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Effect of *Spathodea campanulata* Ethanol Leaf Extract on Hematology and Liver Function of *Salmonella*-infected and Paracetamol-induced Swiss Albino Mice

Fred. Coolborn AKHARAIYI* , Arthur Chinedu OKAFOR**

Effect of Spathodea campanulata Ethanol Leaf Extract on Hematology and Liver Function of Salmonella-infected and Paracetamol-induced Swiss Albino Mice

Spathodea campanulata Yaprağı Etanol Ekstresinin Salmonella ile Enfekte Olan ve Parasetamol ile İndüklenmiş Swiss Albino Farelerin Hematolojisi ve Karaciğer Fonksiyonu Üzerine Etkisi

SUMMARY

Herbal remedies for healing is basically on the existing traditional methods, which is different from one tradition to the other. Liver performs useful functions that maintain health in humans but it can be affected to become malfunction if not guided or protected against some chemical substances contained in some foods, hard drugs and drinks. Effect on hematology and hepatoprotective activity of *Spathodea campanulata* ethanol leaf extract was studied using an animal model. Group I mice served as the positive control, group II mice as negative control, and groups III – XII mice as satellite groups which were treated with 200, 400, 800, 1000, and 2000 mg/kg of extract after respective *Salmonella typhi* infection and paracetamol inducement. Overdose of mice with paracetamol caused changes in the mice's physiology status. In hematology parameters of mice, red blood cell mean count was higher in the negative control (7.6 ± 70.92 million/ mm^3) than the positive control (4.36 ± 0.12 million/ mm^3) and lower white blood cells mean count of 3.50 ± 0.18 thousand/ mm^3 in the negative control than positive control with a value of 9.62 ± 0.39 thousand/ mm^3 . However, in biochemical evaluation, albumin (2.21 ± 0.60 mg/dL) and bilirubin (2.11 ± 0.63 mg/dL) were higher in the positive control than negative control with values of 4.90 ± 0.11 and 1.08 ± 0.10 mg/dL, respectively. These abnormalities in the mice's physiological status were reversed on treatment with extract concentrations of 200 to 2000 mg/mL for five days. *S. campanulata* ethanol leaf extract can be used as traditional medicine for the treatment of liver diseases.

Key Words: Liver function, *Spathodea campanulata*, paracetamol, *Salmonella typhi*.

ÖZ

Tedavi için bitkilerin kullanımı, temelde bir gelenekten diğerine farklı olmakla birlikte mevcut geleneksel yöntemlere dayanmaktadır. Karaciğer, insan sağlığını koruyan fonksiyonlarda görev alır, ancak bazı yiyeceklerde, içeceklerde ve ilaçlarda bulunan çeşitli kimyasal maddelere karşı karaciğerde hasar oluşabilir. *Spathodea campanulata* yapraklarının etanol ekstresinin hematoloji ve hepatoprotektif aktivitesi üzerindeki etkisi hayvan modeli kullanılarak incelenmiştir. Grup I fareler pozitif kontrol, grup II fareler negatif kontrol olarak ve grup III - XII fareler uydu grupları olarak kullanılmış, *Salmonella typhi* enfeksiyonu ve parasetamol indüksiyonundan sonra 200, 400, 800, 1000 ve 2000 mg/kg dozda ekstre ile uygulama yapılmıştır. Parasetamolün aşırı dozu, farelerin fizyolojik durumunda değişikliklere neden olmuştur. Farelerin hematoloji parametreleri incelendiğinde, ortalama kırmızı kan hücresi sayısı negatif kontrolde ($7,6 \pm 70,92$ milyon/ mm^3) pozitif kontrolden ($4,36 \pm 0,12$ milyon/ mm^3) daha yüksek ve ortalama beyaz kan hücresi sayısı negatif kontrolde ($3,50 \pm 0,18$ bin/ mm^3) pozitif kontrole göre ($9,62 \pm 0,39$ bin/ mm^3) daha düşük gözlenmiştir. Ancak biyokimyasal değerlendirmede albümin ($2,21 \pm 0,60$ mg/dL) ve bilirubin ($2,11 \pm 0,63$ mg/dL) negatif kontrole göre sırasıyla $4,90 \pm 0,11$ ve $1,08 \pm 0,10$ mg/dL değerlerinde daha yüksek bulunmuştur. Farelerdeki fizyolojik değişikliklerdeki bu anormallikler, beş gün süreyle 200-2000 mg/mL konsantrasyonlarda ekstre uygulaması ile tersine çevrilmiştir. *S. campanulata* yapraklarının etanolü ekstre, karaciğer hastalıklarının tedavisi için geleneksel ilaç olarak kullanılabilir.

Anahtar Kelimeler: Karaciğer fonksiyonu, *Spathodea campanulata*, parasetamol, *Salmonella typhi*.

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* ORCID: 0000-0001-5605-5543, Microbiology Department, Edo State University Uzairue, KM 7 Auchi-Abuja Road, Iyamho, Edo State, Nigeria

** ORCID: 0000-0002-6819-4724, Microbiology Department, Edo State University Uzairue, KM 7 Auchi-Abuja Road, Iyamho, Edo State, Nigeria

° Corresponding Author; Fred. Coolborn AKHARAIYI

Phone: +234 8066982772, e-mail: akharaiyi.fred@edouniversity.edu.ng

INTRODUCTION

The majority of urban and rural dwellers prioritize in managing diseases with the accessible and available herbal remedies within their localities. This type of practice is adaptable because herbal plants are effective in the management of diseases. It is certain that all over the world, there exist plants that produce defensive secondary metabolites against microbial infections. As reported by World Health Organization (WHO), herbalism is of high percentage in continents like Asia and Africa, where herbal medicines serve primary health care (Khan, 2016). The traditional medicine relied upon by a large population of the world is derived from plant materials (Deshwal, 2011; Arbab, 2016).

The liver is an organ that performs vital functions for the maintenance of health in humans. However, the food we consume and some environmental hazards do affect the liver to relieve or reduce its operations. Outside these, some chemicals such as anti-tuberculosis drugs, paracetamol, carbon tetrachloride (CCl_4), and alcohol have been found injurious to the liver in high dosage (Arbab, 2016). The setting in of such disorders in liver need medical attention as well as protection to avoid infection. Hepatic diseases are recognized by international public health, and there has not been a reliable and suitable drug for their cure. Based on this fact, the need to search and develop effective alternative drugs for liver protection requires urgent attention. For the fact that liver malfunction will reflect vividly in the biochemical physiology and hematological profiles, this study, therefore, was focused on how the plant remedy can be employed to manage or cure the liver infection.

Spathodea campanulata P. Beauv species belong to the Bignoniaceae family. Phytochemicals such as tannins, sterols, vanilic acid, verminoside, triterpenoids, ferulic acid, sterol spathodol, chlorogenic acid, maldivin, quercetin, caffeic acid, flavonoids, steroids, alkaloids, phenol, terpenoids, saponins, anthraquinones, phlobatannins have been identified from the plant (Ngouela, 2001; Akharaiyi, 2015). Report by Adriana et al. (2007) stated that preparations from stem bark and leaves of the plant have value in tradi-

tional medicine to treat inflammation, herpes, kidney diseases, diarrhea, antidote against animal poisoning, urethra inflammations, fungus skin diseases and stomachaches. Leaf extract of the plant has anti-plasmodial activity, anti-HIV, hypoglycemic, analgesic, antimalarial, and antioxidant properties (Niyonzima, 1999).

MATERIALS AND METHODS

Collection of plant samples

Apparently healthy leaves of *S. campanulata* were collected from a forest in Akure, Ondo State of Nigeria. The plant leaf was identified by Prof Oyun M. B. of Forestry and Wood Technology Department, Federal University of Technology, Akure, Ondo State, Nigeria, and the voucher specimen was deposited in the University herbarium with number AF 1508.

Leaf extract preparations

At room temperature of 25 ± 2 °C, leaves of *S. campanulata* were air-dried for seven days. The dried plant leaves were ground to smooth powder with a grinder (Thomas Wiley machine, model 5, USA). Two hundred grams (200 g) of the powder was extracted with 500 ml of ethanol at room temperature. The extract was concentrated in vacuo and stored in a refrigerator for use.

Acute toxicity test

With the criteria of WHO guideline for evaluation of efficacy and safety of herbal medicine (WHO, 2000) and Organization of Economic Co-operation and Development (OECD) guideline for testing chemicals (OECD, 2010), the extracts were tested *in vivo* for toxicity. Thirty-five mice of both sexes were purchased and quarantined for a week. Before the test, the mice were fasted for six h and divided into seven groups of five. Group 1 mice were each orally dosed with 10 ml/kg body weight of normal saline, while the mice in groups 2 – 7 were each dosed with 200, 500, 1000, 2000, 3000, and 4000 mg/kg body weight of extract, respectively. Toxic symptoms, according to the criteria of Lorke, (1983) were observed on the mice for 28 days, and Lethal Dose₅₀ (LD₅₀) of the quote was estimated by using the method of Miller and Tainter (1994). The LD cut-off of the quote was at 3000 mg/kg

body weight, and the therapeutic extract dose for this study was between 200 to 2000 mg/kg body weight. The performed experimental procedures on the animals were approved by Nigerian National Health Research Ethics Committee with the assigned number NHREC/08/2016.

Test bacteria species

Clinical *Salmonella typhi* was obtained from the research laboratory of the Microbiology Department, Federal University of Technology, Akure. The bacteria species was purified and cultured on Salmonella-Shigella Agar for confirmation of essential cultural characteristics. The pure isolate was Gram-stained and identified with biochemical tests for verification before stored on agar slant for use.

Experimental animals

Sixty Swiss albino mice of between 4 to 5 months with the body weight of between 23 to 35 grams were used. The mice were acclimatized for two weeks by feeding them with regular rat feed and water. After which, they were denied food for 18 h but with access to water only and conducted in compliance with the NIH guide.

Experimental design

Twelve groups of five mice each were conducted for the experiment. Group, I was allowed access to feed and water as the negative control. Group II mice were dosed with 1 g/kg body weight of paracetamol (positive control) three times daily for three days. Groups III-VII were each infected orally in a single dose per day for three days with 1 ml of 10^3 CFU/mL of *Salmonella typhi* and treated with 200, 400, 800, 1000, and 2000 mg/kg body weight of extract concentrations respectively for three days. Groups VIII-XII were orally dosed with 1 g/kg body weight of paracetamol in a single dose for 3 days and treated with 200, 400, 800, 1000, and 2000 mg/kg body weight of extract concentrations respectively for three days. After the experimental procedures, mice in each group were anesthetized, and the jugular vein of each mouse was cut with head held downwards and allowed to bleed into a vacutainer blood collection tube. The blood collection tubes were labeled according to groups of mice. For liver function test and histopa-

thology, the mice in each group were dissected, and liver tissues were collected for analysis.

Hematology of experimental mice

Red blood cells and white blood cells, leucocytes differential counts, neutrophil, monocyte, eosinophil, and lymphocyte differential counts were estimated by the criteria of Dacie and Lewis (2002) with the automated hematologic analyzer SYSMEX KX21 (SYSMEX Corporation, Japan), hemoglobin was evaluated with the use of Sahli's Hemoglobinometer by standard procedures according to the criteria of Wintrobe et al. (1961); D'Amour et al. (1967), albumin was by the technique of Doumas et al. (1971) total cholesterol by the technique of Abel et al. (1953), urea with the technique described by Fenech and Tommasini (1952), creatinine by the method of Lustgarten and Wenk, (1972) and total bilirubin by the process of Watson and Rogers (1961).

Histopathology of liver tissues

Mice from the controls, extract-treated, bacterial treated, and satellite groups, liver samples were collected and washed with normal saline. Small pieces of liver was cut from each treatment and dehydrated in grades of ethanol. Xylene was used to clear traces of ethanol and water from the tissues after dehydration before impregnating then in paraffin wax for 1 h at a controlled temperature of 60 °C. The tissues were after that embedded in molten paraffin wax and sectioned with a microtome (Bright, England) at 4 - 6 μ m. The sectioned tissues were floated in a water bath regulated at 35 °C and picked with slides previously robbed with egg albumin. The tissues were then de-waxed with xylene, hydrated, cleared with xylene, stained with hematoxylin and eosin, and mounted with Dibutylphthalate Polystyrene Xylene (DPX). The prepared slides were then allowed to dry and photographed, which were then observed with a binocular microscope for the level of damages or safety.

Statistical analysis

Obtained results from this study were expressed as Mean \pm SD. Differences were compared by One-way Analysis of Variance (ANOVA) and were followed by Dunnett's Multiple Comparison Test using SPSS version 16.

RESULTS AND DISCUSSION

Effect of *S. campanulata* on hematology of mice

In this study, a mice model was used to study the hepatotoxic and hepatoprotective effects of *S. campanulata* ethanol leaf extract at different concentrations. Before the administration of the quotes to the paracetamol-induced mice and bacteria-infected, the LD₅₀ of the plant extract was investigated and was found safe at 2800 mg/kg. Figure 1 represents the hematological profiles of positive control, negative control, bacterial infected, and paracetamol-induced (toxicant) mice and co-administered with the various extract concentrations (satellite). The red blood cells' mean count in the negative control was 7.6±70.92 million/mm³, which was higher than the positive control with a value of 4.36±0.12 million/ mm³. A lower WBC means count of 3.50±0.18 thousand/ mm³ in negative control than the positive control group (9.62±0.39 thousand/ mm³) was recorded. Also, a higher value above the permissible level (11-19%) was obtained in the hemoglobin of the positive control (20.11%) than the negative control (11.50%).

The plant extract concentrations of between 200 - 2000 mg/mL administered to groups of mice were to ascertain any physiological change that might result from the plant toxin. However, the results obtained suggest that *S. campanulata* ethanol leaf extract was not toxic to the mice at the evaluated concentrations. Also, the physiological changes exerted on the hematology profile of the mice due to bacterial infection, and paracetamol toxicity were improved by the extract's potency on dose dependent. Groups III-VII was proposed; hence drug is not administered without illness. The leaf extract of *S. campanulata* has been found to possess antimicrobial activity, and to ascertain its non-effect on organs for safe use in ailments, *S. typhi* infection was allowed to manifest illness in the mice. Outside it that physiological changes in mice were compared with the negative and positive control groups, the hematological, biochemical, and histopathological evaluations hold it safe for use; hence organs such as kidney and intestine were as well protected.

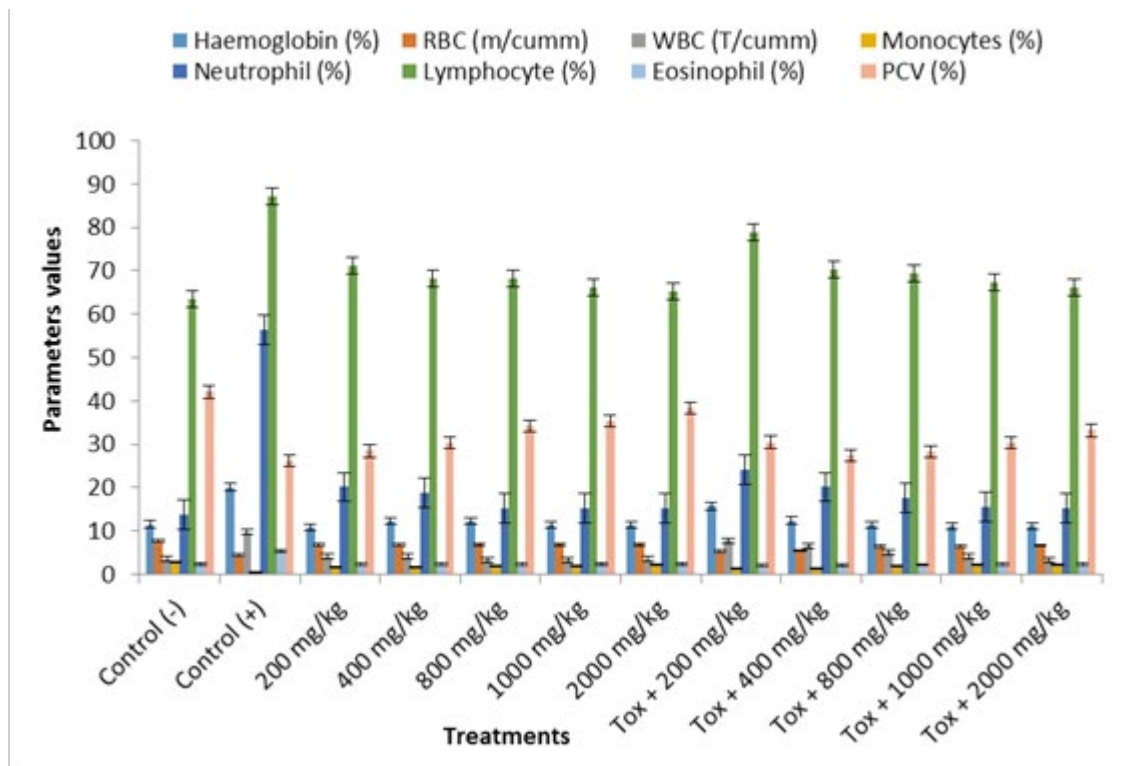


Figure 1. Effect of ethanol leaf extract of *S. campanulata* on hematology of albino mice

Effect of *S. campanulata* on biochemical in liver function

The liver is a multipurpose organ that helps to break down toxins in the body; therefore its damage by any hepatotoxic agent is of necessary consequence (Ansah, 2013). *S. campanulata* ethanol leaf extract effectiveness in biochemical markers to ascertain safety and toxicity of its therapeutic use is presented in Table 1. Total protein was 7.15 ± 0.45 and 5.18 ± 0.60 g/dL, respectively for, negative and positive controls. This significant decrease in the positive control mice suggests depletion of protein which is having the potential of increasing hepatic damage that can result in severe liver necrosis. However, the rise in total protein contents in the groups treated with 200-2000 mg/kg^{bw} of the extract was 6.34 ± 0.40 , 6.48 ± 0.28 , 6.53 ± 0.28 , 6.56 ± 0.26 , and 6.61 ± 0.34 g/dL respectively. This observed result suggests improvement in total protein alongside extract concentrations. Also observed in the satellite groups was the increase in protein towards normalcy alongside extract concentrations with values of 4.78 ± 0.03 , 5.22 ± 0.14 , 5.38 ± 0.24 , 5.42 ± 0.06 , and 5.61 ± 0.30 g/dL in the toxicant treated and co-administered with 200-2000 mg/kg^{bw} of extract. Treatment with the plant extracts protected protein depletion initiated by paracetamol toxins in the mice and was confirmed by the observed gradual increase in protein level. This could be possible because of the essential phytochemicals present in the leaf extract. Bhadauria et al. (2007) have reported that the presence of various flavonoids and esters present in plants might be responsible for the stimulation of protein biosynthesis.

Urea level in the biochemical marker of the liver function of mice treated with the extracts was 18.15 ± 1.50 mg/dL in the negative control and 23.40 ± 0.16 mg/dL in the positive control. These results emphasized adverse impact on the liver with the induced paracetamol and positive effects on health status with regular rat meal and water. Recorded result in the treated mice with 200 - 2000 mg/kg^{bw} of

the extract was in decreasing order of 22.65 ± 0.42 , 22.44 ± 0.23 , 21.60 ± 0.28 , 21.43 ± 1.18 , and 21.24 ± 1.03 mg/dL, respectively. In the satellite mice groups induced with toxicant and co-administered with extracts, an increase in urea values alongside extract concentrations from 26.24 ± 0.46 - 22.18 ± 0.02 mg/dL was observed. This observation definitely illustrates urea reduction in circulation with the extract treatments. A decrease in values to normalcy, as suggested by the results obtained from the negative control, was also observed in the uric acid, creatinine, and cholesterol parameters. So from all observations of the biochemical parameters involved in the liver function of the mice with the leaf extract, indicates recovery of the injured liver from 200 mg/mL extract concentration. The wide difference in these values exists between the satellite groups of mice, where values of between 200 - 2000 mg/kg^{bw} of the extract are 59.56 ± 1.68 , 56.00 ± 3.64 , 56.06 ± 1.16 , 55.04 ± 1.75 , and 55.68 ± 2.65 U/L, respectively. Despite this, the effectiveness of the quote on liver recovery from the sustained injuries imposed by paracetamol was evidenced in the decreased values alongside extract concentrations when compared with the positive control value. A similar trend of decrease in values to normal condition in the release of bilirubin into circulation to predict of non-toxicity of the extract even at 2000 mg/mL concentration was magnificent. Total albumin was found higher in the negative control (4.90 ± 0.11 g/dL) than the positive control (2.21 ± 0.60 g/dL).

While the results of bacteria/extract-treated mice ranged from 2.14 ± 0.25 g/dL in the low dose of 200 mg/kg^{bw} of extract to 3.88 ± 0.09 g/dL in the medium dose of 400 mg/kg^{bw} extract, it was 4.28 ± 0.27 g/dL in the high amount of 800 mg/kg^{bw} of extract and 4.55 ± 0.36 g/dL in the overdose of 2000 mg/kg^{bw} of extract. However, the decrease in value towards meeting up with the negative value with extract concentrations as observed in total protein, urea, and total albumin, was also the order in bilirubin, uric acid, creatinine, and cholesterol levels. Paracetamol administration at overdose to the mice initiated increase in cholesterol,

bilirubin, urea, uric, acid and creatinine levels. On the other hand, the lower values in protein and total albumin observed in the negative group are promising signs of liver function and integrity. The significant increase in the paracetamol-induced mice values in cholesterol, bilirubin, urea, uric acid, and creatinine levels indicated various damages such as hepatic, myocardial, and renal damage and; are responsible for skeletal muscle alteration (Mada, 2014). The ability of overdose with paracetamol to effect damages on liver tissues is connected with the observed varieties of alteration in hematological parameters; and indeed of sufficient injuries to manifest intracellular constituents into circulation. The amount of these in circulation as recorded is a prediction of hepatocellular damages. Stabilization activities of the hematological parameters investigated with the treatment of the plant extract concentrations manifested distinct improvement in the functional status of liver cells. They could be due to free radical scavenging action of the extract. Bilirubin, in its nature, is toxic and was recorded in circulation as a result of a breakdown of hemoglobin. This bilirubin in circulation, would have

been carried to the liver for detoxification and excretion but was unable to bind to albumin as its function was also adversely affected by the toxicant. Gagliano et al. (2007), have reported that overdose of paracetamol elicits injury to hepatic parenchyma that will cause a high increase of bilirubin in circulation. The extracts of *S. campanulata* prevented the severity of liver damage caused by paracetamol, as evidenced by the low level of bilirubin in the serum. A similar result was reported by Panchal et al. (2013). Albumin level was shallow; hence the liver that produces it was defective. The low level of bilirubin in the extract-treated mice, further confirmed that the damages observed in the positive control mice were as a result of a high dosage of paracetamol. The results obtained in this study is following with Gowda et al. (2010), whose report stated that biochemical markers play an important role in accurate diagnosis and also for assessing risk and adopting therapy that improves clinical outcomes and also with Mohamed et al. (2010), who stated that popularity of herbal remedies is increasing globally and at least one-quarter of patients with liver disease use ethnobotanicals.

Table 1. Effect of ethanol leaf extract of *S. campanulata* on biochemical parameters in liver functions of albino mice.

Group	Bilirubin (mg/dL)	Total albumin (mg/dL)	Total protein (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)
Control (-)	1.08±0.10	4.90±0.11	7.15±0.45	18.15±1.50	6.05±0.60	1.38±0.30	113.70±0.50
Control (+)	2.11±0.63	2.21±0.60	5.18±0.60	23.40±0.16	8.02±1.10	1.96±0.09	218.14±0.64
200mg/kg ^{bw}	1.65±0.10	2.14±0.25	6.34±0.41	22.65±0.42	8.36±0.14	1.78±0.44	156.03±6.05
400mg/kg ^{bw}	1.56±0.34	3.88±0.34	6.48±0.28	22.44±0.23	8.22±0.63	1.75±0.18	151.42±1.15
800mg/kg ^{bw}	1.35±0.18	4.28±0.27	6.53±0.28	21.60±0.28	7.30±1.07	1.62±0.65	142.13±0.47
1000mg/kg ^{bw}	1.26±0.21	4.35±0.16	6.56±0.26	21.43±1.18	7.21±0.13	1.51±0.18	133.21±0.13
2000mg/kg ^{bw}	1.12±0.03	4.55±0.36	6.61±0.34	21.24±1.03	6.18±0.45	1.47±0.28	130.16±0.18
TOX+200mg/kg ^{bw}	1.45±0.26	2.84±0.26	4.78±0.03	26.24±0.45	8.40±0.23	2.40±0.14	222.43±0.56
TOX+400mg/kg ^{bw}	1.37±0.44	2.88±0.34	5.22±0.14	26.18±0.21	8.26±0.26	2.28±1.03	163.07±1.13
TOX+800mg/kg ^{bw}	1.28±0.31	3.72±0.18	5.38±0.26	25.31±0.17	7.48±1.17	1.66±0.24	157.14±0.84
TOX+1000mg/kg ^{bw}	1.20±0.11	3.60±0.18	5.42±0.16	23.43±0.11	6.40±0.12	1.53±0.33	130.21±1.63
TOX.+2000g/kg ^{bw}	1.15±0.40	3.65±0.09	5.61±0.30	22.18±0.02	6.33±0.27	1.50±0.26	125.08±1.13

Legend: Tox = Toxicant (paracetamol) co-treated with extract concentrations

Histopathology of experimental mice liver

The histopathological sections of the liver tissue as seen under the light microscope on the bacteria/extract-treated mice, the positive, negative controls, and the satellite-co-administered with extracts treatment are shown in Figures 2a and 2b.

