greater than 99%, particularly to albumin and alpha(1)-acid glycoprotein. It is specifically distributed to the liver and its hepatic uptake is presumed to be mediated by OATP1B1 and OATP1B3. The elimination half-life of pitavastatin is 12 hours and the mean volume of distribution is 133 L. CYP2C9 and CYP2C8 play a minor role in pitavastatin metabolism. Although pitavastatin is a CYP3A4 substrate, cyclopropyl group increases the bioavailability of pitavastatin by inhibiting its metabolism by the cytochrome P450 system (Duggan, 2012; Carella, 2016; Saito, 2011).

Evaluation of the concomitant use of grapefruit juice and atorvastatin or pitavastatin showed that no significant change was observed in the pharmacokinetics of pitavastatin, while the atorvastatin concentration increased significantly. This observation supports the fact that pitavastatin is a better treatment option (Ando, 2005). In another study, grapefruit juice caused a modest increase in pitavastatin levels in the blood. The $AUC_{0\text{-}48h}$ of pitavastatin acid and pitavastatin lactone increased by 14%, while $t_{1/2}$ values remained unchanged. Apparent oral clearance (CL/F) and C_{max} of pitavastatin acid decreased by 10% and 12%, respectively. The reductions in these values for pitavastatin lactone are 15% and 13%, respectively (Hu, 2013). Investigation of the role of OATPs and P-gp (MDR1) in intestinal absorption of pitavastatin confirmed that pitavastatin is the substrate of human OATP1A2, OATP2B1, MDR1 and rat Oatp1a5, Oatp2b1, Mdr1a. When pitavastatin was co-administered with naringin (OATP and MDR1 inhibitor) and/or elacridar (MDR1 inhibitor), rat intestinal permeability of pitavastatin decreased at low naringin concentration while increased at high naringin concentration. Permeability of pitavastatin was increased when elacridar was used alone, but decreased when elacridar and naringin were used together. The results of this study showed that OATP/Oatp and MDR1/Mdr1 have effects on the intestinal absorption of pitavastatin (Shirasaka, 2010). Pitavastatin has been reported to be safer than other statins. It is poorly metabolized by the CYP450 system and no inhibitory effect of the lactone form on CYP3A4 was found. In isolated rat liver microsomes, the HMG-CoA reductase inhibitory effect of pitavastatin was 2.4 and 6.8 times greater than simvastatin and pravastatin, respectively. In human microsomes, the intrinsic clearance of lovastatin,

simvastatin, atorvastatin, and fluvastatin was 100, 50, 8, and 30 times greater than that of pitavastatin, respectively. Based on these results, pitavastatin has a relatively low intrinsic clearance and is much less metabolized than other statins. In addition, pitavastatin produces a clinical response equivalent to atorvastatin, the most preferred and most potent statin. Due to its favorable pharmacokinetic properties, the possibility of drug-drug and/or drug-food interactions is very low, and may be preferred for use in treatment (Kajinami, 2003).

Fluvastatin

Fluvastatin is the first HMG-CoA reductase inhibitor that is entirely synthetic. The pKa of fluvastatin is 5.5, indicating it is a weak acid. At pH 7.0, its octanol/water partition coefficient is 20. Its water solubility at pH 6.0 is 2 g/L. Fluvastatin has two enantiomers due to the presence of two asymmetric centers in the side chain. Commercially available product is a racemic mixture of these enantiomers (Scripture, 2001). Almost all of the orally administered dose is absorbed (98%), but its absolute bioavailability is only around 20% to 30% due to first-pass hepatic metabolism. Fluvastatin has a volume of distribution of 0.35 L/kg and is highly bound to plasma proteins (> 99%). Fluvastatin is mainly metabolized by CYP2C9, but to a lesser extent by CYP3A4 and CYP2D6, and is eliminated in the bile and feces. The elimination half-life is 1.2 hours and total body clearance is 0.97 L/h/kg (Langtry, 1999; Plosker, 1996).

There are no studies evaluating the interaction of fluvastatin with grapefruit juice (Gazzerro, 2012). It has been suggested for use as an alternative to other statins that interact with grapefruit juice because no interaction has been reported (Bailey, 2013).