Observed from the negative control mice are no distortion. The liver cells appeared normal in shape with a prominent nucleus, a precise central vein with typical architectural structure, and a well-preserved cytoplasm. Hepatic cells were arranged in cord-like fashion, which is well separated by sinusoids. The mice dosed with 1g/kg of paracetamol (positive group), has severe hepatocellular degeneration. The representative of liver mice infected with *S. typhi* and co-treated with 200 mg/kg body weight of extract showed no adverse effects in the liver except a hepatocellular necrosis. In the liver of mice induced with paracetamol and co-administered with 200 mg/kg body weight of extract concentrations, recovery from damages was observed as sinusoids, hepatocellular necrosis, portal vein, and dented hepatic sinusoid were seen. The kidney and intestine; sectioned tissues of mice infected with *S. typhi* and co-administered with the plant ex-

tract concentrations are presented in Figure 3.

Evidence of recovery from distortion of the organs were observed in the sectioned kidney and intestine; after treatment. However, the kidney and intestine; sectioned tissues of mice during bacterial infection had no distortions, but distortions were observed in the biochemical and hematology parameters evaluated. The administered concentrations of the plant extracts of between 200-2000 mg/kg, were found not toxic based on values obtained, which were not significantly different from the negative control values. However, they were also found to reduce the elevated importance of hematological parameters in the satellite mice groups towards normality. Activities of the plant extract concentrations in hepatoprotective could be dependent on the antioxidant values of free radical scavenging efficacy of the plant extract (Akharaiyi, 2015). Several plant chemicals have been shown to act synergistically as antioxidants. In this study with *S. campanulata* ethanol extract, it further strengthened the report that without reliable modern drug to manage liver protection, herbs can serve as an alternative to solve liver problems (Buraimoh, 2010; Arifianti, 2020).

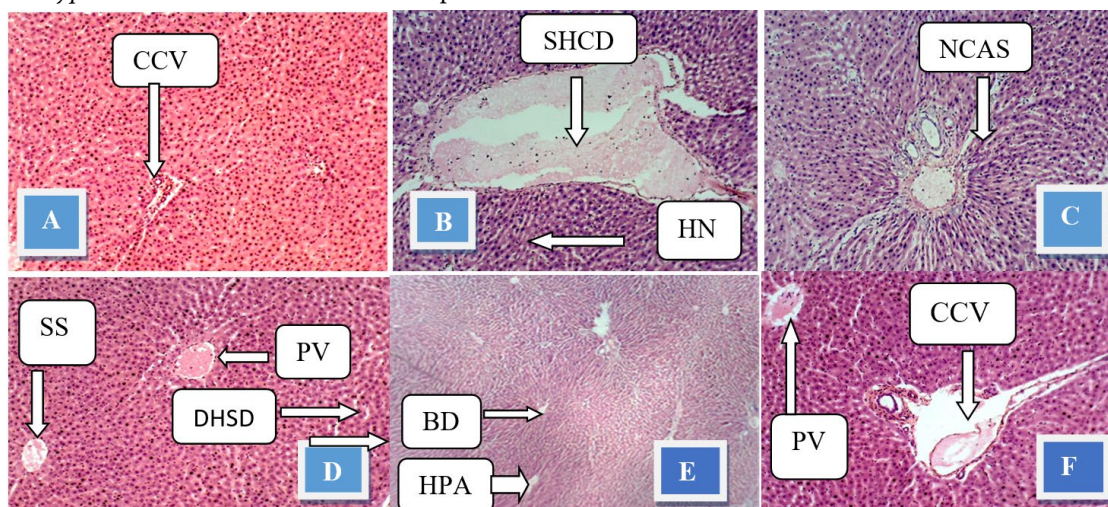


Figure 2a. Histopathological sections of the liver. (A – Mice fed with regular diet and water (-v control), (B - Mice dosed with 1 g/kg body weight of paracetamol without treatment (+v control), (C - Mice infected with bacteria and treated with 200 mg/kg body weight of leaf extracts), (D - Paracetamol induced and co-administered with 200 mg/kg body weight of extract), (E- Mice infected with bacteria and treated with 400 mg/kg body weight of leaf extracts), (F - Paracetamol induced and co-administered with 400 mg/kg body weight of extract).

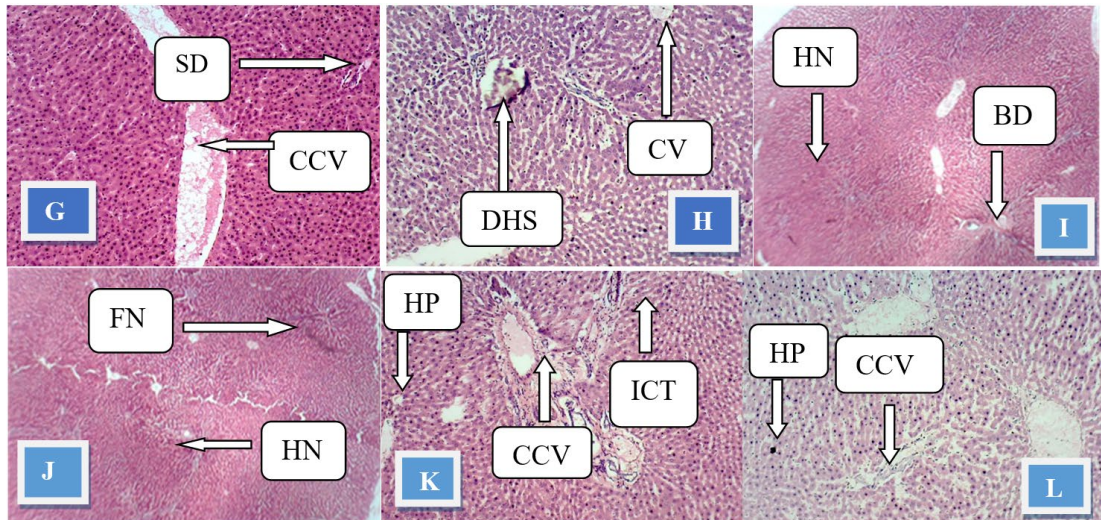


Figure 2b. Histopathological section of the liver. (G - Mice infected with bacteria and treated with 800 mg/kg body weight of leaf extracts), (H - Paracetamol induced and co-administered with 800 mg/kg body weight of extract), (I - Mice infected with bacteria and treated with 1000 mg/kg body weight of leaf extracts), (J - Paracetamol induced and co-administered with 1000 mg/kg body weight of extract), (K- Mice infected with bacteria and treated with 2000 mg/kg body weight of leaf extracts), (L - Paracetamol induced and co-administered with 2000 mg/kg body weight of extract).

Legend: Clear central vein (CCV), Hepatocellular necrosis (HN), Sinusoids (SS), dented hepatic sinusoid (DHSD), Portal vein (PV), Normal cellular architecture (NCAS), Severe Hepatocellular Degeneration (SHCD), Bile duct (BD), Interlobular connective tissue (ICT), Hepatocytes (HP), Central vein (CV), Dented hepatic sinusoid (DHS), Sinusoids (SD), Focal necrosis (FN) and Hepatic artery (HPA)

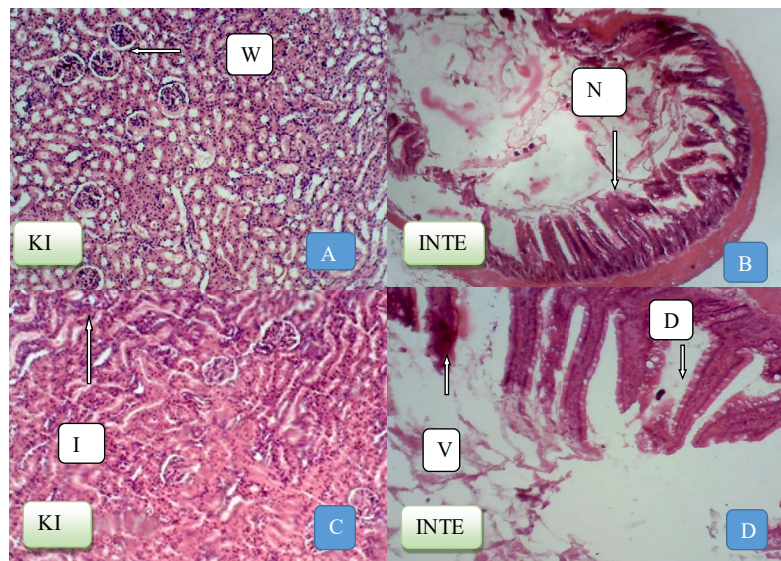


Figure 3. Histopathological sections of Kidney and intestine; of mice (A= Kidney of mice after treatment from *S. typhi* infection, B = Intestine of mice after treatment from *S. typhi* infection, C = Kidney of mice with *S. typhi* infection, D = Intestine of mice with *S. typhi* infection).

Legend: Well structure glomeruli (WSG), Normal villi architectural structure (NVAS), interstitial space (IS), Villi inflammation (VIF), distorted villi structure (DVS).

CONCLUSION

On inducing some mice with an overdose of paracetamol, specific negative changes for liver mal-function were observed. Also, changes from negativity to normality resulting from healing or injury recovery were noticed on a group of mice pretreated (satellite) with the plant extract at varying concentrations. The evaluation of these comparisons was possible because of the negative control group of mice which were given regular mice feed and water only. Most of the pathological changes observed in the mice liver include fibrosis around the central vein and sinusoids which were reduced by the plant extract concentrations in the satellite group of mice. The hepatoprotective effects of *S. campanulata* ethanol leaf extracts highlight their unquestionable antioxidant properties. In the hematological assay, higher values in positive control than negative control was observed in bilirubin and total protein. The rise in these parameters signifies less functional activity of the liver. Apart from the increase in bilirubin and total protein, specific adverse effects were also observed in the urea, uric acid, creatinine, and cholesterol. These alterations indicated skeletal muscle. The hepatoprotective effects of the extract of *S. campanulata* against paracetamol-induced toxicity in this study were further confirmed by the histopathological studies.

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CONFLICT OF INTEREST

There is no conflict of interest among authors of this manuscript.

AUTHOR CONTRIBUTION STATEMENT

Developing the hypothesis, experimentation, first draft of the text, analysis, and interpretation of results (Akharaiyi, F. C.), literature search, statistics, and assistant in the research experimentation (Okafor, A. C.), reviewing the text and approval of the final man-

uscript before sending out for publication (Akharaiyi, F. C., Okafor, A. C.).

REFERENCES

- Abel L. L., Levy B. B., Brodie B. B., Kendall, F. E. (1953). A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *Journal of Biology and Chemistry*, 195(1), 357-66.
- Pianaro, A., Pinto, J. P., Ferreira, D. T., Ishikawa, N. K., Braz-Filho, R. (2007). Iridoidglucoside and antifungal phenoilic compounds from *Spathodea campanulata* roots. Iridoidglucoside and antifungal phenoilic compounds from *Spathodea campanulata* roots. *Ciências Agrárias, Londrin*, 28(2), 251-256.
- Akharaiyi F. C, Boboye B. E., Akpambang V. O., Ade-tuyi F. C. (2015). Phytochemical and antioxidant effect of *Spathodea campanulata* leaf extracts. *International Journal of Biochemistry Research and Review*, 7(3), 148-159. doi: 10.9734/IJB-cRR/2015/16371
- Ansah C, Dadzeasah P. E., Asiamah E. (2013). Aqueous stem bark extract of *Spathodea campanulata* (P. Beauv) modulates carbon tetrachloride induced hepatic damage in rats. *American Journal of Pharmacology and Toxicology*, 8(1), 39-50 doi: 10.3844/ajptsp.2013.39.50
- Arbab A. H, Parvez M. K, Dosari M. S, Rehaily A. J, Ibrahim K. E, Alam P. (2016). Therapeutic efficacy of ethanolic extract of *Aerva javanica* aerial parts in the amelioration of CCl₄- induced hepatotoxicity and oxidative damage in rats. *Food Nutrition Research*. 60:30864. <https://doi.org/10.3402/fnr.v60.30864>
- Arifianti, Lusiana., Sukardiman, Sukardiman., Indriyanti, Niken., Widjowati, Retno. (2020). Anti-cancer property of *orthosiphon stamineus* benth. Extracts in different solvent systems against t47d human breast cancer cell lines. *FABAD Journal of Pharmaceutical Sciences*, 45, 3, 187-194
- Bhadauria, M., Satendra, N., Sanyam, S. (2007). Hepatoprotective efficacy of propolis extract: A biochemical and histopathological approach. *Irani Journal of Parmacology and Therapeutics*, 6(2), 145-154.

- Buraimoh, A. A. Bako, I. G., Ibrahim, F. B. (2010). Hepatoprotective effect of ethanolic leaves extract of *Moringa oleifera* on the histology of paracetamol induced liver damage in Wistar rats. *International journal of Animal and Veterinary Advances*, 3(1), 10-13
- Dacie, J. V., Lewis, S.M. (2002). *Practical Haematology*. 11th Edn., Elsevier, London, UK; pp: 380-382.
- D'Armour, F. E., Blood, F. R., Belden, D. A. (1965). *The Manual for Laboratory Work in Mammalian Physiology*. 3rd ed. Illinois Chicago, The University of Chicago Press, 4-6.
- Deshwal N, Sharma A. K., Sharma P. (2011). Review on hepatoprotective plants. *International Journal of Pharmaceutical Sciences Review and Research*, 7, 15-26.
- Doumas, B. T., Watson, W. A., Biggs H. G. (1971). Albumin standards and the measurement of serum albumin with bromocresol green. *Clinica Chimica Acta*, 31(1), 87-96. doi: 10.1016/s0009-8981(96)06447-9
- Fenech, G., Tommasini A. (1952). Method of colorimetric determination of urea. *Bollettino Chimico Farmaceutico*, 91(10), 391-395.
- Gagliano, N., Grizzi, F., Annoni G. (2007). Mechanism of aging and liver functions. *Digest Discovery Science*, 25, 118-123. doi: 10.1159/000099475
- Gowda, S., Desai, P. B., Kulkarni, S. S., Hull, V. V., Math, A. A. K., Vernekar, S. N. (2010). Markers of renal function tests. *North American Journal of Medical Science*, 2(4), 170-173.
- Lorke, D. A (1983). new approach to acute toxicity testing. *Architect Toxicology*. 54, 275-287. doi: 10.1007/bf01234480
- Lustgarten, J. A, Wenk R. E. (1972). Simple, rapid, kinetic method for serum creatinine measurement. *Clinical Chemistry*, 18(11), 1419-1422.
- Khan, M. Z., Jogeza, N., Tareen, J. K., M., Imran Shabbir. M., Malik, M. A., Khan, A. R. (2016). Compilation on medicinal plants with hepatoprotective activity. *SRA Medical Journal*, 8(3), 196-202
- Mada, S. B., Inuwa, H. M., Abarshi, M. M., Mohammed, H. A., Aliyu, A. (2014). Hepatoprotective effect of *Momordica charantia* extract against CCl₄ induced liver damage in rats. *British Journal of Pharmaceutical Research*, 4(3):368-380. doi: 10.9734/BJPR/2014/4885
- Mohamed, A. A., Khalil, A. A., El-Beltagi, H. E. S. (2010). Antioxidant and antimicrobial properties of kaff maryam (*Anastatica hierochuntica*) and doum palm (*Hyphaene thebaica*). *Grasas Y Aceites*. 61(1):67-75. doi: 10.3989/gya.064509
- Miller, L. C., Tainter M. C. (1994). Estimation of LD50 and its error by means of logarithmic-probit graph paper. *Proceeding of the Society of Experiment Biology and Medicine*, 57, 261-264.
- Ngouela, S., Tsamo, E., Sondengam, B. L., Connolly, J. D. (2001). Spathodol a new polyhydroxysterol from the leaves of *Spathodea campanulata*. *Journal of Natural Products*, 54(3), 873-876.
- Niyonzima, G., Laekeman, G., Witvrouw, M., Van Poel, B., Pieters, L., Paper, D., De Clercq, E., Franz, G., Vietinck, A. J. (1999). Hypoglycemic, anticomplement and anti-HIV activities *Spathodea campanulata* stem bark. *Phytomedicine*, 6(1), 45-49. doi: 10.1016/S0944-7113(99)80034-8
- Panchal, C. V., Jyotiram, A., Sawale, B., Poul, N. Khandelwal, K. R. (2013). Hepatoprotective activity of *Lageneria sicerarla* (MOLINA) Standley fruits against paracetamol induced hapatotoxicity in mice. *International Journal of Pharmaceutical Science Research*, 4(1), 371-377. doi: 10.13040/IJPSR.0975-8232.4(1).371-77
- The Organization of Economic Cooperation and Development (OECD). (2010). The OECD guideline for testing of chemical: 420 Acute Oral Toxicity. France.
- Watson, D., Rogers, J. A. (1961). A study of six representative methods of plasma bilirubin analysis. *Journal of Clinical Pathology*, 14, 271-278.
- Wintrobe, M. M., Lee, G. R., Boggs, D. R., Bithel, T. C., Athens, J. W., Foerester J. (1961). *Clinical Hematology*, 5th ed. Philadelphia, Les and Febiger, p.326.
- World Health Organization (2000). General guidelines for methodologies on research and evaluation of traditional medicine. Switzerland.

Synthesis, Characterization and *In Vitro* Evaluation for Antimicrobial and Anthelmintic Activity of Novel Benzimidazole Substituted 1,3,4-Thiadiazole Schiff's Bases

Saravanan KALIYAPERUMAL* , Priyabrata PATTANAYAK**^o

Synthesis, Characterization and In Vitro Evaluation for Antimicrobial and Anthelmintic Activity of Novel Benzimidazole Substituted 1,3,4-Thiadiazole Schiff's Bases

Yeni Benzimidazol Süstitüe 1,3,4-Tiyadiazol Schiff Bazlarının Sentezi, Karakterizasyonu ve Antimikrobiyal ve Anthelmintik Aktivitesinin In Vitro Değerlendirmesi

SUMMARY

Benzimidazoles, 1,3,4-Thiadiazoles, and Schiff bases have shown many properties against different types of diseases, including bacterial infection and helminthiasis. Because of the need for new antimicrobial and anthelmintic agents, novel benzimidazole substituted 1,3,4-thiadiazole Schiff's bases were designed and synthesized. The synergy arising from the successful incorporation of benzimidazole ring, thiadiazole ring, and Schiff's base in one pharmacophore was exploited in this work. Eleven such derivatives were synthesized and investigated for their *in vitro* antimicrobial and anthelmintic properties. 1H-benzo[d]imidazole-2-carboxylic acid was first prepared by the oxidation of 2-methyl-1H-benzo[d]imidazole with alkaline potassium permanganate. 1H-benzo[d]imidazole-2-carboxylic acid was then converted to N-arylidene-5-(1H-benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-amine by reacting with an aqueous solution of thiosemicarbazide in the presence of few drops of concentrated sulphuric acid. Finally, different benzimidazole substituted 1,3,4-thiadiazole Schiff's bases were prepared by reacting thiadiazole substituted benzimidazole with suitable aryl aldehyde. Compound PP-4 was found to be more potent than the standard drug in causing the death of nematodes, which took an average time of 13.22 and 19.00 min against *Perionyx excavatus* and *Pheretima posthuma*, respectively. Compounds PP-4, PP-6, and PP-8 containing electron-withdrawing groups (4-nitro, 2-bromo, 4-chloro) exhibited antimicrobial activity with the zone of inhibition ranging from 8-27 mm comparable to Ampicillin with the value ranging from 22-27 mm for all the tested strains.

Key Words: Schiff base, Benzimidazole, 1,3,4-Thiadiazole, Anthelmintic activity, Helminthiasis, Antibacterial

ÖZ

Benzimidazoller, 1,3,4-Tiyadiazoller ve Schiff bazları çeşitli bakteriyel enfeksiyonlar ve helmintiyazis gibi hastalıklara karşı farklı özelliklere göstermektedir. Yeni antimikrobiyal ve antihelmintik bileşiklere duyulan ihtiyaç göz önüne alınarak yeni benzimidazol-süstitüe 1,3,4-tiyadiazol Schiff bazları tasarlanmış ve sentezlenmiştir. Bu araştırmada benzimidazol halkası, tiyadiazol halkası ve Schiff bazı farmakoforlarının başarılı bir şekilde bir araya gelmesiyle elde edilecek sinerjiden yararlanılması planlanmıştır. Bu amaçla, 11 türev sentezlenip, *in vitro* antimikrobiyal ve antihelmintik özellikleri açısından araştırılmıştır. Öncelikle, 1H-benzo[d]imidazol-2-karboksilik asit, 2-metil-1H-benzo[d]imidazolün alkali potasyum permanganat ile oksidasyonu ile hazırlanmıştır. Daha sonra, 1H-benzo[d]imidazol-2-karboksilik asit birkaç damla konsantre sülfürik asit varlığında tiyosemikarbazidin sulu çözeltisi ile muamele edilerek N-ariliden-5-(1H-benzo[d]imidazol-2-yl)-1,3,4-tiyadiazol-2-amin'e dönüştürülmüştür. Son olarak, farklı benzimidazol-süstitüe Schiff bazları uygun arilaldehit ile tiyadiazol-süstitüe benzimidazol halkasının reaksiyonu ile hazırlanmıştır. Bileşik PP-4'ün *Perionyx excavatus* and *Perionyx posthuma*'ya karşı nematodları öldürme etkisinin, sırasıyla 13.22 ve 19.00 dakika süreler ile standart ilaçtan daha güçlü olduğu bulunmuştur. Elektron çekici gruplar (4-nitro, 2-bromo, 4-kloro) içeren PP-4, PP-6 ve PP-8 bileşikleri tüm suşlarda 8-27 mm inhibisyon alanı ile 22-27 mm inhibisyon değeri gösteren Ampisilin ile karşılaştırılabilir antimikrobiyal aktivite sergilemiştir.

Anahtar Kelimeler: Schiff bazı, Benzimidazol, 1,3,4-Tiyadiazol, Antihelmintik aktivite, Helmintiyazis, Antibakteriyel

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* ORCID: 0000-0002-8859-8099, Department of Pharmacy, Bhagwant University, Sikar Road, Ajmer, Rajasthan, India, 305004.

** ORCID: 0000-0003-4035-1182, Department of Pharmacy, Bhagwant University, Sikar Road, Ajmer, Rajasthan, India, 305004.

^o Corresponding Author; Priyabrata Pattanayak
Phone: +91 9438269361, e-mail: Priyabrata2005@gmail.com

INTRODUCTION

Antimicrobial resistance (AMR) to antibiotics is one of the global threats to public health standards, and it has reduced the efficacy of antibacterial drugs, making the treatment of patients difficult, costly, or even impossible. This raises the need to search for new potent antimicrobial agents with reduced resistance to pathogens and having a broad spectrum of biological activity. On the other side, infections with parasitic helminths are important causes of morbidity and mortality globally. Anthelmintics are anti-parasitic drugs that expel parasitic worms from the human body without causing significant damage to the host. Resistance to benzimidazoles used to treat helminthiasis, has been reported. There are genetic factors in parasitic helminths that favor the development of anthelmintic resistance (Ahn et al., 1993). Frequent usage of the same group of anthelmintic drugs, using anthelmintics in sub-optimal doses, prophylactic mass treatment of domestic animals, and continuous use of a single drug has contributed to the overall development of anthelmintic resistance (El-Zemity et al., 2006).

In the last few decades, the chemistry of five-membered heterocyclics like 1, 3, 4 - thiadiazole and five - membered fused heterocyclics like benzimidazoles are reported to show a wide spectrum of biological activity. Benzimidazoles like albendazole, thiabendazole, and flubendazole (anthelmintic) (Figure 1), omeprazole, and lansoprazole (anti ulcerative), and astemizole (antihistaminic) are in use. The chemistry and pharmacology of benzimidazole have been of great interest to medicinal chemist because its derivatives possessed various biological activities such as antioxidant (Gurer-Orhan et al., 2006), antimicrobial (Ozkay et al., 2010), anticancer (Zienab et al., 2011), antihypertensive (Kumar et al., 2006), anti-inflammatory (Lazer et al., 1987), analgesic (Achar et al., 2010), antiprotozoal (Katiya et al., 1994), anti-hepatitis (Li et al., 2006), antiulcer (Cho et al., 2001), antifungal (Sanja et al., 2007) and anticonvulsant activity (Shingalpur et al., 2010). Apart from the above activities, benzimidazole derivatives also have reported anthelmintic activities (Mahama et al., 2011; Beatriz et al., 2013; Faruk et al., 2014; Katti et al., 2019).

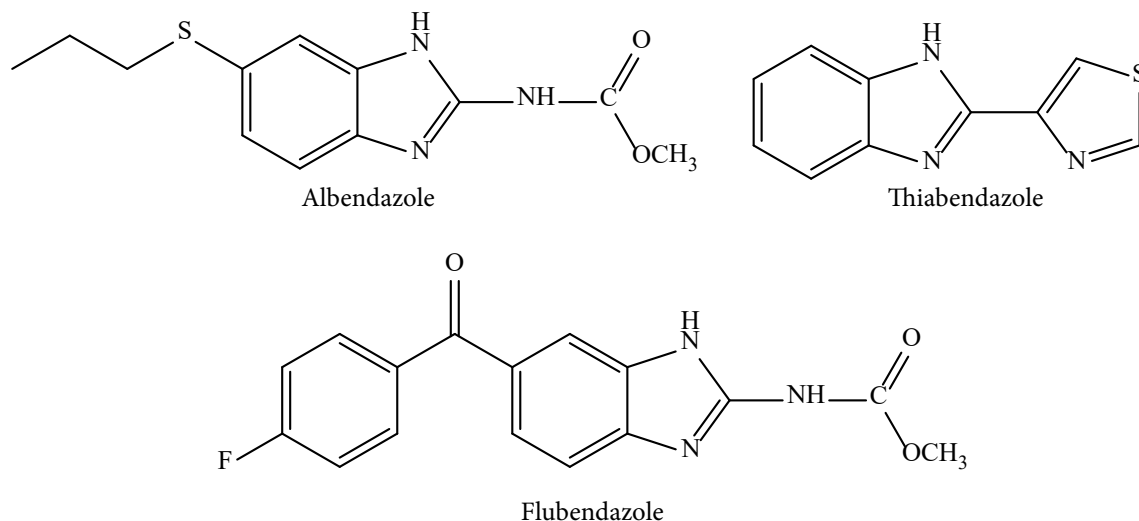


Figure 1. The structure of some marketed benzimidazole anthelmintics

The 1,3,4-thiadiazole pharmacophore has extensive pharmacological significance. The derivatives of 1,3,4- thiadiazole possess an array of biological activities due to azomethine linkage. A variety of 1,3,4-thiadiazole is in use, like Acetazolamide (diuretic), Cefazolin, Cefazedone (antibiotics), Megazol (antiprotozoal), Timolol maleate (NSAIDs), Methazolamide

(carbonic anhydrase inhibitor), and Sulphamethizole (antibacterial). 1,3,4-thiadiazole derivatives also have reported anthelmintic activities (Marin et al., 1992; Somnath et al., 2015).

Schiff's bases are an important class of organic compounds with imine or azomethine ($-C=N-$)

functional group. Schiff's bases are reported to possess a wide variety of pharmacological actions, including potential anti-inflammatory, antibacterial, antitubercular, antiviral, anticonvulsant, and anthelmintic activities. It was proposed that a wide spectrum of biological activities of Schiff's bases could be due to interaction of nitrogen atom of azomethine with the active centers of cell constituents by forming a hydrogen bond, and thus, it interferes in normal cell processes (Venugopala et al., 2003). Schiff's bases also reported as a promising anthelmintic (Rao et al., 2014; Varshney et al., 2014; Reddy and Kumar 2014; Husaina et al., 2018; Satyajit, 2011; Balaji et al., 2017) with some derivatives possessing an activity better than that of albendazole and piperazine citrate.

Because of the need for new antibacterial and anthelmintic agents, novel benzimidazole substituted 1,3,4-thiadiazole Schiff's bases were designed and synthesized. The synergy arising from the successful incorporation of benzimidazole ring, thiadiazole ring, and Schiff's base pharmacophore was exploited in this research. Since the individual pharmacophores have antibacterial and anthelmintic activity, it was expected that, their successful incorporation in one molecule would improve the antibacterial and anthelmintic activity of the compound.

MATERIALS AND METHODS

General

All the chemicals and reagents used in this study are purchased from Himedia, Fischer & Merck chemicals and are self-funded. The melting points for the compounds were determined in an open glass capillary using a Kjeldahl flask containing paraffin and are uncorrected. All the synthesized compounds were characterized by CHN (Carbon, Hydrogen, and Nitrogen) analysis, IR spectral data, ¹H NMR, and some selected compounds were characterized by mass spectroscopy. The IR spectra were recorded using analytical technologies FT-IR spectrophotometer 2202. ¹H-NMR spectra was recorded on Bruker 300 MHz in DMSO. Mass spectra were scanned on Bruker MICR QTOF-QII, ESI mass spectrophotometer. C, H, N elemental analyses were recorded on Heraeus CHN rapid analyzer, and the values were found within ± 0.4% of the theoretical values. The Purity of the compounds was checked by TLC (thin layer chromatog-

raphy) on Merck silica gel 60 F₂₅₄ pre-coated sheets in a chloroform/methanol mixture solvent system, and spots were located using an iodine chamber or a UV chamber.

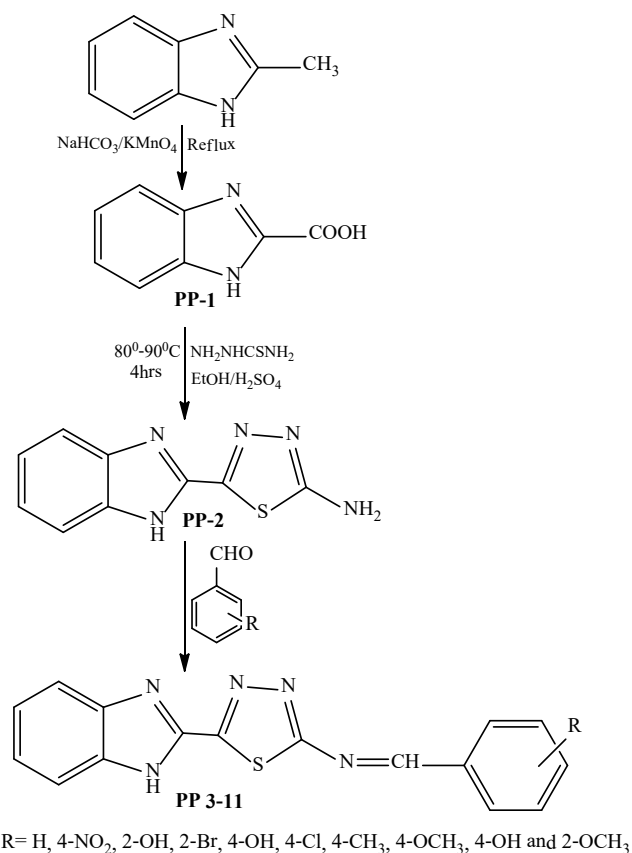


Figure 2. The scheme of Protocol for synthesis of benzimidazole substituted 1,3,4-thiadiazole Schiff's bases.

Chemistry

Synthesis of 1H-benzo[d]imidazole-2-carboxylic acid (PP-1): 2-methyl-1H-benzo[d]imidazole (0.01mole) was added to a solution of sodium bicarbonate (0.01mole) and potassium permanganate (0.01mole) in water (Agrawal et al., 1982; Norris, 1924), then the reaction mixture was refluxed for 15 hrs (Figure 2). The reaction mixture was cooled and acidified with conc. HCl and the product was collected and recrystallized from ethanol. Yield: 82%; m.p. 176-180°C; IR(cm⁻¹): 3426(-NH), 3080 (C-H aromatic), 2971(OH acid) 1742, 1631(C=N),1721(C=O acid); ¹H NMR(DMSO-d₆, δ, ppm): 12.34 (1H, -COOH), 7.49-7.11 (4H, Ar-H), 4.7 (1H, NH); MS (EI, m/z): 162(M⁺), 118 (100%).

Synthesis of 5-(1*H*-benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-amine (PP-2): An ethanolic solution of 1*H*-benzo[d]imidazole-2-carboxylic acid (Compound PP-1, 0.01 mole), was added to aqueous solution of thiosemicarbazide (0.02 moles) with stirring, few drops of conc. sulphuric acid (Hussain et al., 2005) was added and heated for 4 hrs at 80-90°C. With completion of the reaction (TLC), reaction mixture was cooled and poured into ice-cold water, basified with 10% Na₂CO₃ solution, filtered, dried and recrystallized from ethanol. Yield: 74%; m.p. 184-185°C; IR(cm⁻¹): 3426 (-NH), 3250 (-NH₂), 1742, 1631 (C=N), 1020 (C-S); ¹H NMR(DMSO-d₆, δ, ppm): 7.60-7.20 (4H, Ar-H), 7.51 (2H, NH₂) 4.7 (1H, Benzimidazole); MS (EI, *m/z*): 218(M⁺); Anal. % Calc/ found for C₉H₇N₅S (M.W. 217.25): C, 49.76/49.87; H, 3.25/3.71; N, 32.24/32.97.

General synthesis of *N*-Arylidene-5-(1*H*-benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-amine (PP 3-11): The corresponding aryl aldehyde (0.1 moles) was added to a solution of the thiadiazole substituted benzimidazole derivative (compound PP-2, 0.1 moles) in absolute ethanol (30 ml) and the mixture was refluxed for 2 hrs (Varshney et al., 2014; Husaina et al., 2018). The reaction mixture was cooled and kept for 24 hrs. The crystals found were filtered, dried, and recrystallized from ethanol.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-benzylidene-1,3,4-thiadiazol-2-amine (PP-3): Yield: 92%; m.p. 174-176°C; IR(cm⁻¹): 3378(-NH), 3078(Ar-H), 2970-2855(C-H aliphatic), 1604(C=C), 1632(CH=N), 690.47 (C-S-C); ¹H NMR (DMSO-d₆, δ, ppm): 4.69 (1H, NH-Benzimidazole), 7.2-7.8(9H, Ar), 8.24 (1H, -N=CH); MS (EI, *m/z*): 305 (M⁺); Anal. % Calc/ found for C₁₆H₁₁N₅S (M.W. 305.36): C, 62.93/62.87; H, 3.63/3.71; N, 22.93/22.97.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-nitrobenzylidene)-1,3,4-thiadiazol-2-amine (PP-4): Yield: 82% m.p. 168-169°C; IR(cm⁻¹): 3379(-NH), 3024 (Ar-H), 2923(C-H aliphatic), 1517, 1313 (NO₂); ¹H NMR (DMSO-d₆, δ, ppm): 4.7 (1H, NH-benzimidazole), 7.9-7.4(8H, Ar), 8.67 (1H, -N=CH); MS (EI, *m/z*): 349.5 (M⁺); Anal. % Calc/ found for C₁₆H₁₀N₆O₂S (M.W. 350.35): C, 54.85/54.88; H, 2.88/2.85; N, 23.99/24.04.

2-(((5-(1*H*-Benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-yl)imino)methyl)phenol (PP-5): Yield: 88% m.p. 171-173°C; IR(cm⁻¹): 3307(-NH), 3263(-OH), 3036(Ar-H); ¹H NMR (DMSO-d₆, δ, ppm): 4.7 (1H, NH-benzimidazole), 7.4-7.0(8H, Ar), 4.51 (1H, OH), 8.39 (1H, -N=CH); MS (EI, *m/z*): 321 (M⁺); Anal. % Calc/ found for C₁₆H₁₁N₅OS (M.W. 321.36): C, 59.80/59.88; H, 3.45/3.38; N, 21.79/21.74.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(2-bromobenzylidene)-1,3,4-thiadiazol-2-amine (PP-6): Yield: 76%; m.p. 181-183°C; IR(cm⁻¹): 3065 (C-H_{arom}), 2900-2960 (C-H_{aliph}), 1620(C=N), 686-515(C-Br); ¹H NMR (DMSO-d₆, δ, ppm): 4.7 (1H, NH-benzimidazole), 7.2-7.7(8H, Ar), 9.33 (1H, -N=CH); MS (EI, *m/z*): 384 (M⁺); Anal. % Calc/ found for C₁₆H₁₀N₅BrS (M.W. 384.25): C, 50.01/49.88; H, 2.62/2.68; N, 18.23/18.28.

4-(((5-(1*H*-Benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-yl)imino)methyl)phenol (PP-7): Yield: 83%; m.p. 174-175°C; IR(cm⁻¹): 3379 (-NH), 3211 (-OH), 3078 (C-H_{arom}), 2854 (C-H_{aliph}), 1631 (C=N); ¹H NMR (DMSO-d₆, δ, ppm): 4.69 (1H, NH-benzimidazole), 7.78 (2H, Ar), 7.61 (2H, Ar), 7.20 (2H, Ar), 6.85 (2H, Ar), 4.51 (1H, OH), 9.54 (1H, -N=CH); Anal. % Calc/ found for C₁₆H₁₁N₅OS (M.W. 321.36): C, 59.80/59.86; H, 3.45/3.43; N, 21.79/21.83.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-chlorobenzylidene)-1,3,4-thiadiazol-2-amine (PP-8): Yield: 74%; m.p. 189-192°C; IR(cm⁻¹): 3380(-NH), 3065 (C-H_{arom}), 1701(C=N), 815(C-Cl); ¹H NMR (DMSO-d₆, δ, ppm): 5.0 (1H, NH-benzimidazole), 7.2-7.7(8H, Ar), 7.90 (1H, -N=CH); MS (EI, *m/z*): 341 (M⁺); Anal. % Calc/ found for C₁₆H₁₀N₅ClS (M.W. 339.80): C, 56.55/56.49; H, 2.97/2.98; N, 20.61/20.68.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-methylbenzylidene)-1,3,4-thiadiazol-2-amine (PP-9): Yield: 81%; m.p. 187-189°C; IR(cm⁻¹): 3024 (C-H_{arom}), 2923(-CH₃), 2855(=CH), 1699 (C=N); ¹H NMR (DMSO-d₆, δ, ppm): 4.79 (1H, NH-benzimidazole), 7.22-7.7 (8H, Ar), 8.02 (1H, -N=CH-), 2.35(3H, CH₃); MS (EI, *m/z*): 320 (M⁺); Anal. % Calc/ found for C₁₇H₁₃N₅S (M.W. 319.38): C, 63.93/63.87; H, 4.10/4.16; N, 21.93/22.00.

5-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-methoxybenzylidene)-1,3,4-thiadiazol-2-amine (PP-10): Yield: 70%; m.p. 173-174°C; IR(cm⁻¹): 3070 (C-H_{arom}), 1699(-C=N), 1234(-OCH₃); ¹H NMR (DMSO-d₆, δ,

ppm): 4.79 (1H, NH-benzimidazole), 7.0-7.8(8H, Ar), 9.32(1H, N=CH-), 3.99(3H, -OCH₃); MS (EI, *m/z*): 336 (M⁺); Anal. % Calc/found for C₁₇H₁₃N₅OS (M.W. 335.38): C, 60.88/60.87; H, 3.91/4.02; N, 20.88/20.80.

4-(((5-(1H-Benzo[d]imidazol-2-yl)-1,3,4-thiadiazol-2-yl)imino)methyl)-3-methoxyphenol (PP-11): Yield: 68%; m.p. 166-168°C; IR(cm⁻¹): 3434(-OH), 3037, 2882 (C-H_{arom}), 2853(C-H_{aliph.}), 1148(-OCH₃), 687(C-S); ¹H NMR (DMSO-d₆, δ, ppm): 4.79 (1H, NH-benzimidazole), 7.2-7.7(5H, Ar-H), 6.41-6.48(2H, Ar), 5.34(1H, -OH), 9.33(1H, N=CH-) 3.83 (3H, -OCH₃); MS (EI, *m/z*): 352 (M⁺); Anal. % Calc/found for C₁₇H₁₃N₅O₂S (M.W. 351.38): C, 58.11/58.17; H, 3.73/3.68; N, 19.93/19.86.

In vitro Antimicrobial Activity

Antibacterial activity of the newly synthesized compounds was carried out using three gram-positive bacterial strains; *S. aureus* and *B. cereus* and *S. epidermidis*, and three gram-negative bacterial strains; *E. coli*, *S. typhi*, and *K. pneumonia*, by standard disc diffusion method (Cruickshank et al., 1975; Sahoo et al., 2010). Standard inoculums (1/100 mL of medium) with suspension (105 CFU/mL) were introduced into the surface of sterile agar plates, and an even distribution of the inoculum was achieved by using a sterile bent glass spreader. The paper disks prepared from Whatman paper (grade no. 1), measuring 6 mm in diameter and 2 mm thickness, were sterilized by dry heat for 1 h. Three paper discs impregnated with each test samples (PP 1-11) in a concentration of 25 µg/mL in DMF (dimethyl formamide), one standard disc of drug- Ampicillin (20 µg/disc) and one negative control disc impregnated with solvent- DMF were placed at different places in a nutrient agar plate medium having a pH (7.2±0.2). All the Petri dishes were then inverted and kept in an incubator for a period of 24 h at 37±2°C. Inhibition zones (in mm) were measured and the average zone diameter of test samples was obtained in triplicate sets. Inhibition zones of the test samples were compared with the inhibition zone of the standard drug.

Anthelmintic Studies

The newly synthesized benzimidazole substituted 1,3,4-thiadiazole Schiff's bases were tested for anthelmintic activity against two different worms species; *Pheretima posthuma* and *Perionyx excavatus*,

at a 2 mg/mL concentration (Dahiya et al., 2007). Earthworms collected from local marshy areas were washed with normal saline water to remove adhering soil and fecal matter. Suspensions of the synthesized compounds (100 mg) were prepared by triturating with Tween 80 (0.5%) and normal saline solution and stirring the resulting mixtures for 30 min. These suspensions were suitably diluted to obtain conc. of 0.2% w/v of the test samples. The suspension (0.2% w/v) of the standard drug albendazole was prepared in the same manner. Three sets of five earthworms of almost similar sizes (approx. 2 inches in length) were placed in Petri dishes of 4 inches diameter containing 50 mL of a suspension of prepared test samples and albendazole. Another petri dish containing 50 mL suspension of distilled water and tween 80 (0.5%) was kept as control and a set of five earthworms was placed in it. The paralyzing and death times for each synthesized compound and standard drug were noted, and their mean was calculated for triplicate sets. The death time was ascertained by placing the earthworms in warm water (50°C) which stimulated the movement, if the worm was alive.

Statistical Evaluation

Antibacterial activity of the titled compounds were analyzed by mean ± standard deviation (SD) (n=3) and compared with the standard. Similarly, anthelmintic activity of the test compounds was analyzed by mean ± SD (n=5) and compared with reference drug Albendazole.

RESULT AND DISCUSSION

Chemistry

A novel series of benzimidazole heterocyclic compounds incorporated with thiadiazole Schiff bases were prepared as per literature with little modification. The compounds were obtained in moderate to good yield ranging from 68-92%. Elemental analysis data of the synthesized compounds are within ±0.4% of the theoretical values. In general, IR spectra of compounds PP 3-11 exhibited the presence of absorption bands for C=N stretching between 1630-1700 cm⁻¹ and a C-S-C linkage in thiadiazole at around 690cm⁻¹. The ¹H NMR also confirms the presence of shift value at 8.24-9.37 for CH=N groups. The reported spectral data have given sufficient evidence for the successful synthesis of desired compounds.

Biological Studies

All the newly synthesized benzimidazole derivatives showed moderate to good antibacterial activity against all the tested strains. The results of antibacterial studies are presented in “Table 1”, and a comparison of their activity in “Figure 3”. While performing the antimicrobial studies by disc diffusion method,

it was noticeable that compounds (PP 3-11) having varied substitutions at *ortho*- and *para*- position of the aryl ring have different antimicrobial activity spectrum. Compounds PP-4, PP-6 and PP-8 having electron-withdrawing groups (4-nitro, 2-bromo, 4-chloro) exhibited inhibitory effect comparable to Ampicillin. Compound PP-2 containing a free $-NH_2$ group on the thiadiazole ring also showed significant antibacterial activity compared to the standard.

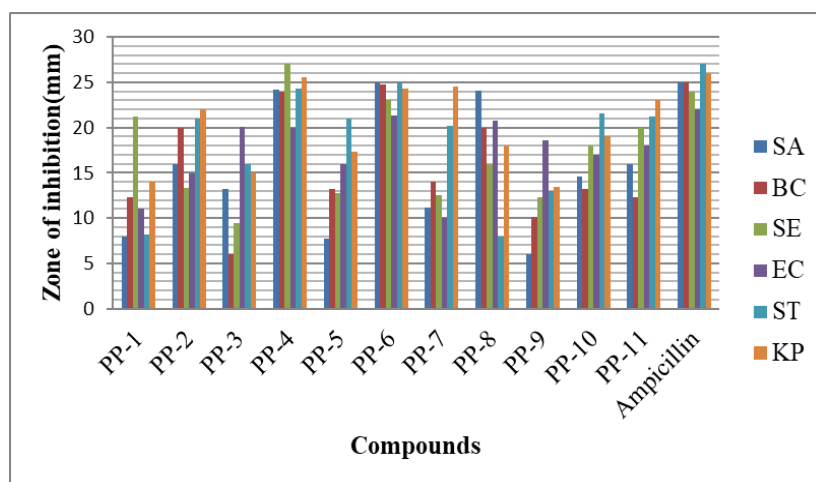


Figure 3. Comparison of antimicrobial activity of the compounds.

SA = *Staphylococcus aureus* (ATCC 11633), BC = *Bacillus cereus* (ATCC 11778), SE = *Staphylococcus epidermidis* (ATCC 155), EC = *Escherichia coli* (ATCC10536), ST = *Salmonella typhi* (MTCC 733), KP = *Klebsiella pneumonia* (ATCC 10031).

Table 1. Antibacterial activity of the synthesized compounds

Compounds	Antibacterial activity (Zone of inhibition in mm)*					
	Gram (+) bacteria			Gram (-) bacteria		
	SA	BC	SE	EC	ST	KP
PP-1	8±0.00	12.32±0.23	21.21±0.32	11±0.00	8.2 ±0.24	14 ± 0.00
PP-2	16±0.00	20±0.00	13.32±0.32	14.9±0.15	21±0.00	22 ± 0.00
PP-3	13.2±0.26	6 ±0.00	9.42±0.25	20.1±0.41	16±0.00	15±0.00
PP-4	24.16±0.12	24±0.00	27±0.00	20±0.32	24.36±0.28	25.6±0.17
PP-5	7.77±0.22	13.24±0.32	12.76±0.27	16±0.00	21±0.00	17.3±0.20
PP-6	25 ±0.00	24.76±0.2	23.1±0.43	21.29±0.41	25±0.00	24.34±0.12
PP-7	11.2±0.26	14±0.00	12.5±0.31	10±0.00	20.2±0.26	24.52±0.3
PP-8	24.1 ±0.32	20±0.00	16±0.00	20.8±0.24	8±0.00	18.0±0.00
PP-9	6±0.00	10.1±0.52	12.3±0.43	18.6±0.4	13±0.00	13.48±0.30
PP-10	14.6±0.10	13.22±0.42	18±0.00	17±0.00	21.6±0.47	19±0.00
PP-11	16±0.00	12.33±0.2	20±0.00	18±0.00	21.25±0.38	23±0.00
Ampicillin	25±0.00	25±0.00	24±0.00	22±0.00	27±0.00	26±0.00
DMF	-	-	-	-	-	-

*Data are given as mean ± S.D (n = 3); SA = *Staphylococcus aureus* (ATCC 11633); BC = *Bacillus cereus* (ATCC 11778); SE = *Staphylococcus epidermidis* (ATCC 155); EC = *Escherichia coli* (ATCC10536);ST = *Salmonella typhi* (MTCC 733); KP = *Klebsiella pneumonia* (ATCC 10031).

However, the pattern of the result of anthelmintic activity of the test compounds was quite different from their antibacterial activity. Some of the compounds were found to show anthelmintic activity comparable to the standard drug albendazole. The results of anthelmintic studies are reported in "Table 2", and a comparison of their activity in "Figure 4". The mean paralyzing time (min) of tested compounds against *P. excavatus* and *P. posthuma*, was observed to be 9.10-18.23 and 13.11-22.31 min in comparison to 9.70 and 12.20 min shown by albendazole. The mean death time (min) of tested compounds against *P. excavatus* and *P. posthuma*, ranged from 13.22-28.36 and 19.00-28.32 min in comparison to 14.80 and 20.70 min shown by albendazole. The most and the least potent anthelmintic compound in terms of mean paralyzing time against *P. excavatus* was noted to be

PP-4 (9.10 min) and PP-9 (18.23 min), while against *P. posthuma*, PP-4 (11.10 min) and PP-1 (28.32 min) had the similar spectrum of activity. Compound PP-4 was found to be more potent than the standard drug in causing the death of nematodes, which took an average time of 13.22 and 19.00 min against *P. excavatus* and *P. posthuma*, respectively. Apart from compound PP-4, other compounds like PP-6 and PP-11 had comparable anthelmintic activity to that of albendazole. Compounds with electron-withdrawing substituent at *ortho* position led to a considerable increase in activity. Again, the compound with hydroxyl substituent in *para* position and methoxy substituent in *ortho* position also shows significant increase in the anthelmintic activities. Hence, there is a significant role of electron-withdrawing group on the anthelmintic activity.