Rosuvastatin

Rosuvastatin is a synthetic HMG-CoA inhibitor. In addition to the statin-specific pharmacophore group in its structure, the hydrophilic methane sulfonamide group provides low lipophilicity. The log D value at pH 7.4 is -0.33. Rosuvastatin has an absolute bioavailability of 20%. Food decreases the absorption rate of rosuvastatin by 20%, but the extent of absorption is not affected. Rosuvastatin is used in daily doses of 5-40 mg. The C_{max} of 6.1 μg/L is reached 5 hours after a single oral 20 mg dose, while the C_{max} of 19–25 μg/L is reached 3-5 hours after a single oral 40 mg dose. The mean volume of distribution of rosuvastatin is 134 L and plasma protein binding is 88%. Rosuvastatin is not extensively metabolized in humans. *In vitro* studies have shown that CYP2C9 and 2C19 are primary metabolic enzymes. Its $t_{1/2}$ varies between 18-24 hours depending on age (White, 2002; Scott, 2004; Carswell, 2002).

Rosuvastatin has been shown to be a substrate of OATP1B1, 1B3, 2B1, and 1A2 in studies (Ho, 2006). There is no known interaction between rosuvastatin and grapefruit juice (Bailey, 2010). However, given the inhibitory effect of grapefruit juice on OATPs, such an interaction is possible.

CONCLUSION

According to the reviewed studies, flavonoids (naringin, naringenin) and furanocoumarins (bergamottin, dihydroxybergamottin) in grapefruit juice are the main components that cause drug interactions. Grapefruit juice-drug interactions occur in different ways. CYP3A4 inhibition is the most accepted and investigated mechanism. When drugs that are metabolized by this enzyme are given together with grapefruit juice, their plasma concentration values increase. Other mechanisms are inhibition of absorptive (OATPs) and efflux transporters (P-gp) that regulate drug absorption in the gut. When OATPs are inhibited, blood level and bioavailability of substrate drugs are reduced. On the other hand, P-gp inhibition increases the bioavailability of substrates. Statins are a class of drugs that have a well-known interaction with grapefruit juice. While most statins interact with grapefruit juice via CYP3A4 inhibition, no interaction has been demonstrated for CYP2C9 substrates, fluvastatin and rosuvastatin. Although CYP450 enzymes do not play an important role in the metab-

olism of pitavastatin and pravastatin, the interaction of these statins with grapefruit juice occurs through OATP and/or P-gp inhibition. Except for cerivastatin and pitavastatin, statins have a maximum oral bioavailability of 30%. Interactions of statins, which are CYP3A4 and/or P-gp substrates, with grapefruit juice may require dose adjustment as they will increase the bioavailability of drug. Conversely, bioavailability will be further reduced when statins, which are OATP substrates, are co-administered with grapefruit juice. Therefore, when statins and grapefruit juice are used together, the mechanism of interaction should be known and clarified.

The grapefruit juice-statin interaction is also clinically important and depend on many factors such as genetic polymorphism, variability of grapefruit juice components, and the patient's sensitivity to side effects. Long-term and/or use of large amount of grapefruit juice makes interactions more likely. Grapefruit juice enhances the effect of statins (especially atorvastatin, lovastatin, simvastatin) by increasing plasma drug levels. Thus, they further reduce LDL levels and the risk of ischemic heart disease. Although the risk of rhabdomyolysis, one of the most important side effects of statins, increases, this increase is not significant. The inhibitory effects of flavonoids and furanocoumarins can last for several hours. The interaction is minimized by giving statins and grapefruit juice at least 4 hours apart. Patients taking atorvastatin, lovastatin, or simvastatin should be informed to avoid or drink too much grapefruit juice. The interaction of pitavastatin and pravastatin with grapefruit juice is more limited, and there are no available studies showing the interaction for fluvastatin and rosuvastatin. Therefore, the possibility of interaction can be reduced by choosing statins (pravastatin, pitavastatin, fluvastatin, rosuvastatin) not metabolized primarily by CYP3A4.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Choose the subject (S.S.), literature search and preparation of the manuscript (M.A.), evaluation and final editing of the review (S.S.)

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