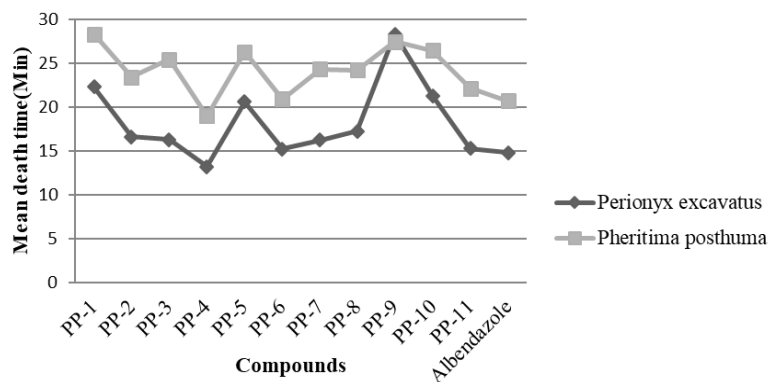


Figure 4. Comparison of anthelmintic activity of the compounds

Table 2. Anthelmintic activity of the title compounds PP1-11

Compounds	Earthworm Species			
	<i>Perionyx excavates</i>		<i>Pheritima posthuma</i>	
	Mean paralyzing time (min) ^a	Mean death time (min) ^a	Mean paralyzing time (min) ^a	Mean death time (min) ^a
PP-1	17.43±0.28	22.32±0.13	21.11±0.92	28.32±0.30
PP-2	11.86±0.30	16.62±0.11	14.87±0.68	23.42±0.42
PP-3	11.5±0.28	16.30±0.35	15.67±0.21	25.48±0.53
PP-4	9.10±0.32	13.22±0.25	11.10±0.72	19.00±0.10
PP-5	15.12±0.36	20.62±0.15	21.23±0.12	26.30±0.22
PP-6	10.24±0.47	15.20±0.32	13.42±0.56	20.91±0.15
PP-7	12.21±0.11	16.22±0.30	15.78±0.56	24.36±0.13
PP-8	12.31±0.16	17.22±0.41	16.11±0.75	24.23±0.28
PP-9	18.23±0.19	28.36±0.25	19.67±0.81	27.48±0.91
PP-10	16.23±0.32	21.32±0.71	22.31±0.43	26.47±0.18
PP-11	10.12±0.33	15.30±0.24	13.15±0.53	22.16±0.63
Albendazole	9.70±0.19	14.80±0.42	12.20±0.35	20.70±0.21
Control	No paralysis	No death	No paralysis	No death

^aData are given as mean ± S.D (n = 5)

CONCLUSION

Various benzimidazole substituted 1,3,4-thiadiazole Schiff base derivatives were prepared with the objective of developing better antibacterial and anthelmintic moiety. The procedure reported herein is simple, economical, efficient, and environmentally friendly. Additionally, the derivatives were precipitated in their analytical grade without the necessity for chromatographic purification. Three of the eleven new compounds had a comparable antibacterial and anthelmintic activity to that of standard and, as such, could be further developed as alternative antimicrobial and anthelmintic agents for the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTION STATEMENT

Initial literature survey, experimental design, antimicrobial and anthelmintic assay, statistical analysis, data acquisition, writing the manuscript (Pattanayak, P.), data interpretation, spectra analysis, approval of the final manuscript (Savanan, K.).

REFERENCES

- Achar, K. C., & Hosamani, K. M. (2010). *In-vivo* analgesic and anti-inflammatory activities of newly synthesized benzimidazole derivatives. *European Journal of Medicinal Chemistry*, 45, 2048-2054. doi: 10.1016/j.bioorg.2016.11.014
- Agrawal, R. K., & Sharma, S. (1982). A new synthesis of substituted benzimidazole-2-one. *Indian Journal of Chemistry*, 21(b), 967-968.
- Ahn, Y. J., Kwon, M., Yoo, J. K., Byun, S. J. (1993). Toxicity of flufenoxuron alone and in mixture with alphacypermethrin or fenbutanin oxide to *Tetranychus urticae* and *Panonychus ulmi*. *Journal of Economic Entomology*, 86, 1334-1338. doi: /10.1093/jee/86.5.1334.
- Balaji, P. N., Ranganayakulu, D., Reddy, G. V. (2017). Synthesis, in vitro evaluation for anthelmintic and antimicrobial activity for the novel thiazolidine-4-one incorporate substituted chloro-quinoline. *Asian Journal of Pharmacy and Pharmacology*, 3, 9-15.
- Beatriz, M., Pablo, M., Romina, E., Andrea L. (2013). Synthesis and Anthelmintic Evaluation of Novel Valerolactam-Benzimidazole Hybrids. *Letters in Drug Design & Discovery*, 10, 1007-1014. doi: 10.2174/15701808113109990028.
- Bhinge, S. D., Chature, V., Sonawane, L. V. (2015). Synthesis of some novel 1,3,4-thiadiazole derivatives and biological screening for anti-microbial, antifungal and anthelmintic activity, *Pharmaceutical Chemistry Journal*, 49, 367-372. doi: /10.1007/s11094-015-1287-8
- Cho, S. Y., Kang, S. K., Kim, S. S., Yum, E. M. (2001). Synthesis and SAR of benzimidazole derivatives containing oxycyclic pyridine as a gastric ATPase inhibitors. *Bulletin of the Korean Chemical Society*, 22, 1217-1223.
- Cruickshank, R., Duguid, J.P., Marion, B.P., Swain, R.H.A. (1975). *Medicinal microbiology*, 12th ed.; Churchill Livingstone: London, Vol. 2, 196-202.
- Dahiya, R., Pathak, D. (2007). Synthetic studies on novel benzimidazole peptides with antimicrobial, cytotoxic and anthelmintic potential. *European Journal of Medicinal Chemistry*, 42, 772-98. doi: /10.1016/j.ejmech.2006.11.015.
- El-Zemity, S., Badawy, M. E., Khattab M. M., Marei, A. E. (2006). Structure and Acaricidal Activity Relationship of Some Sulfonamide Derivatives Against the Two-spotted Spider Mite, *tetranychus urticae* (Koch). *International Journal of Agricultural Biology*, 8, 661-665.
- Faruk, A., Biplab, K. D., Sharma, K. (2014). Synthesis, antimicrobial and anthelmintic activity of some novel benzimidazole derivatives. *International Journal of Drug Research and Technology*, 4, 31-38.

- Gurer-Orhan, H., Suzen, S., Buyukbingol, E., (2006). Synthesis and evaluation of *in vitro* antioxidant capacities of some benzimidazole derivatives. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 21, 241-247. doi: 10.1080/14756360600586031
- Husaina, A., Munendra, M. V., Versha, P. (2018). Nalidixic Acid Schiff Bases: Synthesis and Biological Evaluation, *Letters in Drug Design & Discovery*, 15, 103-111. doi: 10.2174/1570180814666170710160751
- Hussain, A., Sharba, K., Al-Bayati, R., Rezki, N., Aouad, M. R. (2005). Synthesis of Thiadiazoles and 1,2,4-Triazoles derived from cyclopropane dicarboxylic acid, *Molecules*, 10, 1153-1160. doi: 10.3390/10091153
- Katiya, S. K., Gordon, V. R., Edlind, T. D. (1994). Antiprotozoal activities of benzimidazoles and correlations with beta-Tubulin sequence. *Antimicrobial Agents and Chemotherapy*, 38, 2086-2090. doi: 10.1128/aac.38.9.2086
- Katti, S. A., Desai, K. S., Loharkar, S. V. (2019). Synthesis, molecular docking & evaluation of anthelmintic activity of 2-(2-amino ethyl)-1H-benzimidazole derivatives. *World Journal of Pharmaceutical Research*, 8, 1141-1151. doi: 10.20959/wjpr201911-15922
- Kumar, J. R., Jawharand, J., Pathak, D. P. (2006). Synthesis of benzimidazole derivatives as anti-hypertensive agents. *Journal of Chemistry*, 3, 278-285. doi: 10.1155/2006/765712
- Lazer, E. S., Matteo, M. R., Possanza, G. J. (1987). Benzimidazole derivatives with atypical anti-inflammatory activity. *Journal of Medicinal Chemistry*, 30, 726-729. doi: 10.1021/jm00387a026
- Li, Y. F., Wang, G. F., Zhu, F. H. (2006). Synthesis and anti-Hepatitis-B virus activity of novel benzimidazole derivatives. *Journal of Medicinal Chemistry*, 49, 4790-4794. doi: 10.1021/jm060330f
- Mahama, O., Drissa, S., Mamidou W. K. (2011). Synthesis and anthelmintic activity of some hybrid Benzimidazolyl-chalcone derivatives. *Tropical Journal of Pharmaceutical Research*, 10, 767-775. doi: 10.4314/tjpr.v10i6.10
- Marin, A., Valls, N., Berenguer, F. J., Alonso, M. T., Martinez, A. R. (1992). Synthesis and anthelmintic activity of carbamates derived from imidazo[2,1-b][1,3,4]thiadiazole and imidazo[2,1-b]thiazole. *Farmaco*, 47, 63-75. PMID: 1616578.
- Norris, J. F. (1924). Preparation of Terephthalic acid from p- Toluic Acid. In *Experimental Organic Chemistry*, 2nd ed.; Mc Graw-Hill Book Company, Inc.: New York, NY, USA.
- Ozkay, Y., Tunai, Y., Karaka, H., Isikdag, I. (2010). Antimicrobial activity and a SAR study of some novel benzimidazole bearing hydrazone moiety. *European Journal of Medicinal Chemistry*, 45, 3293-3298. doi: 10.1016/j.ejmech.2010.04.012
- Rao, R., Reddy, K.R., Mahendra, K.N. (2014). Synthesis, characterization, antibacterial, antifungal and anthelmintic activities of a new 5 - nitroisatin Schiff base and its metal complexes. *Bulgarian Chemical Communications*, 46, 11-17.
- Reddy, D. R. S., Kumar, K. H. (2014). N-substituted fluoro benzothiazolo Schiff's bases: Synthesis and characterisation of new novel anthelmintic agents. *International Journal of Pharmaceutical and Clinical Research*, 6, 71-75.
- Sahoo, P.K., Sharma, R., Pattanayak, P. (2010). Synthesis and evaluation of 4-amino-5-phenyl-4H-[1,2,4]-triazole-3-thiol derivatives as antimicrobial agents. *Medicinal Chemistry Research*. 19, 127-135. doi: 10.1007/s00044-009-9178-8
- Sanja, O., Dijana, J. B., Dragaljub, D. (2007). QSAR of some 1-benzyl benzimidazole derivatives as antifungal agent. *APTEFF*, 38, 139-147. doi:10.2298/APT0738139P
- Satyajit, D. (2011). Studies on synthesis and anthelmintic activities of some N-Benzylidenepyridin-2-amines. *FABAD Journal of Pharmaceutical Sciences*, 36, 189-195.
- Shingalpur, R. V., Keri, R. S., Hugar, M. H. (2010). Derivatives of benzimidazole pharmacophore: synthesis, anticonvulsant, antidiabetic studies. *European Journal of Medicinal Chemistry*, 4, 1735-1759. doi: 10.1016/j.ejmech.2010.01.007

- Varshney, M. M., Husain, A., Parcha, V. (2014). Synthesis, characterization, *in vitro* antimicrobial, and anthelmintic evaluations of 2-(4-chloro-3-methylphenoxy)-N'-[5'-(substituted aryl)-furan-2'-yl]-methylidene]-acetohydrazides. *Medicinal Chemistry Research*, 23, 4034–4041. doi: /10.1007/s00044-014-0982-4
- Venugopala, K. N., & Jayashree, B. S. (2003). Synthesis of carboxamides of 2'-amino-4'-(6-bromo-3-coumarinyl) thiazole as analgesic and anti-inflammatory agents. *Indian Journal of Heterocyclic Chemistry*, 12, 307–310.
- Zienab, M., Nofal, A., Soliman S. S., Sroor S.S. (2011). Novel benzimidazole derivatives as expected anticancer agents. *Acta Polonica Pharmaceutica*, 68, 519-534. PMID: 21796934.

Serum Type Hyaluronic Acid Formulations: *In vitro* Characterization and Patch Test Study

Serdar TORT* , Alptug KARAKUCUK**,*,°

Serum Type Hyaluronic Acid Formulations: *In vitro* Characterization and Patch Test Study

SUMMARY

Hyaluronic acid is a natural polymer that provides moisture to the skin and supports the skin's elasticity by helping to keep it supple. Hyaluronic acid-containing serum, semi-solid and injectable formulations are available commercially. In this study, serum type formulations containing hyaluronic acid were prepared. The final formulation containing 1% hyaluronic acid was selected from the prepared formulations and stability tests, protective efficacy tests (ISO 11930), and *in vivo* allergic irritation tests of this formulation were performed. The pH of the 1% hyaluronic acid formulation was adjusted to 5.5. Microbial analysis using *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* strains showed that the final formulation does not pose a contamination risk. In addition, it has been proven in the protective efficacy test of the final formulation that it has an antimicrobial effect of up to 28 days. According to the patch-shaped irritation test results in 15 subjects between the ages of 22-70, no allergic reaction was observed in the subjects for one week. No change was observed in the physicochemical properties of the final formulation at 25°C 65% relative humidity. In conclusion, the hyaluronic acid serum formulation has been evaluated as a product that can be used safely for moisturizing the skin.

Key Words: Hyaluronic acid, skin moisturizing, serum type formulation, *in vivo* allergy test, cosmetic product

Hyaluronik Asit Serum Formülasyonları: *In vitro* Karakterizasyon ve Yama Testi Çalışması

ÖZ

Hyaluronik asit, cildin nemli ve esnek kalmasına yardımcı olarak cildin elastikiyetini destekleyen doğal bir polimerdir. Hyaluronik asit içeren serum, yarı katı ve enjektabl formülasyonlar ticari olarak bulunmaktadır. Bu çalışmada, hyaluronik asit içeren serum tipi formülasyonlar hazırlanmıştır. Hazırlanan formülasyonlardan %1 hyaluronik asit içeren formülasyon sonuç formülasyon olarak seçilerek, stabilite testleri, korucuyu etkinlik testleri (ISO 11930) ve *in vivo* alerjik irritasyon testleri yapılmıştır. Sonuç formülasyonunun pH'sı 5,5 olarak ayarlanmıştır. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* ve *Candida albicans* suşlarının kullandığı mikrobiyal analiz, sonuç formülasyonunun kontaminasyon riski oluşturmadığını göstermiştir. Ayrıca sonuç formülasyonunun koruyucu etkinlik testinde 28 güne kadar antimikrobiyal etkiye sahip olduğunu kanıtlamıştır. 22-70 yaş arası 15 kişide yama şeklindeki alerjik irritasyon testi sonuçlarına göre, deneklerde 1 hafta süreyle herhangi bir alerjik reaksiyon görülmemiştir. 25°C %65 bağıl nemde sonuç formülasyonunun fizikokimyasal özelliklerinde bir değişiklik olmamıştır. Sonuç olarak hyaluronik asit serum formülasyonu cildi nemlendirmede güvenle kullanılacak bir ürün olarak değerlendirilmiştir.

Anahtar Kelimeler: Hyaluronik asit, cilt nemlendirici, serum tipi formülasyon, *in vivo* alerji testi, kozmetik ürün

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* ORCID: 0000-0003-4945-5420, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Turkey.

** ORCID: 0000-0002-9061-2427, Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara Medipol University, Ankara, Turkey

° Corresponding Author; Alptug KARAKUCUK

Phone: +90-533-6388331, e-mail: karakucuk@gazi.edu.tr, alptug.karakucuk@ankaramedipol.edu.tr

INTRODUCTION

Loss of epidermal polarity by chronic sun exposure, keratinocyte atypia, and reduction of collagen are the main reasons for skin aging (Lee, 2015). Crow's feet wrinkles are associated with skin aging. One of the reasons for the appearance of them is the reduction in the epidermal content of hyaluronic acid (Y. J. Lee, 2019). Hyaluronic acid (HA) is a natural polysaccharide that is a primary component of the extracellular matrix of human tissues and the endogenous component of human skin. It has a vital water-binding capacity (6 L water in 1 g) (Jegasothy, 2014). It is used to overcome skin wrinkles, provide skin elasticity and moisture (Choi, 2017). Because of the biocompatibility, biodegradability, non-immunogenicity, and viscoelasticity properties of HA, it is ideal to use in cosmetic, medical, or pharmaceutical purposes, especially to fabricate injectable fillers (Brown and Jones 2005; J. S. Lee, 2019). HA is commercially produced by synovial fluid, umbilical cord, or skin of the animals, from rooster comb, or bacteria by fermentation or isolation (Brown and Jones, 2005).

There are various forms of HA such as hydrogels, scaffolds, creams, films, foams, gels, serum, lotion, implants, etc. (Bukhari, 2018). The general approach to delivering HA through the skin is the injection of HA filler; however, this method is invasive, painful, and usually have side effects (J. S. Lee, 2019). Therefore, it may be the more promising approach to deliver HA into the skin with serum type formulation to provide skin moisture. However, because the HA has a hydrophilic characteristic, the lipophilic stratum corneum roles as a natural barrier against HA. Nevertheless, HA in serum or cream formulations plays a transitory water holding and smoothing effect on the skin (Wu, 2020).

HA formulations can be classified as low molecular weight (500–1000 kDa), medium molecular weight (1200–4500 kDa), and high molecular weight (6000–7000 kDa) (Iturriaga, 2021). The water absorption capacity and skin moisturizing properties of HA depend on the molecular weight of HA. The higher molecular weight provides a more robust water

absorption capacity and also has more resistance for degradation by hyaluronidase (Jang, 2020). However, HA with low molecular weight (20-300 kDa) passes more effectively through the skin in comparison with high molecular weight HA (1000-1400 kDa) (Essendoubi, 2016).

The primary purpose of this study is to develop low molecular weight (400 kDa) HA serum for topical application. Viscosity, surface tension, and pH measurements were evaluated as *in vitro* characterization studies. Physical and microbiological stability tests and protective efficacy tests were carried out. *In vivo* allergic irritation test was performed with 15 subjects regarding patch-shaped irritation test.

According to the Cosmetics Regulation, the final product was declared by the Republic of Turkey Ministry of Health has been commercially launched by Fiber Farma Drug and Cosmetics Co. with the brand of ResCare® Hyaluronic Acid Serum since 2017.

MATERIALS AND METHODS

Materials

HA (Mw. 400 kDa) was purchased from Kadioğlu Medical Ltd. (Ankara, Turkey). 2-Phenoxyethanol and lactic acid were purchased from Tekkim (Istanbul, Turkey). All other chemicals were of analytical grade.

Preparation of serum formulations

HA has a gelling property related to its molecular weight. Therefore, three different concentrations (0.5, 1, and 2 %) of HA were selected for preparing serum formulations. HA was added to purified water and mixed until completely dissolved at room temperature. Then, 1% of 2-phenoxyethanol was added and mixed. Finally, the pH of the solution was adjusted to 5.5 with lactic acid.

Viscosity and surface tension measurements

The viscosity of serum formulations was measured with a cone-plate rheometer (Brookfield Rheometer DV-III) using a CP-52 spindle at room temperature. Shear rate (1/s) -viscosity (cP.s) curves were evaluated in terms of viscosity measurements.

Surface tension is a critical value for cosmetic products. Therefore, surface tension values of formulations were analyzed using an optical tensiometer (ThetaLite Optical Tensiometer). The pendant drop method was used with 10 µL of sample solution (Özden and Avcı, 2017).

Protective efficacy tests (Challenge tests)

The protective effectiveness of 2-phenoxyethanol was evaluated with standard challenge tests (ISO 11930). For this purpose, five different microorganisms (*Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404)) were used. The final serum formulation was held at room temperature and evaluated on the 7th, 14th, and 28th days after inoculation. At each time point, colonies were counted, and the log reduction of each microorganism was calculated.

Stability tests

The physical stability test of the final serum formulation was made at 25°C 65% relative humidity (RH) for six months. The appearance, weight variation, and pH values of test samples were determined at 30-day intervals.

In vivo allergy testing

In vivo allergy tests were conducted according

to the Regulation of the European Parliament and Council Regulation (EC) No 1223/2009 of November 30, 2009; Cosmetics Europe- The Personal Care Association Guidelines, Product test Guidelines for the Assessment of Human Skin Compatibility 1997; and WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. Dermatological tests were performed following the COLIPA Guidelines for the Assessment of Human Skin Compatibility. The patch test was performed at Skin Lab International, Cracow, Poland, with a test number of 20/12/17/D/7. Fifteen women aged 22 – 70 years were selected for the dermatological tests of the product. All of the patch samples selected for testing met the requirements for inclusion in the study, signed an agreement to participate in the study, and were informed about the study’s purpose, how it is carried out, and the possible side effects. The test was conducted using the Jodassohn-Bloch model (Lachapelle and Maibach, 2009) by the International Contact Dermatitis Research Group (ICDRG). Patch testing was made with standard IQ chambers. A small drop of serum formulation was applied to the patients’ forearm for 48 hours and then removed (Figure 2). After 30 min, 72 h, 96 h, and one-week readings were recorded and evaluated according to a graphic scale consistent with the generally accepted clinical dermatological scale.

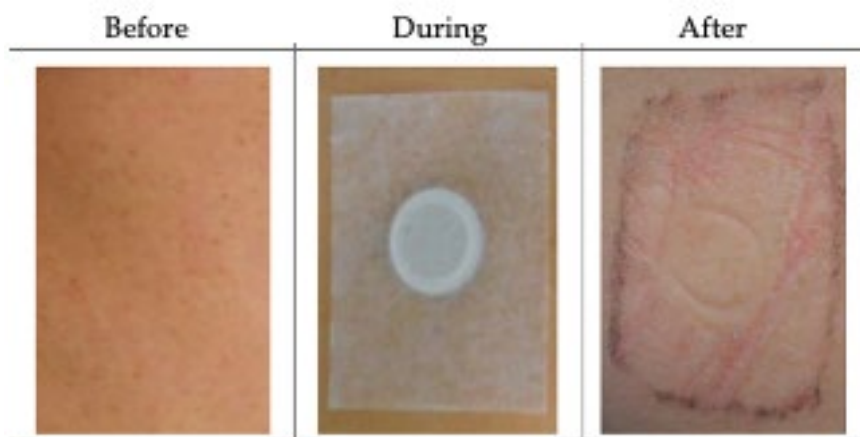


Figure 1. Application of test HA gel formulations to human skin

Statistical analysis

Statistical differences for viscosity and surface tension were analyzed with one- way ANOVA test following Tukey HSD post-hoc test using GraphPad Prism Version 8. Results were expressed as the mean \pm standard deviation, and evaluation was carried out with a significance level of 0.05.

RESULTS AND DISCUSSION

Preparation of HA serum

HA serum formulations were prepared by dissolving different concentrations of HA in distilled water in the existence of 1% 2-phenoxyethanol as a stabilizer (Dreno, 2019). A minimum number of excipients were added to avoid facial skin irritation, where some fragranced ingredients were reported to cause allergy or sensitizations (Panico, 2019). A high concentration of HA in serum allows that the formulation remains on the skin surface to provide long-acting skin moisturization. In this way, topical HA helps skin regeneration and shows the anti-wrinkle effect (Choi, 2017). Similar to this study, the anti-wrinkle efficacy of HA-based topical cream has investigated by Poetschke et al., and the researchers found that daily application of HA cream resulted in a significant reduction in the depth of wrinkles improvement of skin elasticity and tightness

(Poetschke, 2016). Jegasothy et al. applied topical lotion, serum, and cream of HA for 8-weeks to 33 women and found a significant effect on improving moisturizing, skin elasticity, and skin roughness (Jegasothy, 2014).

Viscosity and surface tension measurements

Viscosity is a critical parameter for topical formulations (Karakucuk, 2021). Topical solutions with low viscosity have faster clearance than viscous solutions. In addition, highly viscous solutions can have an undesirable effect on the skin. In terms of penetration, solutions with lower viscosity should penetrate better than a thicker control serum (Surini, 2018). The viscosity of solutions affected directly by polymer concentration and molecular weight of the polymer. In this study, HA with 400 kDa, which could be classified as intermediate, was used. Although solutions with 2% HA showed shear thinning behavior, solutions with 1% and 0.5 % concentration showed Newtonian flow (Figure 2). When the concentration of HA increased from 0.5 to 1 and 2 %, the viscosity solution increased significantly four times and 20 times at 50 rpm ($p < 0.05$). A similar result can be seen in the literature. Budiasih et al., prepared argan oil containing serum formulations with 214-351 pa (Budiasih, 2018).

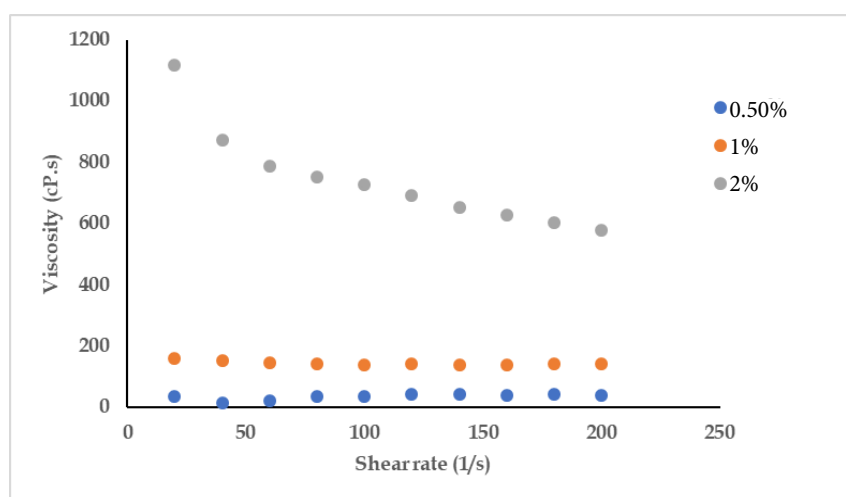


Figure 2. Shear rate – viscosity curve of HA gel serum formulations

The surface tension of formulation can play a critical role in wetting the skin surface. The surface tension of 1% HA containing solution was 67.35 ± 0.12 mN/m, and it was a significantly higher value than 0.5% HA serum which had a surface tension of 66.83 ± 0.15 mN/m ($p < 0.05$). Increasing HA concentration to 2% decreased the surface tension significantly to 63.88 ± 0.33 mN/m ($p < 0.05$). Although cleaning cosmetic products have very low surface tension values, cosmetic products for hydration should not have low surface tension values. The main effect that provides moistening is related to the material's structure rather than the surface tension. HA can create hydration film on the skin to moisturize the stratum corneum (Xie, J. 2018). The surface tension of solutions could be decreased with surfactant addition. However, surfactants have skin irritation in cosmetic products according to the amounts (Seweryn, A. 2018).

Protective efficacy tests (Challenge tests)

For cosmetic products, at least one preservative agent should be added to the formulation. Preservative agents have a critical effect on product quality and also

on consumer health. In addition, microorganisms can affect the product quality after opening the product. Therefore, the effectiveness of protective agents should be performed with challenge tests. For this purpose, cosmetic challenge test standards are based on five different microorganisms. Products without water or alcohol-based products (more than 20%) do not need this test procedure. Phenoxyethanol is a safe and commonly used preservative for cosmetic products. This preservative can be used between 0.1 to 1 % concentrations (Dreno, 2019). An essential advantage of Phenoxyethanol is that it does not change the appearance and smell of the formulations. However, the possibility of skin irritation is a disadvantage (Farage 2019). In this study, phenoxyethanol was added to formulations at a 1% concentration. It was shown that this concentration successfully protected the product from selected bacteria and fungus in Table 1. Phenoxyethanol provided more than 5 log and 4 log reduction towards bacteria and fungi, respectively, for 28 days. It was shown that phenoxyethanol has an efficient bactericidal and fungicidal effect in the final product.

Table 1. Challenge tests result for 2-Phenoxyethanol

Microorganism	Inoculation (CFU)	Number of CFU/g				Log reduction		
		Day 0	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
<i>P. aeruginosa</i>	2.7×10^7	2.7×10^7	<10	<10	<10	5.18	5.18	5.18
<i>E. coli</i>	2.3×10^7	2.3×10^7	<10	<10	<10	5.18	5.18	5.18
<i>S. aureus</i>	2.5×10^7	2.5×10^7	<10	<10	<10	5.48	5.48	5.48
<i>C. albicans</i>	2.2×10^8	2.2×10^8	<10	<10	<10	4.48	4.48	4.48
<i>A. niger</i>	3.0×10^8	3.0×10^8	<10	<10	<10	4.48	4.48	4.48

Stability tests

Physical stability tests of the final product were made at 25°C, 65% RH. Final products were determined as transparent, colorless and no color change was observed during storage time. The samples showed the same results as the first weighing (approximately $12 \text{ g} \pm 0.1$) at the end of the study, and no weight variation was observed. The pH of solutions

was found stable at 5.5 during the test period. At the end of the study, microbiological and physical stability were protected.

In vivo allergy testing

Patch testing for in vivo allergy is the gold standard to identify skin compatibility for cosmetic products and cause allergic contact dermatitis and diagnose

a delayed type of hypersensitivity (Kasemsarn and Boonchai 2012). Patch testing allows test substance application on skin surface and causes of reaction of the immune system by a characteristic pattern of erythema and edema develops from 6 h and reaches a maximum by 36- 48 h (Friedmann and Ardern-Jones 2010). Patch testing was performed for HA

serum formulations, and the results were evaluated according to a graphic scale that was consistent with the generally accepted clinical dermatological scale. Results were evaluated by the recommendations of the International Contact Dermatitis Research Group (ICDRG) (Lachapelle and Maibach, 2009) (Table 2).

Table 2. Identification of the patch test reactions

Record	Diagnosis	Interpretation
-	Negative reaction	No skin lesions
?	Doubtful reaction	Faint erythema only
+	Weak positive reaction	Palpable erythema, infiltration, possibly papules
++	Strong positive reaction	Erythema, infiltration, papules, vesicles
+++	Extreme positive reaction	Intense erythema, infiltration, and coalescing vesicles, bullous or ulcerative reaction
IR	The irritant reaction of different types	Discrete patchy erythema without infiltration.

There were no allergic reactions or hypersensitivity of the formulation on female skin during the study (Table 3). Similarly, Kong et al., (2011) reported that HA-based nanoemulsions did not cause skin irritation

in the dermis and skin surface. Choi et al., (2017) also reported the non-irritant effect of HA-containing microneedle patches.

Table 3. The clinical dermatological scale of patch testing on female subjects

No	Identification number	Sex (Female – F)	Age	Test result			
				48 h	72 h	96 h	One week
1	20/12/17/D/7-1	F	22	(-)	(-)	(-)	(-)
2	20/12/17/D/7-2	F	24	(-)	(-)	(-)	(-)
3	20/12/17/D/7-3	F	23	(-)	(-)	(-)	(-)
4	20/12/17/D/7-4	F	62	(-)	(-)	(-)	(-)
5	20/12/17/D/7-5	F	70	(-)	(-)	(-)	(-)
6	20/12/17/D/7-6	F	27	(-)	(-)	(-)	(-)
7	20/12/17/D/7-7	F	44	(-)	(-)	(-)	(-)
8	20/12/17/D/7-8	F	26	(-)	(-)	(-)	(-)
9	20/12/17/D/7-9	F	23	(-)	(-)	(-)	(-)
10	20/12/17/D/7-10	F	23	(-)	(-)	(-)	(-)
11	20/12/17/D/7-11	F	61	(-)	(-)	(-)	(-)
12	20/12/17/D/7-12	F	49	(-)	(-)	(-)	(-)
13	20/12/17/D/7-13	F	23	(-)	(-)	(-)	(-)
14	20/12/17/D/7-14	F	24	(-)	(-)	(-)	(-)
15	20/12/17/D/7-15	F	48	(-)	(-)	(-)	(-)

CONCLUSION

HA is one of the most successful cosmetic ingredients using in anti-aging products because of its compatibility with skin and capacity for hydration. HA serum formulations were prepared for a topical application to provide skin moisture in this study. The in vitro characterization studies showed that the formulation is smooth and easy for application, increasing consumers' compliance. The final formulation stayed physically and microbiologically stable during the storage time. Patch testing was performed to evaluate any skin reactions of the products, and there were no adverse effects such as hypersensitivity or erythema. The benefits of using HA and the safety of the final formulation promise that the product, which is available commercially as a cosmetic product in Turkey, can be safely used as skin moisturizing.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept (Tort, S., Karaküçük, A.), Design (Tort, S., Karaküçük, A.), Supervision (Karaküçük, A.), Resources (Tort, S., Karaküçük, A.), Materials (Tort, S., Karaküçük, A.), Data Collection and Processing (Tort, S., Karaküçük, A.), Analysis, and Interpretation (Tort, S., Karaküçük, A.), Literature Search (Tort, S., Karaküçük, A.), Writing (Tort, S., Karaküçük, A.), Critical Reviews (Tort, S., Karaküçük, A.).

REFERENCES

- Brown, M. B., and Jones S. A. (2005). Hyaluronic acid: A unique topical vehicle for the localized delivery of drugs to the skin. *Journal of the European Academy of Dermatology and Venereology*, 19(3), 308–318. doi: 10.1111/j.1468-3083.2004.01180.x.
- Budiasih, S., I. Masyitah, K. Jiyuddin, M. Kaleemullah, A. D. Samer, A. Mohd Fadli, and Eddy Yusuf. (2018). Formulation and Characterization of Cosmetic Serum Containing Argan Oil as Moisturizing Agent. *Proceedings of BROMO Conference (BROMO 2018)*, 297–304.
- Bukhari, S. N. A., Roswandi, N. L., Waqas, M., Habib, H., Hussain, F., Khan, S., ... Hussain, Z. (2018). Hyaluronic acid, a promising skin rejuvenating biomedicine: A review of recent updates and pre-clinical and clinical investigations on cosmetic and nutricosmetic effects. *International Journal of Biological Macromolecules*, 120, 1682–1695. doi: 10.1016/j.ijbiomac.2018.09.188.
- Choi, S. Y., Kwon, H. J., Ahn, G. R., Ko, E. J., Yoo, K. H., Kim, B. J., ... Kim, D. (2017). Hyaluronic acid microneedle patch for the improvement of Crow's feet wrinkles. *Dermatologic Therapy*, 30(6), 1–5. doi: 10.1111/dth.12546.
- Dréno, B., Zuberbier, T., Gelmetti, C., Gontijo, G., Marinovich, M. (2019). Safety review of phenoxyethanol when used as a preservative in cosmetics. *Journal of the European Academy of Dermatology and Venereology*, 33, 15-24.
- Essendoubi, M., Gobinet, C., Reynaud, R., Angiboust, J. F., Manfait, M., Piot O. (2016). Human skin penetration of hyaluronic acid of different molecular weights as probed by Raman Spectroscopy. *Skin Research and Technology*, 22(1):55–62. doi: 10.1111/srt.12228.
- Farage, M. A. (2019). The prevalence of sensitive skin. *Frontiers in medicine*, 6, 98.
- Friedmann, P. S., & Ardern-Jones, M. (2010). Patch testing in drug allergy. *Current Opinion in Allergy and Clinical Immunology*, 10(4), 291–296. doi: 10.1097/ACI.0b013e32833aa54d.
- Iturriaga, V., Vasquez, B., Bornhardt, T., del Sol, M. (2021). Effects of low and high molecular weight hyaluronic acid on the osteoarthritic temporomandibular joint in rabbit. *Clinical Oral Investigations*, 25(7), 4507-4518. doi: 10.007/s00784-020-03763-x.
- Jang, M., S. Baek, G. Kang, H. Yang, S. Kim, and H. Jung. (2020). Dissolving microneedle with high molecular weight hyaluronic acid to improve skin wrinkles, dermal density and elasticity.. *International Journal of Cosmetic Science*, 42(3), 302–309. doi: 10.1111/ics.12617.

- Jegasothy, S. M., Zabolotniaia, V., and Bielfeldt, S. (2014). Efficacy of a new topical nano-hyaluronic acid in humans. *Journal of Clinical and Aesthetic Dermatology*, 7(3), 27–29.
- Karakucuk, A., Tort, S., Han, S., Oktay, A.N., Celebi, N. (2021). Etodolac nanosuspension based gel for enhanced dermal delivery: in vitro and in vivo evaluation, *Journal of Microencapsulation*, 38(4), 218-232. doi: 10.1080/02652048.2021.1895344
- Kasemsarn, P., & Boonchai, W. (2012). Usefulness of patch testing in dermatology. *Siriraj Medical Journal*, 64(2), 73–77.
- Kong, M., Chen, X. G., Kweon, D. K., and Park, H. J. (2011). Investigations on skin permeation of hyaluronic acid-based nanoemulsion as transdermal carrier. *Carbohydrate Polymers*, 86(2), 837-843.
- Lachapelle, J. M., & Maibach, H. I. (2009). Patch testing and prick testing. 2nd ed. International Contact Dermatitis Research Group (ICDRG), Springer, 33- 70.
- Lee, D. H., Oh, I. Y., Koo, K. T., Suk, J. M., Jung, S. W., Park, J. O., ... Choi, Y.M. (2015). Improvement in skin wrinkles using a preparation containing human growth factors and hyaluronic acid serum. *Journal of Cosmetic and Laser Therapy*, 17(1), 20–23. doi: 10.3109/14764172.2014.968577.
- Lee, J. S., Cho, J. H., An, S., Shin, J., Choi, S., Jeon, E. J., Cho, S. W. (2019). In situ self-cross-linkable, long-term stable hyaluronic acid filler by gallol autoxidation for tissue augmentation and wrinkle correction. *Chemistry of Materials*, 31(23), 9614–9624. doi: 10.1021/acs.chemmater.9b02802.
- Lee, Y. J., Kim, H. T., Lee, W. J., Chang, S. E., Lee, M. W., Choi, J. H., and Won, C. H. (2019). Anti-Aging and Hydration Efficacy of a Cross-Linked Hyaluronic Acid Microstructure Patch. *Dermatologic Therapy*, 32(3), 2–5. doi: 10.1111/dth.12888.
- Özden, P., & Avcı, N. (2017). Asılı ve tek damla yöntemiyle liyotropik sıvı kristallerde yüzey gerilimlerinin belirlenmesi. *Balikesir Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 19(3), 129-134. doi: 10.25092/baunfbed.366210
- Panico, A., Serio, F., Bagordo, F., Grassi, T., Idolo, A., De Giorgi, M., ... and De Donno, A. (2019). Skin safety and health prevention: an overview of chemicals in cosmetic products. *Journal of preventive medicine and hygiene*, 60(1), E50.
- Poetschke, J., Schwaiger, H., Steckmeier, S., Ruzicka, T., and Gauglitz, G. G. (2016). Anti-wrinkle creams with hyaluronic acid: How effective are they? *MMW-Fortschritte Der Medizin*, 158(4), 1–6.
- Seweryn, A. (2018). Interactions between surfactants and the skin—theory, and practice. *Advances in Colloid and Interface Science*, 256, 242-255.
- Surini, S., Mubarak, H., and Ramadon, D. (2018). Cosmetic serum containing grape (*Vitis vinifera* L.) seed extract phytosome: Formulation and in vitro penetration study. *Journal of Young Pharmacists*, 10(2), s51–55. doi: 10.5530/jyp.2018.2s.10.
- Xie, J., Ji, Y., Xue, W., Ma, D., & Hu, Y. (2018). Hyaluronic acid-containing ethosomes as a potential carrier for transdermal drug delivery. *Colloids and Surfaces B: Biointerfaces*, 172, 323-329.
- Wu, X., Zhang, H., He, S., Yu, Q., Lu, Y., Wu, W., ... Qi, J. (2020). Improving dermal delivery of hyaluronic acid by ionic liquids for attenuating skin dehydration. *International Journal of Biological Macromolecules*, 150, 528–535. doi: 10.1016/j.ijbiomac.2020.02.072.

Association Between *TP53* Gene Polymorphism and Obesity

Mehmethan CİHAN* , Hakan BULUŞ** , Onur DİRİCAN*** ,
Serpil OĞUZTÜZÜN**** , Doğan ÖZTÜRK***** , Abdulkadir ÜNSAL***** ,
Ahmet Oğuz ADA***** , Mümtaz İŞCAN*****

Association Between *TP53* Gene Polymorphism and Obesity

TP53 Gen Polimorfizmi ve Obezite Arasındaki İlişki

SUMMARY

Obesity is a chronic disorder with increasing prevalence worldwide and occurs when energy intake is greater than energy expenditure. Obesity is one of the factors that cause oxidative stress and arises from an imbalance between the reactive oxygen species (ROS) and the cell's antioxidant defense system. Increasing ROS in obesity, influencing the hypothalamic neurons, affects hunger and satiety control, so correspondingly on body weight control. When ROS amount increases, through DNA, protein and lipid oxidation, cell damage, necrosis, and apoptosis take place. Tumor protein p53, the guardian of the genome, is responsible for the regulation of genes involved in apoptosis as well as energy generating metabolic pathways. In our study, we investigated the *TP53* (Arg72Pro) polymorphism in 151 patients diagnosed with obesity. *TP53* mutation (rs1042522) was determined by real-time PCR. In 8 patients, the *TP53* mutation was identified as carrying heterozygous (Arg72Pro) and in 143 patients carrying homozygous (wild type) (Arg72Arg). No individual with a homozygous mutant (Pro72Pro) genotype was found in the studied group. Associations between *TP53* genotypes and clinical obesity parameters such as body mass index, thyroid stimulating hormone, glucose, postprandial blood sugar, triglyceride and cholesterol levels were compared statistically. According to the results of statistical analysis, it was observed that *TP53* polymorphism was associated with insulin level. Genotype frequencies were also compared with previous studies performed in control populations and found to be different. This study shows that there may be a relationship between *TP53*(Arg72Pro) polymorphism and obesity.

Key Words: Obesity, Oxidative stress, *TP53*, Polymorphism.

ÖZ

Obezite, alınan enerjinin, harcanan enerjiden fazla olmasından kaynaklanan, tüm dünyada prevalansı endişe verici şekilde artan kronik bir hastalıktır. Obeziteye neden olan etkenlerden biri olan oksidatif stres, reaktif oksijen türleri (ROT) ile hücrenin antioksidan savunma sistemi arasındaki dengesizlikten ortaya çıkar. Obezitede artış gösteren ROT'lar hipotalamik nöronlar üzerinde etkili olarak, açlık ve tokluğun kontrolünde ve buna bağlı olarak vücut ağırlığının kontrolünde etkili olurlar. ROT arttığında, DNA, protein ve lipitlerin oksidasyonu yoluyla hücre zedelemesi, nekroz ve apoptoz oluşur. Genomun koruyucusu olan tümör proteini p53, enerji üreten metabolik yolların yanı sıra apoptozda yer alan genlerin düzenlenmesinden sorumludur. Çalışmamızda obezite tanısı almış 151 hastada *TP53* (Arg72Pro) polimorfizmi araştırıldı. *TP53* mutasyonu (rs1042522), gerçek zamanlı PCR ile belirlendi. 8 hastada *TP53* mutasyonu heterozigot taşıyan (Arg72Pro) ve 143 hastada homozigot taşıyan (yabanıl tip) (Arg72Arg) olarak tanımlandı. Çalışılan grupta homozigot mutant (Pro72Pro) genotipine sahip birey bulunamadı. *TP53* genotipleri ile vücut kitle indeksi, tiroit stimüle edici hormon, glukoz, tokluk kan şekeri, trigliserit ve kolesterol düzeyleri gibi klinik obezite parametreleri arasındaki ilişkiler istatistiksel olarak karşılaştırıldı. İstatistiksel analiz sonuçlarına göre *TP53* polimorfizminin insülin düzeyi ile ilişkili olduğu gözlemlendi. Ayrıca genotip frekansları kontrol popülasyonlarında gerçekleştirilen önceki çalışmalarla karşılaştırıldı ve farklı olduğu bulundu. Bu çalışma, *TP53* (Arg72Pro) polimorfizmi ile obezite arasında ilişki olabileceğini göstermektedir.

Anahtar Kelimeler: Obezite, Oksidatif stres, *TP53*, Polimorfizm.

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* ORCID: 0000-0001-8701-754X, University of Health Sciences; Keçiören Training and Research Hospital, General Surgery Department; Ankara/Turkey

** ORCID: 0000-0001-7439-8099, University of Health Sciences; Keçiören Training and Research Hospital, General Surgery Department; Ankara/Turkey

*** ORCID: 0000-0003-0511-6611, Kırıkkale University Faculty of Science; Department of Biology, Kırıkkale/Turkey

**** ORCID: 0000-0002-5892-3735, Kırıkkale University Faculty of Science; Department of Biology, Kırıkkale/Turkey

***** ORCID: 0000-0003-1754-9246, University of Health Sciences; Keçiören Training and Research Hospital, General Surgery Department; Ankara/Turkey

***** ORCID: 0000-0002-7989-4232, University of Health Sciences; Keçiören Training and Research Hospital, General Surgery Department; Ankara/Turkey

***** ORCID: 0000-0001-9987-0572, Ankara University Faculty of Pharmacy Department of Toxicology; Ankara/Turkey.

***** ORCID: 0000-0001-5839-4987, Cyprus International University, Faculty of Pharmacy, Lefkoşe, Turkish Republic of Northern Cyprus.

INTRODUCTION

The prevalence and incidence of obesity, a public health problem that has gained importance recently, increases day by day. Obesity badly affects life expectancy (Mensah, 2004). Obesity both reduces the quality of life and shortens its duration. Because of that, diet, exercise and medical treatments have been applied, and the lack of success has become the focus of researches on hormones, mediators and genes that may be the source of surgical interventions (Bertakis&Azari, 2005). Obesity is accepted as an increasing disease in the world and in our country, which occurs as a result of the interaction of genetic and environmental factors. Obesity is the result of the body's fat mass to lean mass, and the body weight is higher than the expected level according to the height fit. Body Mass Index (BMI) is classified as 25-29,9 kg/m² overweight, 30-34,9 kg/m² obese, over morbidity obesity according to the World Health Organization (WHO) (Aydemir, 2006). BMI can be easily calculated by dividing body weight in kilograms by the square of the neck in meters (Body weight / height²) and its unit is kg / m². BMI can be easily calculated by dividing body weight in kilograms by the square of the neck in meters (Body weight/height²) and its unit is kg/m². Obesity is increasingly becoming an epidemic problem. The MONICA study carried out by WHO in 6 different regions of Asia, Africa and Europe and lasting for 12 years, it was reported that an increase in the prevalence of obesity between 10 and 30% in 10 years (Silventoinen, 2004). According to WHO, it is estimated that there are over 1.9 billion overweight and 650 million obese adults worldwide in 2016. The risk of cardiometabolic disease increases significantly in obese and overweight patients. Obesity, even if carbohydrate metabolism is normal, endothelial dysfunction, dyslipidemia, hypertension (HT) and vascular inflammation may develop due to insulin resistance and increased adipokines. All these pathogenetic changes contribute to the development of atherosclerosis. The atherosclerotic process becomes more severe and accelerated with the decline of car-

bohydrate metabolism. The frequency of cardiometabolic diseases and other systemic problems increases in proportion to the severity and duration of obesity. These accompanying diseases with obesity increase the risk of developing complications and causes some difficulties in the process of regulation of treatment (Artham, 2009). The International Cancer Research Agency announced the relationship between obesity and many types of cancer in 2002. Particularly noteworthy cancers are colon, postmenopausal breast, endometrial, kidney and esophageal cancers. A cohort study containing 900,000 cases in the USA showed the contribution of obesity to 11% in postmenopausal colon cancer, 9% in breast cancer, 39% in endometrial cancer, 25% in kidney cancer, and 37% in esophageal cancer. It has also been shown that the risk of cancer increases as the degree of obesity increases (Calle et al, 2004; Birmingham, 2009). The mechanism of the relationship associated with obesity is multifactorial. Increasing insulin activates the IGF-1 pathway, causing an increase in cancer cells. Also, adipocytokines are thought to play a role in the mechanism. Colon, prostate and breast cancer leptin levels positive with endometrial, breast, colon and prostate because there is a negative correlation between cancer. Also, those associated with obesity are hypoxia, genetic predisposition and increased inflammation are also accused factors in the obesity-cancer relationship. Obese cases should be followed up in terms of cancer risk besides metabolic diseases and they should be supported about this issue (Hursting, 2010; Basen-Engquist, 2011).

Although oxygen is essential for human life, it is produced during normal metabolism. Some types of reactive oxygen have the potential to intense harm to the body (Diplock, 1998). Reactive oxygen species (ROS), mostly formed by free radicals, with normal oxygen molecules are oxygen forms with higher chemical reactivity (Nawar, 1996). It is well known that ROS increases obesity. ROS act on hypothalamic neurons effective in controlling hunger and satiety and, consequently, body weight. The increase of

ROS also causes cell damage, necrosis and apoptosis through the oxidation of DNA, proteins and lipids (Buyukuslu & Yigitbasi, 2015). The unpaired electrons in free radicals give them huge reactivity that damage protein, lipid, DNA and nucleotides. This harm promotes aging to these components of the body and causes degenerative diseases such as, cardiovascular diseases, various types of cancers, cataracts, weakened immunity and nervous system disorders. (Diplock, 1998). Oxygen metabolism in living cells, environmental pollutants, various factors such as radiation, pesticides, various medical treatments, and contaminated waters are inevitably led to the formation of free radicals: single oxygen (O_2), superoxide anion (O_2^-), hydroxy (OH), peroxy (ROO) and alkoxy (RO) radicals (Kaur&Kapoor, 2001). Different natural defense systems in the body keep free radicals under control against the damages of reactive oxygen species. These systems are found in different cells and prevent oxidation caused by free radicals. The substances having the ability to capture and stabilize free radicals are so-called "antioxidants" (Nawar, 1996; Diplock, 1998). Enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase, vitamins such as vitamin C and vitamin E, and compounds such as uric acid, bilirubin and polyphenols are well known antioxidants and generally responsible for limiting free radicals damaging cellular components such as DNA, proteins and lipids (Diplock, 1998; Elliot, 1999; Ou, 2002). Oxidative DNA damage is important in the pathogenesis of many diseases, especially carcinogenesis. It is known to play a role. High reactivity having hydroxyl radicals on oxidative stress and intracellular structures as in lipids and proteins as it is said, H atom to double bonds in DNA bases by adding or from the C-H bonds of 2-deoxyribose and H atom from methyl groups in thymine structure it reacts with the DNA molecule (Breen, 1995). The thymine peroxy radicals formed are reduced and oxidation products such as hydroxy hydroperoxides, thymine glycol, 5-hydroxy methyl uracil, 5-formyl uracil and 5-hydroxy 5-methyl hydantoin. Hydroxyl radicals (OH \cdot) interact at the 8th position in the gua-

nine molecule, leading to oxidation. Undergoing as a result of oxidative damage of DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) has formed. Besides, Cu^{+2} ions have a high affinity for DNA, especially connected guanine bases. By interacting with H_2 and O_2 , they contribute to DNA damage. DNA adduct 8-OHdG is the most known marker of oxidative DNA damage. (Helbock, 1999). Oxidative stress occurs as a result of the disruption between the formation of ROS and the inactivation of these products by the antioxidant defense system. Adipose tissue is one of the main sources for the formation of ROS, and fat accumulation is closely related to increased oxidative stress through NADPH oxidase activation.

P53 is a transcription factor that regulates the cell cycle (Ngo et al. 2010). A central role in the p53 cell cycle is an important tumor suppressor that plays, it acts as a transcription factor. DNA damage, hypoxia, oxidative stress, oncogene cellular activation, such as telomere erosion after the stress signals p53 is activated. TP53 targets genes in cellular aging, angiogenesis and it also plays a role in autophagy (Vousden, 2009; Bieging, 2014). P53 is a powerful tumor suppressor. P53 protein in more than 50% of cancers inactivated due to TP53 gene mutation state. TP53 gene mutation its prevalence is different in various cancers (Leroy, 2014). P53 is shown to be responsible for poor prognosis in most studies (Sheikh, 2003). The *TP53* gene encoding p53 localized in chromosome 17p13.1 is the most common target of genetic change in human tumors. A little over 50% of tumors carry a mutation in this gene. Almost every cancer, including lung, colon, and breast carcinomas, which are the three leading causes of cancer-related deaths, have a homozygous loss in *TP53* gene activity (Baselga & Norton, 2002). Physiological p53 protein has a role in stopping the cell cycle due to DNA damage and apoptosis, and mutation in the *TP53* gene is the most common single gene mutation in human cancers (Cross et al. 1995). Publications are reporting that the presence of mutant p53 protein is associated with poor prognosis in many cancers such as lung, breast, prostate and blad-

der cancers (Quinlan et al. 1992; Moul, 1999). It is known that p53, which plays a suppressor role, has an important role in preventing cancer formation mechanisms (Oguztuzun, 2016).

Obesity accompanying morbidities decreases lifetime and quality. In the USA, 11869 nurses were followed up for 13 years, and an increase in cardiovascular and cancer-related deaths was detected in the fatter group. Overweight women, even within normal limits, have a higher risk of coronary heart disease than those who are not overweight (Kopelman & Stock, 2000). In 8800 men who have been followed up for twenty-six years, the mortality rate due to all causes was 2 times higher in obese and 3.3 times higher due to coronary heart disease (Kırım S., 2005). In a prospective research study in nine hundred thousand men and women who examined cancer-related deaths during the 16-year follow-up period, a positive correlation was found between overweight and mortality due to many types of cancer (Calle et al. 2003). Although it is known that genetic factors are effective in both disorders, the information obtained through intense studies to date still cannot fully explain their genetic basis.

Therefore, in this study it is aimed to reveal the polymorphic condition in the *TP53* gene in obese patients. Besides, it was aimed compare the clinical data of the patients whose *TP53* polymorphic states were revealed in obesity.

MATERIALS AND METHODS

The study group was consisted of 151 obese patients who underwent bariatric surgery in Ankara Keçiören Training and Research Hospital General Surgery Service in 2017. Ethics committee approval of this study was provided by the decision of the Ethics Committee of Keçiören Education and Research Hospital with the decision numbered 2012-KAEK-15/1160.

Blood samples were collected from patients participating in the study after their informed consents. The diagnosis, all information available for the opera-

tion of obesity, and appropriateness of the blood samples collected were made at the same hospital service. The patients consisted of 23 men and 128 women. The average age of the patients was 39, the average BMI of the patients was 46.5 kg/m², the average of the TSH levels was 2.5, the average of the insulin levels was 19.5, the average of glucose levels in the blood was 111, the average of postprandial blood sugar levels was 137.6, the mean of triglycerides was 176, and the cholesterol level averages was 215.6.

DNA isolation from blood samples collected was performed by adhering to the PROMEGA[®] blood DNA isolation kit protocol. *TP53* genotyping was performed by real time PCR LightCycler[®] 480 device. Primers and probes specified in the method of Talseth et al. (2006) were used to determine the genetic polymorphism (rs1042522) in the *TP53* gene (Arg72Pro) encoding the p53 protein. Sequences of primers and probes are shown in Table 1. In order to confirm the efficiency of the primers used in the study and the base size of the studied gene region, the primers were controlled by a 1.5% electrophoresis gel study after conventional PCR. Base sizes were compared for *TP53* using the National Center for Biotechnology Information's (NCBI) international database. During the real time PCR stage, the testing phase of the study was carried out by using FastStart Essential DNA Probes Master (06402682001), Lightcycler 480 Multiwell Plate 96 (04729692001) and Lightsnip *TP53* probe (07330782001) (Roche Applied Science). Components used in the PCR stage are as follows; 12.5µL from 2x OneTaq Quick LoadMaster Mix solution, 1µL from Forward primer (10pM), 1µL from Reverse primer (10pM), 2.5µL from cDNA (50ng/µL), 2.5µL probe, and DNase/RNase free water calculated for in a 25 µL total volume. PCR conditions were performed as follows; Initial Denaturation is 5 minutes at 95°C, 50 cycles of denaturation at 95°C for 10 seconds, Annealing at 58°C for 20 seconds and Extension at 72°C for 20 seconds. 1 cycle, 5 seconds at Acquisition 95°C and 1 minute at 55°C. 10 seconds at Final Extension 40°C.

Table 1. Primers and fluorescent probes used in the study.

Primer/Probe	Sequence
Forward primer	5'-CCAGATGAAGCTCCCAGAATGC-3'
Reverse primer	5'-GCCGCCGGTGTAGGA-3'
Wildtype probe	5'-VIC-TCCCCGCGTGGCC-3'
Mutant probe	5'-FAM-CTCCCCCGTGGCC-3'

The definition of *TP53* R72P polymorphism using the allelic discrimination method was as follows; Samples with a peak at 58°C were interpreted as wild type (G/G) and samples with a peak at 66°C were interpreted as mutant type (C/C). Samples with both degrees

of the peak in each same sample were evaluated as (G/C) Heterozygous. One representative sample from each of the three genotypes was included as the reaction control for each “PCR run” as an internal control. (Figure 1).

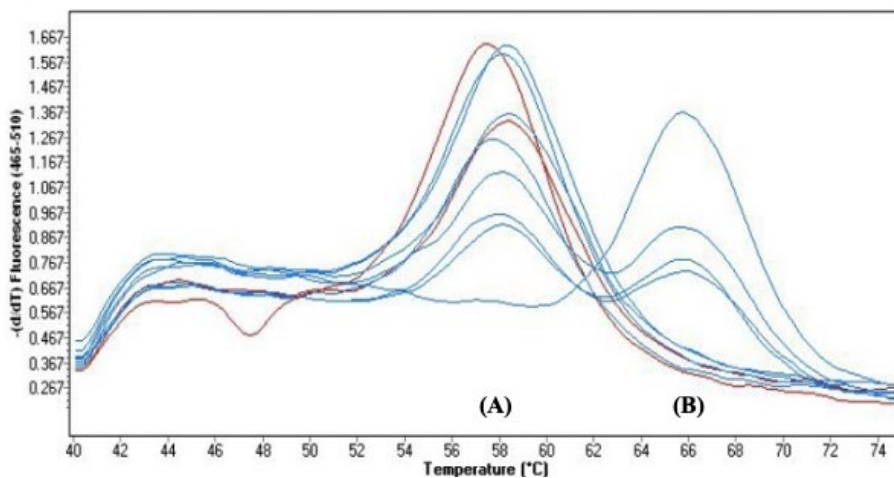


Figure 1. Allelic discrimination of *TP53* (Arg72Pro) polymorphism.

Identification of *TP53* (Arg72Pro) polymorphism by allelic discrimination Overview of a PCR reaction performed using the method. (A): General view of Wild Type (Arg/Arg) samples peaking at 58°C. (B): View of Mutant (Pro/Pro) internal control with a peak at 66°C. (A&B): General view of Heterozygous (Arg/Pro) samples with a peak at both degrees.

Clinical data of patients from whom samples were obtained were also compared with cross statistical data and interpreted by comparing their significance with real time PCR data. Statistical Package for the Social Sciences (SPSS) package software ANOVA analysis were used to compare the clinical data of the patients with the cross-data of the results.

RESULTS AND DISCUSSION

In our study, *TP53* genetic polymorphism in patients diagnosed with obesity were investigated and their relationship to the disease was interpreted. Genotype distributions of 151 obese patients were determined as *TP53* mutation carrying heterozygous (Arg72Pro) in 8 patients and homozygous (wild type) carrying (Arg72Arg) in 143 patients. No individual with a homozygous mutant (Pro72Pro) genotype was found in the studied group. The clinical parameters and genotypes of *TP53* mutation, heterozygous and homozygous obese patients were compared. As seen in Table 2; after statistical analysis, it is seen that the polymorphism in *TP53* is related to the insulin parameter. It

has been observed that the insulin level is higher in heterozygous individuals than in homozygous individuals. In obese patients, no statistically significant relationships were found between BMI, TSH, glucose, postprandial blood sugar, triglyceride and cholesterol levels. In obese individuals, significant changes occur in *TP53* polymorphism. While these changes may occur as an adaptive response, the findings suggest that the oxidative stress observed in obesity may be one of the possible mechanisms underlying this change.

Besides, it is supported that *TP53* variant genotype can contribute to insulin changes. Accordingly, the insulin level made a difference in heterozygous and homozygous obese individuals. The level of insulin was higher in heterozygous individuals than in homozygote individuals. But other parameters were not related. This result is in line with the study of Bonfigli et al., (2013) who found that (Arg72Pro) polymorphism was associated with insulin resistance in type 2 diabetic subjects.

Table 2. Correlation between blood parameters and *TP53* polymorphisms.

<i>TP53</i>	Variance Analysis		ANOVA	
	Levene Statistic	Sig.	F	Sig.
BMI	0.821	0.366	0.021	0.886
TSH	1.358	0.246	1.753	0.187
Insulin	67.629	0.0001	14.311	0.0001
Glucose	0.334	0.564	0.120	0.730
Blood sugar (postprandial)	0.590	0.444	0.330	0.567
Trigliserid	0.330	0.566	0.004	0.949
Cholesterol	0.147	0.702	0.011	0.916

When we compare the results of our study with previous studies conducted for (Arg72Pro) polymorphism, we observed that there are significant differences between genotype frequencies of obese individuals in our study group and genotype frequencies of healthy individuals in “Caucasian” populations (Table 3). Although these comparative studies were studies investigating gastric tissue differentiation, the

control groups were completely composed of healthy individuals. This finding shows that *TP53* (Arg72Pro) polymorphism might be associated with obesity. Our findings can also contribute to the definition of the physiopathology of obesity, and other diseases such as cardiovascular diseases that may develop due to obesity. It is important in preventing complications and developing preventive approaches.

Table 3. The distribution of *TP53* genotypes in Caucasian control populations and in this study.

Study (Reference)	Country/Region	Ethnicity	Total n	Arg72Arg		Arg72Pro		Pro72Pro	
				n	%	n	%	n	%
Capella et al. (2008)	Europe	Caucasian	1056	588	56	399	37.8	69	6.2
De Feo et al. (2009)	Italy	Caucasian	295	169	57.3	102	34.5	24	8.2
Belyavskaya et al. (2006)	Russia	Caucasian	125	60	48	46	36.8	19	15.2
Alpizar-Alpizar et al. (2005)	Spain	Caucasian	47	26	55.3	17	36.2	4	8.5
Zhang et al. (2003)	UK	Caucasian	277	125	45.1	129	46.5	23	8.4
Sul et al. (2006)	USA	Caucasian	134	51	38.1	61	45.5	22	16.4
Engin et al. (2011)	Turkey	Caucasian	108	52	48.1	42	38.8	14	13.1
This study	Turkey	Caucasian	151	143	94.7	8	5.2	0	0

CONCLUSION

In conclusion, regarding the comparative evaluation of the results of the *TP53* polymorphism state obtained in our study with similar studies in the literature, it has been determined that it may be associated with obesity, but further studies are needed to verify this relationship. It was thought that investigating the association of obesity and *TP53* (Arg72Pro) polymorphism in different ethnic groups and larger populations would benefit the emergence of the relationship with metabolic diseases related with obesity. To our knowledge, *TP53* polymorphism was investigated in obese patients for the first time in a Turkish population. In our study, it is hoped that it will be a reference for revealing the genetic backgrounds of obesity, clarifying the molecular mechanism of the disease and developing genetic risk panels for early diagnosis and making this information available in the management of treatment.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Designing the concept and drafted the manuscript (MC, HB and SO). Preparing the figures (SO, OD). Ethical approval and sample collection and clinical data (MC, HB, DÖ, and AÜ). Carried out the laboratory applications of this study (MC, OD). AOA and OD reviewed the existing journal policy. Contributing to the writing of the final version of the manuscript (MC, HB, OD, SO, DÖ, AÜ, AOA, Mİ).

REFERENCES

- Alpizar-Alpizar, W., Sierra, R., Cuenca, P., Une, C., Mena, F., Pérez-Pérez, G. I. (2005). Association of the p53 codon 72 polymorphisms to gastric cancer risk in a high risk population of Costa Rica. *Revista de Biología Tropical*, 53(3-4), 317–324.
- Artham, S. M., Lavie, C. J., Milani, R. V., Ventura, H. O. (2009). Obesity and hypertension, heart failure, and coronary heart disease-risk factor, paradox, and recommendations for weight loss. *The Ochsner Journal*, 9(3), 124–132.
- Aydemir Ö. (2006). Sağlıkla İlgili Yaşam Kalitesinin Klinik Uygulamalarda Kullanımı. *Sağlıkta Birlik*, 2(1), 6–8.
- Baselga, J., Norton, L. (2002). Focus on breast cancer. *Cancer Cell*, 1(4), 319–322. [https://doi.org/10.1016/s1535-6108\(02\)00066-1](https://doi.org/10.1016/s1535-6108(02)00066-1).
- Basen-Engquist, K., & Chang, M. (2011). Obesity and cancer risk: recent review and evidence. *Current Oncology Reports*, 13(1), 71–76. <https://doi.org/10.1007/s11912-010-0139-7>.
- Belyavskaya, V. A., Vardosanidze, V. K., Smirnova, O. Y., Karakin, E. I., Savkin, I. V., Gervas, P. A., Cherdyntseva, N. V., & Voevoda, M. I. (2006). Genetic status of p53 in stomach cancer: somatic mutations and polymorphism of codon 72. *Bulletin of Experimental Biology and Medicine*, 141(2), 243–246. <https://doi.org/10.1007/s10517-006-0139-7>.
- Bertakis, K. D., & Azari, R. (2005). Obesity and the use of health care services. *Obesity Research*, 13(2), 372–379. <https://doi.org/10.1038/oby.2005.49>
- Biegging, K., Mello, S. & Attardi, L. (2014). Unravelling mechanisms of p53-mediated tumour suppression. *Natural Review of Cancer*, 14, 359–370. <https://doi.org/10.1038/nrc3711>.
- Birmingham, J. M., Busik, J. V., Hansen-Smith, F. M., & Fenton, J. I. (2009). Novel mechanism for obesity-induced colon cancer progression. *Carcinogenesis*, 30(4), 690–697. <https://doi.org/10.1093/carcin/bgp04>.
- Bonfigli, A. R., Sirolla, C., Testa, R., Cucchi M., Spazafumo L., Salvioli, S., Ceriello, A., Olivieri, F., Festa, R., Procopio, A. D., Brandoni, G., Boemi, M., Marra, M., Franceschi, C. (2013). The p53 codon 72 (Arg72Pro) polymorphism is associated with the degree of insulin resistance in type 2 diabetic subjects: a cross-sectional study. *Acta Diabetol* 50, 429–436. <https://doi.org/10.1007/s00592-012-0450-x>
- Breen, A. P., & Murphy, J. A. (1995). Reactions of oxyl radicals with DNA. *Free Radical Biology & Medicine*, 18(6), 1033–1077. [https://doi.org/10.1016/0891-5849\(94\)00209-3](https://doi.org/10.1016/0891-5849(94)00209-3).

- Buyukuslu N., Yigitbasi T. (2015). Reaktif oksijen türleri ve obezitede oksidatif stres. *Clinical and Experimental Health Sciences*, 5(3),197-203. <https://dx.doi.org/10.5455/musbed.20150604061607>
- Calle, E. E., Kaaks, R. (2004). Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature Reviews. Cancer*, 4(8),579–591. <https://doi.org/10.1038/nrc1408>.
- Calle, E. E., Rodriguez, C., Walker-Thurmond, K., Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *The New England Journal of Medicine*, 348(17), 1625–1638. <https://doi.org/10.1056/NEJMoa021423>.
- Capellá, G., Pera, G., Sala, N., Agudo, A., Rico, F., Del Giudice, G., Plebani, M., Palli, D., Boeing, H., Bueno-de-Mesquita, H. B., Carneiro, F., Berrino, F., Vineis, P., Tumino, R., Panico, S., Berglund, G., Simán, H., Nyrén, O., Hallmans, G., Martinez, C., ... González, C. A. (2008). DNA repair polymorphisms and the risk of stomach adenocarcinoma and severe chronic gastritis in the EPIC-EURGAST study. *International Journal of Epidemiology*, 37(6), 1316–1325. <https://doi.org/10.1093/ije/dyn145>.
- Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., Reid, B. J. (1995). A p53-dependent mouse spindle checkpoint. *Science*, 267(5202), 1353–1356. <https://doi.org/10.1126/science.7871434>.
- De Feo, E., Persiani, R., La Greca, A., Amore, R., Arzani, D., Rausei, S., D’Ugo, D., Magistrelli, P., van Duijn, C. M., Ricciardi, G., Boccia, S. (2009). A case-control study on the effect of p53 and p73 gene polymorphisms on gastric cancer risk and progression. *Mutation Research*, 675(1-2), 60–65. <https://doi.org/10.1016/j.mrgentox.2009.02.009>.
- Diplock, A. (1998). “Healthy Lifestyles Nutrition and Physical Activity: Antioxidant Nutrients,” *ILSI Europe Concise Monograph Series*, Belgium, p. 59.
- Elliot, J.G. 1999. Application of antioxidant vitamins in foods and beverages. *Food Technology*. 53(2); 46-48.
- Engin, A. B., Karahalil, B., Karakaya, A. E., & Engin, A. (2011). Association between XRCC1 ARG-399GLN and P53 ARG72PRO polymorphisms and the risk of gastric and colorectal cancer in Turkish population. *Arhiv Za Higijenu Rada I Toksikologiju*, 62(3), 207–214. <https://doi.org/10.2478/10004-1254-62-2011-2098>.
- Helbock, H. J., Beckman, K. B., & Ames, B. N. (1999). 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. *Methods in Enzymology*, 300, 156–166. [https://doi.org/10.1016/s0076-6879\(99\)00123-8](https://doi.org/10.1016/s0076-6879(99)00123-8).
- Hursting, S. D., & Berger, N. A. (2010). Energy balance, host-related factors, and cancer progression. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 28(26), 4058–4065. <https://doi.org/10.1200/JCO.2010.27.9935>.
- Jones, J. S., Chi, X., Gu, X., Lynch, P. M., Amos, C. I., & Frazier, M. L. (2004). p53 polymorphism and age of onset of hereditary nonpolyposis colorectal cancer in a Caucasian population. *Clinical cancer research: an official journal of the American Association for Cancer Research*, 10(17), 5845–5849. <https://doi.org/10.1158/1078-0432.CCR-03-0590>
- Katkoori, V. R., Manne, U., Chaturvedi, L. S., Basson, M. D., Haan, P., Coffey, D., & Bumpers, H. L. (2017). Functional consequence of the p53 codon 72 polymorphisms in colorectal cancer. *Oncotarget*, 8(44), 76574.
- Kaur, C. and Kapoor, H.C., 2001. Antioxidants in fruits and vegetables-the millennium’s health. *Int. J. Food Sci. Tech.* 36; 703-725.
- Kırım S., (2005). Obez hastalarda diyet, egzersiz ve ilaç tedavisinin homosistein düzeylerine etkisi. Cukurova Üniversitesi Tıp Fakültesi İç Hastalıkları A.D. Adana.

- Kopelman P.G, Stock J.M. (2000). Klinik obezite. 1. baskı, And Yayıncılık, İstanbul,124-156.
- Leroy, B., Anderson, M., Soussi, T. (2014). TP53 mutations in human cancer: database reassessment and prospects for the next decade. *Human Mutation*, 35(6), 672–688. <https://doi.org/10.1002/humu.22552>.
- Mensah, G. A., Mokdad, A. H., Ford, E., Narayan, K. M., Giles, W. H., Vinicor, F., & Deedwania, P. C. (2004). Obesity, metabolic syndrome, and type 2 diabetes: emerging epidemics and their cardiovascular implications. *Cardiology Clinics*, 22(4), 485–504. <https://doi.org/10.1016/j.ccl.2004.06.005>
- Metropolitan Height and Weight Tables. Metropolitan Life Foundation, statistical bulletin 1983; 64(1): 2-9.
- Moul J. W. (1999). Angiogenesis, p53, bcl-2 and Ki-67 in the progression of prostate cancer after radical prostatectomy. *European Urology*, 35(5-6), 399–407. <https://doi.org/10.1159/000019916>.
- Nawar, W.W. 1996. Lipids. *Food Chemistry*, 225-319.
- Ngo, NT., Tan, E., Tekkis, P. (2010) Differential expression of p53 and p504s in hyperplastic polyp, sessile serrated adenoma and traditional serrated adenoma. *Int J Colorectal Dis*,25, 1193–1200. <https://doi.org/10.1007/s00384-010-1007-5>.
- Oguztuzun, S., Ada, A. O., Kilic, M., Cakir, E., & Yilmaz, A. (2013). Prognostic Significance of Caspase-3, Bcl-2, P53 and GSTPI Expressions in Lung Adenocarcinoma/Akciger adenokanserlerinde kaspaz-3, bcl-2, p53 ve GSTPI ekspresyonlarının prognostik önemi. *FABAD Journal of Pharmaceutical Sciences*, 38(2), 83.
- Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural and Food Chemistry*, 50(11), 3122–3128. <https://doi.org/10.1021/jf0116606>
- Quinlan, D. C., Davidson, A. G., Summers, C. L., Warden, H. E., & Doshi, H. M. (1992). Accumulation of p53 protein correlates with a poor prognosis in human lung cancer. *Cancer Research*, 52(17), 4828–4831.
- Santos, A. M., Sousa, H., Catarino, R., Pinto, D., Pereira, D., Vasconcelos, A., Matos, A., Lopes, C., & Medeiros, R. (2005). TP53 codon 72 polymorphism and risk for cervical cancer in Portugal. *Cancer Genetics and Cytogenetics*, 159(2), 143–147.
- Sheikh, R. A., Min, B. H., Yasmeen, S., Teplitz, R., Tesluk, H., Ruebner, B. H., Tobi, M., Hatfield, J., Fligel, S., & Lawson, M. J. (2003). Correlation of Ki-67, p53, and Adnab-9 immunohistochemical staining and ploidy with clinical and histopathologic features of severely dysplastic colorectal adenomas. *Digestive Diseases and Sciences*, 48(1), 223–229. <https://doi.org/10.1023/a:1021727608133>.
- Silventoinen, K., Sans, S., Tolonen, H., Monterde, D., Kuulasmaa, K., Kesteloot, H., Tuomilehto, J., & WHO MONICA Project (2004). Trends in obesity and energy supply in the WHO MONICA Project. *International journal of obesity and related metabolic disorders: Journal of the International Association for the Study of Obesity*, 28(5), 710–718. <https://doi.org/10.1038/sj.ijo.0802614>.
- Sousa, H., Santos, A. M., Pinto, D., & Medeiros, R. (2007). Is the p53 codon 72 polymorphism a key biomarker for cervical cancer development? A meta-analysis review within European populations. *International Journal of Molecular Medicine*, 20(5), 731–741.
- Sul, J., Yu, G. P., Lu, Q. Y., Lu, M. L., Setiawan, V. W., Wang, M. R., Guo, C. H., Yu, S. Z., Mu, L., Cai, L., Kurtz, R. C., & Zhang, Z. F. (2006). P53 Codon 72 polymorphisms: a case-control study of gastric cancer and potential interactions. *Cancer Letters*, 238(2), 210–223. <https://doi.org/10.1016/j.canlet.2005.07.004>.

- Talseth, B. A., Meldrum, C., Suchy, J., Kurzawski, G., Lubinski, J., & Scott, R. J. (2006). Age of diagnosis of colorectal cancer in HNPCC patients is more complex than that predicted by R72P polymorphism in *TP53*. *International Journal of Cancer*, 118(10), 2479–2484. <https://doi.org/10.1002/ijc.21661>.
- Vousden, K. H., & Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. *Cell*, 137(3), 413–431. <https://doi.org/10.1016/j.cell.2009.04.037>.
- World Health Organization. WHO fact sheet on overweight and obesity. <http://www.who.int/media-centre/factsheets/fs311/en/> Date of Access: June 13, 2020.
- Zhang, Z. W., Newcomb, P., Hollowood, A., Feakins, R., Moorghen, M., Storey, A., Farthing, M. J., Alderson, D., & Holly, J. (2003). Age-associated increase of codon 72 Arginine p53 frequency in gastric cardia and non-cardia adenocarcinoma. *Clinical cancer research: an official journal of the American Association for Cancer Research*, 9(6), 2151–2156.

Molecular Investigation of Carbapenem and Colistin Resistance Mechanisms in *Klebsiella pneumoniae* Bloodstream Isolates

Neslihan GENİŞEL* , Nida ÖZCAN** , Kadri GÜL*** , Nezahat AKPOLAT**** , Selahattin ATMACA***** , Levent KENAR***** , Nurten ALTANLAR***** , Tuba DAL*****

Molecular Investigation of Carbapenem and Colistin Resistance Mechanisms in Klebsiella pneumoniae Bloodstream Isolates

Klebsiella Pneumoniae Kan İzolatlarında Karbapenem ve Kolistin Direnç Mekanizmalarının Moleküler Olarak İncelenmesi

SUMMARY

Carbapenem-Resistant *Klebsiella pneumoniae* (CRKp) infections are worrying health problems due to decreasing treatment options. This study investigates the carbapenemase (OXA-23,24, 48, 51, 55, 58, KPC, NDM-1, VIM, IMP) and *mcr-1* genes of the CRKps isolates. A total of 33 CRKp isolates isolated from patient blood samples from the Dicle University Medical Faculty Hospital, intensive care units (ICUs) between February 2020 and June 2020, were included in the study. The presence of carbapenemase encoding genes -including all CRKp isolates, *bla* OXA-23, 24, 48, 58, *bla* KPC, *bla*NDM-1, *bla* VIM, *bla* IMP- were investigated by multiplex Polymerase Chain Reaction (PCR). CRKp isolates were tested for *mcr-1* gene and *bla* OXA-51, *bla* OXA-55 genes by monoplex PCR. All CRKp isolates studied with Kirby Bauer Disc Diffusion Method (DDM) (100%) were resistant to ertapenem, 9 (27.27%) resistant to imipenem, and 23 (69.70%) were resistant to meropenem. 20 (60.61%) of the isolates were found resistant to colistin. *bla* OXA-48, *bla* NDM-1 and *bla* OXA-24 genes were found in 75.76% (n = 25), 6.06% (n = 2) and 3.03% (n = 1) isolates, respectively. Both *bla* OXA-48 and *bla* NDM-1 genes were detected in two (6.06%) isolates and *mcr-1* gene in 16 (48.48%) isolates. While the mean hospitalization was 20.3 days in 13 patients with a colistin minimum inhibitory concentration (MIC) of 2 µg/ml, it was 33.9 days in 20 patients with a colistin MIC of > 2 µg/ml. The average length of stay in the hospital was 21.8 days in *mcr-1* negative patients and 35.7 days in *mcr-1* positive patients. Carbapenemase and *mcr-1* positivities were found at dramatically high rates in Diyarbakır, Turkey. It was indicated that plasmid-mediated antimicrobial resistance in Kp isolates was problematic. Each hospital should monitor the colistin and carbapenem resistance mechanisms by molecular methods. Colistin resistance should be confirmed by the broth microdilution method (BMD).

Key Words: *Klebsiella pneumoniae*, carbapenemase, *bla* OXA-48, *mcr-1*, multiplex PCR, broth microdilution.

ÖZ

Karbapenem Dirençli *Klebsiella pneumoniae* (KDKp) enfeksiyonları azalan tedavi seçenekleri nedeniyle endişe verici sağlık sorunları oluşturmaktadır. Bu çalışmada, KDKp izolatlarının karbapenemaz (OXA-23,24, 48, 51, 55, 58, KPC, NDM-1, VIM, IMP) ve *mcr-1* genleri araştırılmaktadır. Dicle Üniversitesi Tıp Fakültesi Hastanesi yoğun bakım ünitelerinden (YBÜ) Şubat 2020 ile Haziran 2020 tarihleri arasında alınan hasta kan örneklerinden izole edilen toplam 33 KDKp izolatu çalışmaya dahil edildi. Tüm KDKp izolatları, *bla* OXA-23, 24, 48, 58, *bla* KPC, *bla*NDM-1, *bla* VIM, *bla* IMP dahil olmak üzere karbapenemaz kodlayan genlerin varlığı multiplex Polimeraz Zincir Reaksiyonu (PZR) ile araştırıldı. KDKp izolatları monoplex PZR ile *mcr-1* geni ve *bla* OXA-51, *bla* OXA-55 genleri için test edildi. Kirby Bauer Disk Difüzyon Test (DDT) (%100) ile çalışılan tüm KDKp izolatları ertapenem'e dirençliydi; 9'u (%2.27) imipeneme, 23'ü (%69.70) meropeneme dirençliydi. İzolatların 20'si (%60,61) kolistine dirençli bulundu. *bla* OXA-48, *bla* NDM-1 ve *bla* OXA-24 genleri sırasıyla %75.76 (n=25), %6.06 (n=2) ve %3.03 (n=1) izolatında bulundu. İki (%6.06) izolatta hem *bla* OXA-48 hem de *bla* NDM-1 genleri, 16 (%48,48) izolatta *mcr-1* geni saptandı. Kolistin minimum inhibitör konsantrasyonu (MİK) değeri 2 µg/ml olan 13 hastada ortalama yatış süresi 20.3 gün iken, kolistin MİK değeri > 2 µg/ml olan 20 hastada 33.9 gündü. Hastanede ortalama kalış süresi *mcr-1* negatif hastalarda 21.8 gün, *mcr-1* pozitif hastalarda 35.7 gündü. Karbapenemaz ve *mcr-1* pozitiflikleri Diyarbakır, Türkiye'de çarpıcı biçimde yüksek oranlarda bulundu. Kp izolatlarında plazmit aracılı antimikrobiyal dirençin sorunlu olduğu belirtildi. Her hastane moleküler yöntemlerle kolistin ve karbapenem direnç mekanizmalarını izlemelidir. Kolistin direnci sıvı mikrodilüsyon yöntemi ile doğrulanmalıdır.

Anahtar Kelimeler: *Klebsiella pneumoniae*, karbapenemaz, *bla* OXA-48, *mcr-1*, multiplex PZR, sıvı mikrodilüsyon.

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* ORCID: 0000-0002-2579-1932, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Dicle University, Diyarbakir, Turkey

** ORCID: 0000-0001-6898-7516, Department of Medical Microbiology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

*** ORCID: 0000-0002-4642-0276, Department of Medical Microbiology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

**** ORCID: 0000-0002-8653-6046, Department of Medical Microbiology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

***** ORCID: 0000-0002-2730-5790, Department of Medical Microbiology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

***** ORCID: 0000-0002-6613-1308, Department of Medical CBRN Defense, University of Health Sciences, Ankara, Turkey

***** ORCID: 0000-0003-2977-2269, Department of Pharmaceutical Microbiology, University of Ankara, Ankara, Turkey

***** ORCID: 0000-0002-4245-1534, Department of Clinical Microbiology, Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara,

* Corresponding Author; ; Neslihan GENİŞEL

Tel: +90 412 2411000 - 7545; e-mail : ngenisel@gmail.com

INTRODUCTION

Antibiotic resistance of Gram-negative bacteria has become a concern for public health. Patients with Multi-Drug Resistant (MDR) bacterial infections have to undergo intravenous treatment in the hospital, as there are no effective oral medications. Resistance in empirical antibiotic therapy results in increased mortality rates, prolonged hospital stay, difficulty in treatment, and higher costs.

K. pneumoniae is a Gram-negative bacteria belonging to the Enterobacterales order. As being a part of the healthy human microbiome, this microorganism is colonized in the gut and other parts of the human body. It can cause bloodstream, urinary tract infections, and severe pneumoniae, especially in critically ill or immune-compromised patients, neonates, or patients with risk factors in hospital settings. The frequency of Extended-spectrum β -lactamase (ESBL)-producing *K. pneumoniae* (Kp) has raised substantially (Knoth, 1983; Pitout, 2006). ESBL-producing Kp has been reported as resistant to cephalosporins and various antibiotics, including quinolones (Mugnaioli, 2006). Therefore, carbapenems have been proposed for the treatment of ESBL-producing Kp infections. Recently, CRKp increased globally over the years. CRKp infections pose a significant threat to human health, as the appropriate treatment options are limited and the mortality rate is relatively high. Due to the increase in CRKp outbreaks in hospitals worldwide, many studies are carried out to reveal the resistance mechanisms. Two main mechanisms mediate carbapenem resistance. First, CRKp isolates may produce β -lactamase enzymes along with reduced cell wall membrane permeability (Leavitt, 2009). The second mechanism is the synthesis of beta-lactamases which can hydrolyze most beta-lactams, including carbapenems. According to the Ambler classification, these carbapenemases are divided into three groups; class A (*K. pneumoniae* carbapenemase, KPC), class B (Metallo- β -lactamases, NDM), and class D (oxaci-

linases, OXA-48-like) (Pitout, 2015). Under these circumstances, colistin has become the antibiotic of last resort for CRKp infections (Perez, 2013).

Colistin (polymyxin E) is a polypeptide antimicrobial among polymyxin agents (polymyxin B and E) (Bergen, 2006). It has been kept as a backup agent for a long time due to severe nephrotoxicity and neurotoxicity problems, and less toxic antibiotics were preferred (Beringer, 2001). The alarming increase in the prevalence of MDR Gram-negative bacteria has led to the reassessment of colistin as a viable treatment option, especially in critically ill patients. Colistin resistance has been reported worldwide secondary to the increase in the use of colistin in the treatment of MDR bacterial infections (Landman, 2008). Previously, it was known that colistin resistance was coded by chromosomal genes. However, plasmid-mediated mobilized colistin resistance (*mcr*) genes (*mcr-1* and its variants) have been reported to lead to colistin resistance. The original variant *mcr-1* can make horizontal transitions among various strains of a bacterial species. The potential for *mcr* genes to spread rapidly across strains raises concerns regarding the use of colistin as a last-resort therapeutic option. Infections of MDR isolates are frequently encountered in hospitalized patients of ICUs. Infections caused by MDR isolates are frequently encountered, especially in patients hospitalized in intensive care units. If these isolates become resistant to colistin, treatment of serious infections such as bacteremia in intensive care patients will be difficult, mortality will increase, and outbreaks will occur in the hospital (Ling, 2020).

In this study, we aimed to investigate carbapenemase genes responsible for carbapenem resistance and *mcr-1* gene among CRKp isolates. It was also aimed to determine the MIC values of colistin with the BMD method, to compare it with the automated system for ensuring the selection of the appropriate antimicrobial treatment.

MATERIAL AND METHODS

A total of 33 non-duplicated CRKp isolates were included from hospitalized ICU patients in Dicle University Medical Faculty Hospital between February 2020, and June 2020 were included in the study. The Non-Interventional Clinical Research Ethics Committee of Dicle University Faculty of Medicine approved the study (no:56/2020).

The identification of bacteria grown in media was performed using mass spectrometry by Maldi Biotyper version 1.3 (Bruker Daltonics, Germany). Antimicrobial susceptibilities of the identified strains were performed by BD Phoenix 100 (Becton Dickinson, USA) automated microbiology system with Phoenix 100 NMIC panel.

Isolates resistant to at least one of the carbapenems (meropenem, imipenem, ertapenem) were confirmed with DDM. Imipenem (Oxoid, England), meropenem (Oxoid, England), and ertapenem (Oxoid, UK) discs with 10 µg content each were used for DDM. The minimum inhibitory concentration (MIC) values and the zone diameters were evaluated according to the

European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (EUCAST, 2019).

The antimicrobial susceptibility of colistin was performed with the broth microdilution (BMD) method according to EUCAST. The lowest antimicrobial concentration preventing growth was taken as the MIC value. Accordingly, those with MIC values > 2 mg / L were considered resistant to colistin, while those 2 mg / L were deemed to be sensitive to colistin. *E. coli* ATCC 25922, *mcr-1* positive *E. coli* NCTC 13846, and *P. aeruginosa* ATCC 27853 were used as quality control strains according to the recommendations of EUCAST (EUCAST, 2019).

Isolates were stored in Tryptic Soy Broth with 16% glycerol (Merck, Germany) medium at -20 °C until molecular study. DNAs of CRKp isolates were extracted by boiling method. Subsequently, two separate multiplex PCR were performed for *bla* OXA-23, 24, 48,58 genes and for *bla* KPC, *bla* NDM-1, *bla* VIM, *bla* NDM-1, *bla* IMP genes. Monoplex PCR was performed for *bla* OXA-51, *bla* OXA-55, and *mcr-1* gene (12). Table 1 demonstrates the primers used in this study (Table 1.) (Arabaci, 2019).

Table1. The properties of primers used in the study

Oligoname	Base pairs 5'-3'	Amplicon length (bp)
CLR5	F: CGGTCAGTCCGTTTGTTTC R: CTTGGTCGGTCTGTAGGG	305
OXA-23	F:GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCAT	501
OXA-24	F: GGTTAGTTGGCCCCCTTAAA R:AGTTGAGCGAAAAGGGGATT	246
OXA-48	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTGTGATGGC	743
OXA-51	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353
OXA-55	F: CATCTACCTTTAAAATTCCC R: AGCTGTTCCCTGCTTGAGCAC	975
OXA-58	F: AAGTAT TGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	599
IMP	F: CATGGTTTGGTGGTTCTTGT R: ATAATTTGGCGGACTTTGGC	488
VIM	F: ATTGGTCTATTTGACCGCGTC R: TGCTACTCAACGACTGAGCG	780
NDM-1	F: GAGATTGCCGAGCGACTTG R: CGAATGTCTGGCAGCACACTT	497
KPC	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCTTACTGCC	893

PCR conditions were as follows; final volume was 25 µl, initial denaturation at 94° C for 4 minutes, a total of 40 cycles including 94° C for 30 seconds, a final extension step of 40 seconds at 52° C and 50 seconds at 72°C, 10 minutes at 72 C. Gel Doc™ XR + (Bio Rad, USA) device was used for imaging. Colistin resistant *mcr-1* positive *E. coli* NTCC 13846, *K. pneumoniae* NCTC 13443 (NDM-1), *E. coli* NCTC 13476 (IMP), *A. baumannii* NCTC 13301 (OXA-23) and *A. baumannii* ATCC 19606 (OXA-51), *A. baumannii* NCTC 13302 (OXA-58), *K. pneumoniae* NCTC 13442 (OXA-48), *K. pneumoniae* NCTC 13439 (VIM-1) strains were used.

RESULTS AND DISCUSSION

Results of the Patients' Data

Of the 33 CRKp isolates included in the study, 19 (58%) were isolated from females and 14 (42%) from male patients. The average age of the female patients was 55.4, while of the male patients was 47.4. All of the patients were hospitalized in ICUs of the hospital including 8 (24.24%) chest diseases, 7 (21.21%) internal medicine, 5 (15.15%) pediatrics, 4 (12.12%) neurology, 3 (9.09%) hematology, 2 (0.06%) cardiology, 2 (0.06%) anesthesia and reanimation, 1 (0.03%) emergency and 1 (0.03%) gastroenterology departments.

According to the BMD, the mean hospitalization duration in 13 patients with 2 µg / ml colistin MIC value was 20.3 days, in 20 patients with > 2 µg / ml colistin MIC was 33.9 days. The mean hospitalization period of 8 patients with 32 µg / ml MIC values was 37.63 days; 6 patients with a MIC value of 64 µg / ml was 34.2 days. The average length of stay in the hospital was 21.8 days in *mcr-1* negative patients; 35.7 days in *mcr-1* positive patients. Nine (27.3%) patients died

while they were hospitalized, and all of the isolates of these patients were resistant to colistin by BMD. In addition, 5 of the deceased patients had a colistin MIC of 64 µg/ml, 2 of them 32 µg/ml, 1 of them 16 µg/ml and 1 of them 4 µg/ml. Of these 9 patients, 7 were *mcr-1* positive. The mortality rate was 43.75% (n=7) in *mcr-1* positives (n=16) and 11.8% (n=2) in *mcr-1* negatives (n=17). While the mortality rate of colistin-resistant patients (>2 µg/ml) according to BMD was 45% (n=9), no death was detected in susceptible patients.

Results of Antimicrobial Susceptibility Methods

The antibiotic susceptibility testing results by automated system and disk diffusion test:

piperacillin-tazobactam 93.94% (n=31) R (resistant), 6.06% (n=2) S (sensitive); amikacin % 69.70 (n = 23) R, 30.30% (n = 10) S; gentamicin 66.67% (n = 22) R, 33.33% (n = 11) S; ciprofloxacin 84.85% (n = 28) R, 15.15% (n = 5) S; ceftazidime 90.91% R (n = 30), 9.09% (n = 3) S; 87.88% R (n = 29), 9.09% (n = 3) S and 3.03% (n = 1) I for cefepime (moderately sensitive); 87.88% (n = 29) R, 12.12% (n = 4) S for trimethoprim-sulfamethaxazole; 48.48% (n = 16) R for colistin, 51.52% (n = 17) S; and 96.97% (n = 32) R for ertapenem, 3.03% (n = 1); 60.61% (n =20) R, 3.03% (n = 1) S and 36.36% (n = 12) I for imipenem; for meropenem, 84.85% (n = 28) R, 9.09 % (n = 3) S and 6.06 % (n = 2).

Distribution of the numbers of isolates according to colistin MIC values is given in (Figure 1.). The MIC values of colistin by BMD method were 0.25 µg/ml (n=6), 1.00 µg/ml (n=4), 2.00 µg/ml (n=3), 4.00 µg/ml (n=2), 8.00 µg/ml (n=2), 16.00 µg/ml (n=2), 32 µg/ml (n=8), and 64 µg/ml (n=6) (Figure 1).

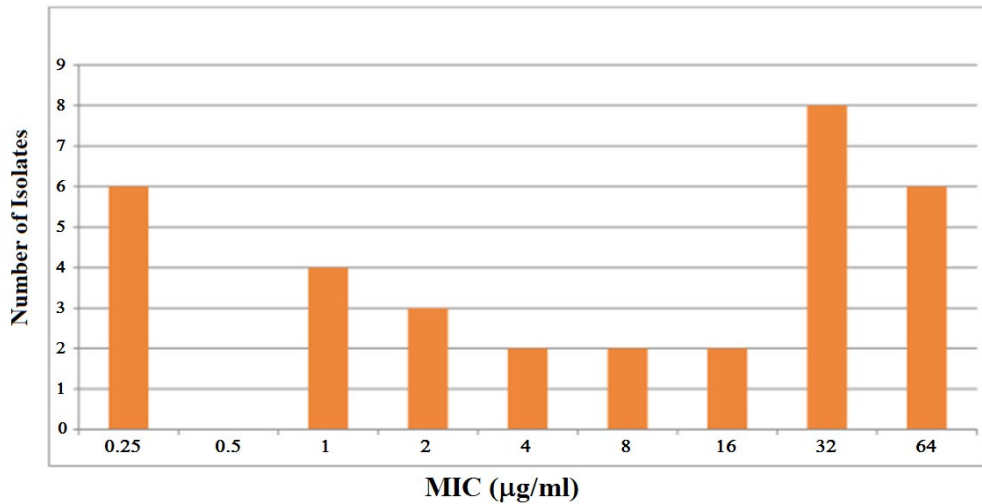


Figure 1. Distribution of isolate numbers according to Colistin MIC values determined by BMD method.

Results of Molecular Methods

PCR Findings of Carbapenemase Genes: The carbapenemase genes detected by PCR method of 33 KDKp isolates studied in total are shown in Figure 2. These rates were 75.76% (n = 25) for *bla* OXA-48, 6.06% for *bla* NDM-1 (n=2), and 3.03% (n=1)

for *bla* OXA-24. Isolates from *bla* OXA-23, *bla* OXA-51, *bla* OXA-55, *bla* OXA-58, *bla* KPC, *bla* VIM, and *bla* IMP was detected. Two (6.06%) isolates and *bla* OXA-48 both *bla* NDM-1 genes, were carrying each other (Figure 2.).

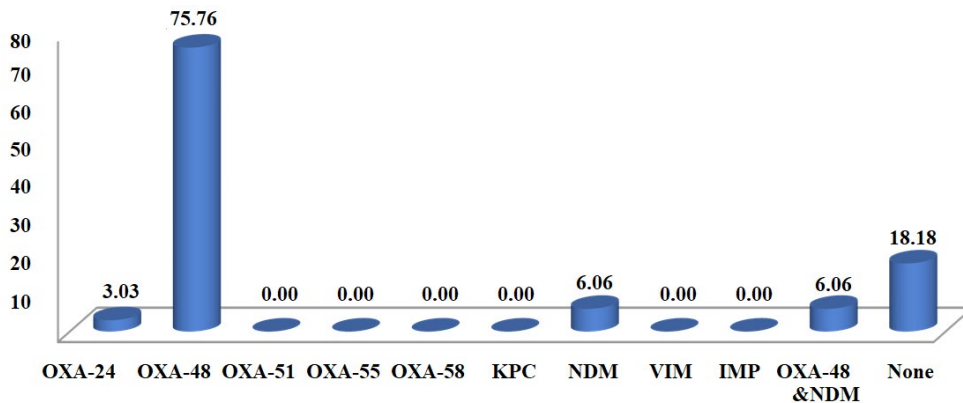


Figure 2. Percentage distribution of carbapenemase genes detected by PCR methods of CRKp 33 isolates.

Of the 33 CRKp isolates, 16 (48.48%) were found to be *mcr-1* positive, and 17 (51.52%) were found to be *mcr-1* negative. The numbers of isolates with *mcr-1*

positivity according to the carbapenemase positivity are shown in (Table 2.).

Table 2. The numbers of isolates with *mcr-1* positivity according to carbapenemase positivity

Carbapenemase	<i>mcr-1</i> (+)	<i>mcr-1</i> (-)
OXA-48	15	10
OXA-24	0	1
NDM-1	0	2
Negative	1	6

Comparison of Phenotypic and Molecular Methods

Among the 25 *bla* OXA-48 gene positive isolates all (100%) were ertapenem, and 11 (44%) were imipenem; 21 (84%) were meropenem, 23 (93.54%) piperacillin-tazobactam, 22 (70.97 %) amikacine, 16 (64%) gentamicin, 23 (92%) ciprofloxacin, 24 (96%) ceftazidime, 24 (96%) cefepime and 14 (56%) colistin resistant. Among the *bla* OXA-48 gene positive isolates, 15 (48.39%) were found to carry the *mcr-1* gene, simultaneously.

Twenty-two (66.67%) of 33 CRKp isolates were susceptible to amikacin and/or gentamicin. Out of the colistin-resistant isolates, 55% (n:11) were susceptible to amikacin and/or gentamicin. Nine (56.35%) of *mcr-1* positive isolates (n = 16) were found sensitive to amikacin and/or gentamicin.

In the study, the number of colistin-resistant isolates by the automated system was 16 and by BMD method was 20. Four isolates that were susceptible to colistin by the automated system were resistant to BMD.

Among 16 *mcr-1* gene-positive isolates, colistin resistance was detected in 14 (87.50%) isolates by BMD. Of 17 the *mcr-1* gene negative isolates, 6 (35.29%) isolates were resistant against colistin by BMD.

Discussion

The *mcr-1* positive results (48.48%) obtained were found to be well above the reported results from other studies (Arabaci, 2019; Yildiz, 2021; Karki, 2021). PCR results may also be false-positive and must be confirmed by sequencing. Besides, this condition can

originate from regional differences in the patients treated in Dicle University Hospitals. In the study, the majority of the patients receiving treatment at Dicle University Hospitals were from Diyarbakir and its surrounding provinces and immigrants from Syria.

Colistin-resistant *Klebsiella pneumoniae* isolates are an important health problem worldwide. In our study, 9 (27.3%) patients died, and all of the isolates of these patients were resistant to colistin. Additionally, eight of the isolates belonging to these patients' colistin MIC was ≥ 16 $\mu\text{g/ml}$, and seven were *mcr-1* positive. In our study, the mortality rate was 45% in colistin-resistant patients and 43.75% in *mcr-1* positives; the average length of stay in the hospital was 33.9 days in patients with MIC > 2 $\mu\text{g/ml}$ Kp isolates and 37.63 days in patients with MIC values are > 32 $\mu\text{g/ml}$ Kp. The average hospital stay was 21.8 days in *mcr-1* negatives; 35.7 days in *mcr-1* positives. Giacobbe et al. reported that in patients with risk factors such as the presence of colistin resistance and hospitalization duration of more than 30 days in ICUs, the mortality rate was 51% (Giacobbe, 2015). Our data and literature data indicated that the length of hospitalization and mortality rate was higher in colistin-resistant Kp and/or *mcr-1* positive Kp isolated patients.

All isolates in our study were ESBL positive, and cefepime resistance was 87.88%. Amikacin and/or gentamicin are antibiotics that can be used as a combined therapy in colistin/carbapenem-resistant Kp infection due to adverse effects, including nephrotoxicity (Vardakas, 2012). Our study showed that the significant majority of the isolates were resistant against amikacin and/or gentamicin, but approximately %30

of the isolates were susceptible. These results indicated that amikacin and/or gentamicin could be an alternative drug in combined therapy in antimicrobial-resistant infections.

Colistin resistance in Kp isolates has been reported worldwide. Colistin resistance rates in Kp isolates were reported as 10.5-20% in Greece, 6.8% in South Korea, 6.3% in Singapore (Ah, 2014). In the last ten years, colistin resistance in Kp isolates from different hospitals has increased tremendously (from 6% to 75.6%) in Turkey (Aris, 2020). In our study, the colistin resistance rate was 60.61% by BMD, and colistin resistance was an important problem in our hospital.

BMD method was recommended for testing the colistin resistance by EUCAST due to false results can be obtained by gradient tests and automated processes (Jayol, 2015). The study of Poirel et al. reported that the rate of false-sensitive results was 15% with the automated system (Poirel, 2017). In our study, four of 16 isolates that were susceptible to colistin by the automated system were found to be resistant to BMD. The false sensitivity finding of 18.75% in our study was compatible with the literature, and the automated system was found to be inadequate in detecting some colistin-resistant strains. Our data clearly indicated that colistin susceptibility should be confirmed by BMD.

In our study, 16 isolates were *mcr-1* positive 17 isolates were *mcr-1* negative. In addition to this, 20 isolates were colistin-resistant by BMD. Of 20 colistin-resistant isolates, 14 isolates were *mcr-1* positive, and 6 were *mcr-1* negative. However, among the 16 *mcr-1* positive isolates, 14 isolates were colistin-resistant, and two isolates were colistin susceptible by BMD. Our data showed that chromosomal resistance mechanisms and other *mcr* variants should be investigated in Kp isolates in further studies (Hadjadj, 2019). Genome analysis studies showed that in strains with *mcr-8* gene, different antibiotic resistance genes

(the beta-lactams, aminoglycosides, sulfonamides, fluoroquinolones) could be seen together (Hadjadj, 2019). *mcr-1* positivity in colistin susceptible Kp isolates showed that *mcr-1* and *mcr* gene variants screening of colistin susceptible Kp isolates could be recommended for controlling of infection and spreading of *mcr-1* gene by the plasmid.

OXA-48-producing *K. pneumoniae* infections are common in Turkey, Europe, Middle East, and Africa with the rate of %10-72 (Aktaş, 2008; Carrër, 2010; Davarcı, 2019). In our study OXA-48 positivity rate was 75.76%. The OXA-48 beta-lactamase enzyme can successfully hydrolyze penicillins but shows weak or no activity against broad-spectrum cephalosporins (such as ceftazidime, cefotaxime, cefepime), carbapenems. In this study, among 25 (93.94%) isolates whose OXA-48 gene was detected, all were resistant to ertapenem, 44% to imipenem, 84% to meropenem, 56% of to colistin, and antibiotic resistance rates were more than 55 % in OXA-48 positive Kp isolates. Neuner et al. In their study, the colistin, amikacin, and gentamicin sensitivities of OXA-48 positive isolated from blood samples of patients hospitalized in ICUs were found to be 86%, 45%, and 22%, respectively (Neuner, 2011).

In two multicenter studies in Turkey NDM-1 ratio was 7.25% and 6.5% (Grundmann, 2017; Çakar, 2016). In the study of Çakar et al., the rate of NDM + OXA-48 coexistence was found to be 2.4% (Çakar, 2016). In our study, this rate was 6.06% and is within similar limits. In EUSCAP study (European Survey on Carbapenemas of Producing Enterobacteriaceae) of Turkey centers in a different region, in 124 Kp isolates, two isolates (1.6%) were IMP positive (Çakar, 2016). KPC-producing isolates have been found mainly in Greece, the USA, Israel, Turkey (24,26, current study). VIM, IMP and KPC were not detected in our study, but this plasmid-mediated enzyme poses a problem for our country.

CONCLUSION

Consequently, *K. pneumoniae* with colistin and carbapenem resistance is a significant public health problem in ICUs and hospitals in Turkey. OXA-48 beta-lactamase is the most commonly reported carbapenemase enzyme type in our country. Colistin resistance and *mcr-1* positivities were the risk factors for extended hospitalization and high mortality. Automated systems may be insufficient to detect colistin sensitivity, and these results should be confirmed by BMD. *mcr-1* gene can also be detected in colistin susceptible isolates. For the controlling of plasmid-mediated spread, screening of the *mcr-1* and *mcr* variant genes is helpful in both colistin-resistant and susceptible isolates.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis and experimenting (Genişel, N., Dal, T., Özcan, N.), preparing the study text and literature research (Genişel, N., Gül, K.), analysis and interpretation of the data (Genişel, N., Dal, T.), designed the concept and drafted the manuscript (Genişel, N., Özcan, N., Dal, T.), prepared the figures (Genişel, N., Kenar, L.), held ethical approval and collected relevant samples and clinical data (Özcan, N., Gül, K., Akpolat, N., and Atmaca, S.), carried out the laboratory applications of this study (Genişel, N., Dal, T.), reviewed the existing journal policy (Altanlar, N., Kenar, L.), contributed to the writing of the final version of the manuscript (Dal, T., Kenar, L.).

REFERENCES

- Ah, Y. M., Kim, A. J., & Lee, J. Y. (2014). Colistin resistance in *Klebsiella pneumoniae*. *International Journal of Antimicrobial Agents*, 44(1), 8–15. <https://doi.org/10.1016/j.ijantimicag.2014.02.016>
- Aktas, Z., & Ay, S. (2016). Investigation of carbapenemases in carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains isolated in 2014 in Turkey. <http://doi.org/10.5578/mb.10695>
- Aktaş, Z., Kayacan, Ç. B., Schneider, I., Can, B., Midilli, K., & Bauernfeind, A. (2008). Carbapenem-hydrolyzing oxacillinase, OXA-48, persists in *Klebsiella pneumoniae* in Istanbul, Turkey. *Chemotherapy*, 54(2), 101-106. <https://doi.org/10.1159/000118661>
- Arabacı, Ç., Dal, T., Başıyigit, T., Genişel, N., & Durmaz, R. (2019). Investigation of carbapenemase and *mcr-1* genes in carbapenem-resistant *Klebsiella pneumoniae* isolates. *The Journal of Infection in Developing Countries*, 13(06), 504-509. <https://doi.org/10.3855/jidc.11048>
- Aris, P., Robotjazi, S., Nikkhahi, F., & Marashi, S. M. A. (2020). Molecular mechanisms and prevalence of colistin resistance of *Klebsiella pneumoniae* in the Middle East region: A review over the last 5 years. *Journal of Global Antimicrobial Resistance*, 22, 625-630. <https://doi.org/10.1016/j.jgar.2020.06.009>
- Bergen, P. J., Li, J., Rayner, C. R., & Nation, R. L. (2006). Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 50(6), 1953-1958. <https://doi.org/10.1128/AAC.00035-06>
- Beringer, P. (2001). The clinical use of colistin in patients with cystic fibrosis. *Current Opinion in Pulmonary Medicine*, 7(6), 434-440.
- Carrër, A., Poirer, L., Yilmaz, M., Akan, O. A., Feriha, C., Cuzon, G., Matar, G., Honderlick, P., Nordmann, P. (2010). Spread of OXA-48-encoding plasmid in Turkey and beyond. *Antimicrobial Agents and Chemotherapy*, 54(3), 1369-1373. <https://doi.org/10.1128/AAC.01312-09>
- Davarcı İ, Şenbayrak, S, Aksaray S, Koçoğlu, M.E., Kuşkucu M.A., Smasti, M, Molecular epidemiology of carbapenem-resistant *Klebsiella pneumoniae* isolates. *Anatolian Clinic the Journal of Medical Sciences*, 24(1), 1-7. <https://doi.org/10.21673/anadoluklin.423081>

- Giacobbe, D. R., Del Bono, V., Trecarichi, E. M., De Rosa, F. G., Giannella, M., Bassetti, M., ... & Tumbarello, M. (2015). Risk factors for bloodstream infections due to colistin-resistant KPC-producing *Klebsiella pneumoniae*: results from a multi-center case-control-control study. *Clinical Microbiology and Infection*, 21(12), 1106-e1. <https://doi.org/10.1016/j.cmi.2015.08.001>
- Grundmann, H., Glasner, C., Albiger, B., Aanensen, D. M., Tomlinson, C. T., Andrasević, A. T., ... & Brown, D. J. (2017). Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study. *The Lancet infectious diseases*, 17(2), 153-163. [https://doi.org/10.1016/S1473-3099\(16\)30257-2](https://doi.org/10.1016/S1473-3099(16)30257-2)
- Hadjadj, L., Baron, S. A., Olaitan, A. O., Morand, S., & Rolain, J. M. (2019). Co-occurrence of variants of *mcr-3* and *mcr-8* genes in a *Klebsiella pneumoniae* isolate from Laos. *Frontiers in microbiology*, 10, 2720. <https://doi.org/10.3389/fmicb.2019.02720>
- Jacoby, G. A., Mills, D. M., & Chow, N. (2004). Role of β -lactamases and porins in resistance to ertapenem and other β -lactams in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, 48(8), 3203-3206. <https://doi.org/10.1128/AAC.48.8.3203-3206.2004>
- Jayol, A., Nordmann, P., Brink, A., & Poirel, L. (2015). Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrobial agents and chemotherapy*, 59(5), 2780-2784. <https://doi.org/10.1128/AAC.05055-14>
- Karki, D., Dhungel, B., Bhandari, S., Kunwar, A., Joshi, P.R., Shrestha, D., Rijal, K.R., Ghimire, P., Banjara, M.R., (2021) Antibiotic resistance and detection of plasmid mediated colistin resistance *mcr-1* gene among *Escherichia coli* and *Klebsiella pneumoniae* isolated from clinical samples, 13, 45 <https://doi.org/10.1186/s13099-021-00441-5>.
- Knothe, H., Shah, P., Krcmery, V., Antal, M., & Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection*, 11(6), 315-317.
- Landman, D., Georgescu, C., Martin, D. A., & Quale, J. (2008). Polymyxins revisited. *Clinical Microbiology Reviews*, 21(3), 449-465. <https://doi.org/10.1128/CMR.00006-08>
- Leavitt, A., Chmelnitsky, I., Colodner, R., Ofek, I., Carmeli, Y., & Navon-Venezia, S. (2009). Ertapenem resistance among extended-spectrum- β -lactamase-producing *Klebsiella pneumoniae* isolates. *Journal of Clinical Microbiology*, 47(4), 969-974. <https://doi.org/10.1128/JCM.00651-08>
- Ling, Z., Yin, W., Shen, Z., Wang, Y., Shen, J., & Walsh, T. R. (2020). Epidemiology of mobile colistin resistance genes *mcr-1* to *mcr-9*. *Journal of Antimicrobial Chemotherapy*, 75(11), 3087-3095. <https://doi.org/10.1093/jac/dkaa205>
- Mugnaioli, C., Luzzaro, F., De Luca, F., Brigante, G., Perilli, M., Amicosante, G., Stefani, S., Torino, A., Rossolini, G. M. (2006). CTX-M-type extended-spectrum β -lactamases in Italy: molecular epidemiology of an emerging countrywide problem. *Antimicrobial Agents and Chemotherapy*, 50(8), 2700-2706. <https://doi.org/10.1128/AAC.00068-06>
- Neuner, E. A., Yeh, J. Y., Hall, G. S., Sekeres, J., Endimiani, A., Bonomo, R. A., Shrestha, N.K., Fraser, T.G., Duin, D. (2011). Treatment and outcomes in carbapenem-resistant *Klebsiella pneumoniae* bloodstream infections. *Diagnostic Microbiology and Infectious Disease*, 69(4), 357-362. <https://doi.org/10.1016/j.diagmicrobio.2010.10.013>
- Perez, F., & Van Duin, D. (2013). Carbapenem-resistant Enterobacteriaceae: a menace to our most vulnerable patients. *Cleveland Clinic Journal of Medicine*, 80(4), 225. <https://doi.org/10.3949/ccjm.80a.12182>

- Pitout, J. D., & Laupland, K. B. (2008). Extended-spectrum β -lactamase-producing Enterobacteriaceae: an emerging public-health concern. *The Lancet Infectious Diseases*, 8(3), 159-166. [https://doi.org/10.1016/S1473-3099\(08\)70041-0](https://doi.org/10.1016/S1473-3099(08)70041-0)
- Poirel, L., Jayol, A., & Nordmann, P. (2017). Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clinical Microbiology Reviews*, 30(2), 557-596. <https://doi.org/10.1128/CMR.00064-16>
- Silva, D. D. C., Rampelotto, R. F., Lorenzoni, V. V., Santos, S. O. D., Damer, J., Hörner, M., & Hörner, R. (2017). Phenotypic methods for screening carbapenem-resistant Enterobacteriaceae and assessment of their antimicrobial susceptibility profile. *Revista da Sociedade Brasileira de Medicina Tropical*, 50, 173-178. <https://doi.org/10.1590/0037-8682-0471-2016>
- Suzuk Yildiz, S., Şimşek, H., Bakkaloğlu, Z., Numanoğlu Çevik, Y., Hekimoğlu, C.H., Kılıç, S., Alp Meşe, E., Ulusal Karbapenemaz Sürveyans Çalışma Grubu (2021) The Epidemiology of Carbapenemases in Escherichia coli and Klebsiella pneumoniae Isolated in 2019 in Turkey, *Mikrobiyol Bul.* 55(1), 1-16. <https://doi.org/10.5578/mb.20124>
- The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. <http://www.eucast.org>.
- Vardakas, K. Z., Tansarli, G. S., Rafailidis, P. I., & Falagas, M. E. (2012). Carbapenems versus alternative antibiotics for the treatment of bacteraemia due to Enterobacteriaceae producing extended-spectrum β -lactamases: a systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*, 67(12), 2793-2803. <https://doi.org/10.1093/jac/dks301>

Neuroprotective Therapy with Citicoline and Piracetam at Acute Cerebrovascular Disease: Clinical and Psychosomatic Effects

Iryna SOKOLOVA[°], Serafima TAZINA^{**}, Oksana ZAKHAROVA^{***}

Neuroprotective Therapy with Citicoline and Piracetam at Acute Cerebrovascular Disease: Clinical and Psychosomatic Effects

Akut Serebrovasküler Hastalıkta Sitikolin ve Pirasetam ile Nöroprotektif Tedavi: Klinik ve Psikosomatik Etkiler

SUMMARY

Contemporary pharmacological market is well developed, suggesting a wide choice of medical preparations for treating various disorders. Particular attention is paid to the group of diseases related to cerebrovascular accidents as the complications and consequences are often unfavorable. A study was conducted in the post-Soviet countries and aimed to determine the effect and efficacy of using neuroprotective drugs in the treatment of cerebrovascular disease, taking into account the psychosomatic effect in patients. Two preparations were chosen for the study, namely, Citicolin and Piracetam. The main purpose was to compare the effectiveness and necessity of these drugs in improving the patients' condition and reducing the effects and mortality. The results of this study and works of other scientists proved a higher efficacy of using Citicolin compared to Piracetam. Among 680 patients (100%) receiving Citicolin as a neuroprotective therapy, 625 (91.9%) patients noted improvement in general condition already after three days. Of 405 patients (100%) receiving Piracetam, the regression of neurological symptoms occurred on the 4th or 5th day of treatment. The improvement of visual functions was noted in 26 patients from Citicolin group and only in 3 patients who received Piracetam as neuroprotective therapy.

Key Words: Ischemic stroke, citicoline, piracetam, neuroprotective therapy, psychosomatic effect

ÖZ

Günümüzün iyi gelişmiş modern ilaç piyasasında çeşitli rahatsızlıkların tedavisi için kullanılacak çok çeşitli tıbbi ilaçlar mevcuttur. Genellikle komplikasyonları ve sonuçları ağır olduğundan, serebrovasküler kazalarla ilişkili hastalık grubuna özellikle dikkat edilmektedir. Sovyet sonrası ülkelerde gerçekleştirilen bu çalışmada hastalardaki psikosomatik etki dikkate alınarak serebrovasküler hastalıkların tedavisinde nöroprotektif ilaç kullanımının etki ve etkinliğinin belirlenmesi amaçlanmıştır. Çalışma için Sitikolin ve Pirasetam olmak üzere iki ilaç seçilmiştir. Temel amaç, bu ilaçların hastaların durumunun iyileştirilmesi ile etki ve ölüm oranlarının azaltılmasındaki etkinliklerinin ve gerekliliklerinin karşılaştırılmasıdır. Bu çalışmanın sonuçları ve diğer bilim insanlarının çalışmaları, Sitikolin'in Pirasetam'a kıyasla daha yüksek bir etkinliği olduğunu kanıtlamıştır. Nöroprotektif tedavi olarak Sitikolin alan 680 hastanın (%100) içerisindeki 625 hastanın (%91,9) üç gün sonra genel durumunda iyileşme kaydedilmiştir. Pirasetam alan 405 hastada (%100) ise, nörolojik semptomlarda gerileme, tedavinin 4. veya 5. gününde meydana gelmiştir. Görsel fonksiyonlarda iyileşme Sitikolin grubundan 26 hastada gerçekleşmesine karşın nöroprotektif tedavi olarak Piracetam alan sadece 3 hastada görsel fonksiyonlarda iyileşme kaydedilmiştir.

Anahtar Kelimeler: İskemik inme, sitikolin, pirasetam, nöroprotektif tedavi, psikosomatik etki

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[°] Orcid ID: 0000-0002-3102-2910, Department of Practical Psychology, Ukrainian Engineering and Pedagogical Academy, Kharkiv, Ukraine;

^{**} Orcid ID: 0000-0003-3676-3467, Department of Therapy, Sechenov First Moscow State Medical University, Moscow, Russian Federation;

^{***} Orcid ID: 0000-0003-0249-5257, Department of Organization and Economics of Pharmacy, Sechenov First Moscow State Medical University, Moscow, Russian Federation

[°] Corresponding Author; Iryna SOKOLOVA

Phone: +380503642304; E-mail: sokolovairr@ukr.net

INTRODUCTION

To date, cerebrovascular diseases are the second leading cause of death worldwide (World Health Organization, 2018). Cerebrovascular pathology (CVP) is considered the most common non-infectious disease and quite a frequent nervous system disorder. The most complex form of vascular disease is an ischemic stroke, which is characterized by focal lesions of the brain resulting from the disruption of its blood supply. This disorder occurs as a complication of such diseases like atherosclerosis and arterial hypertension.

Ischemic stroke is still believed to be the second leading cause of death in developed countries and the first reason for long-term disability in patients who experienced stroke (World Health Organization, 2018). However, there is still no pharmacological treatment for stroke with proven efficacy or with a favorable risk/benefit ratio for the acute phase of the disease.

Treatment of acute and chronic cerebrovascular disorders should be comprehensive. Basic drugs are antithrombotic agents, hypotensive and hypolipidemic preparations. Also, lifestyle modification with the exclusion of the disease risk factors like obesity, endocrine diseases in decompensation stage, and harmful habits such as smoking and alcohol abuse, is required for the higher success of the treatment outcome (Hacke, 2000). The sooner the correct therapy is started, the more chances for a positive result. Besides, to establish whether the disease is ischemic or hemorrhagic is of high importance as it may help to determine the correct treatment tactics.

One of the essential points on the way to recovery is neuroprotective therapy, the main purpose of which is to prevent the development of mechanisms of neuronal death in the ischemic brain tissue. In modern medicine, there is a large number of

drugs with neuroprotective properties (Mildronate, Emoxypinum, Citicoline, Dicynone, etc.) However, scientific recommendations with a sufficiently proven effect of these drugs are missing in the available literature sources being considered ineffective from the perspective of evidence-based medicine. Therefore, studying the effectiveness of various neuroprotective drugs are still relevant today due to the lack of evidence-based action.

Certain neuroprotectants have a positive effect on the course and prognosis of various cerebral vascular pathologies. However, their success depends on several factors: the active ingredient, dose, frequency of administration, duration of use, etc. Often, doctors prescribe 2 or 3 neuroprotective drugs, although there is no specific need and no evidence base for this. On the contrary, their irrational intake can lead to the development of polypragmasy with further negative effects on the body. Therefore, the estimation of the neuroprotective therapy efficiency in the complex treatment of cerebrovascular disorders of different genesis remains an important point in world medicine.

Citicoline is a naturally derived endogenous compound. This compound is an intermediate metabolite involved in phosphatidylcholine synthesis processes (Ortega, 2010). The latter is a major component of the cellular membrane. Citicoline is a complex substance composed of cytidine and choline. There is a diphosphate bridge between them that decomposes following the hydrolysis reaction. In this respect, Citicoline has a very high level of bioavailability - approximately 100% when administered orally. The two components of citicoline pass the haematoencephalic barrier. After passing the barrier, citicoline is synthesized in the brain and then extracted from the body via air and urine (Donmez & Outeiro, 2013). Citicoline is characterized by a membranotropic effect, which promotes the repair of membranes in neurons. This happens because of phosphatidylcholine, as well as due to slower

neuronal membrane damage through the decrease in the activity of enzyme phospholipase A2 (Parfenov, 2012). Citicoline also promotes the preservation of not only phospholipids but also sphingomyelin, as well as standardizes the work of the sodium-potassium pump and the functional capacity of such organelles as mitochondria. Increased acetylcholine synthesis contributes to the standardization of cholinergic neurons function (Secades, 2011). The effect of citicoline is associated with its positive influence on the mechanisms that determine cerebral plasticity, as well as on neuro repair processes. That defines its therapeutic effect, which is why citicoline is used to treat various nervous system dysfunctions like dementia, memory loss, depressive disorders, as well as Parkinson's disease (Polito, 2013).

Piracetam's action mechanism is based on changes in metabolic processes, bioenergy metabolic processes occurring in the neuron, and increased protein biosynthesis. The action of piracetam on the use of O₂ and metabolic processes linked to glucose is based on aerobic or anaerobic conditions, which determine the response. Aerobic conditions facilitate the O₂ uptake by one-third under the influence of piracetam, while anaerobic conditions lead to an increase in glycolysis (Flicker & Evans, 2004). The latter is caused by activation of the pentose-phosphate cycle, which leads to the formation of NADPH, which is the main source of energy in brain metabolism. Under anaerobic (or hypoxic) conditions, the ATP synthesis is more intense, and the ATP-cAMP cycle in neuronal cells is more active. Based on the fact that lactate levels do not increase, anaerobic processes do not play the primary role (PASS II, 2001).

A stroke occurs in more than 15 million people annually worldwide, and almost 5 million people subsequently die (Belova, 2016). The incidence of stroke is 100-200 cases per 100 thousand people in different European countries. According to the World Health Organization, the number of stroke patients will increase by 30% by 2025 (Truelsen, 2006). Over the last decade, the WHO has reassessed the development

of stroke and its consequences. Thus, there are 16 million primary cases of stroke and 5.7 million deaths due to it worldwide. Acute cerebrovascular disorder ranks 3rd by material costs of treatment and 2nd by causes of death in the United States and Europe.

To date, neuroprotective drugs provoke numerous debates among scientists. Some authors argue about the necessity of their use during ischemia zone recovery (Shabanov, 2020). Other scientists believe that neuroprotectors do not have a sufficient evidence base and their effectiveness is minimal (Sharayeva, 2018). Over the past 20 years, great importance has been attached to Citicoline as a strong neuroprotective agent. There are also a large number of articles that testify to the useful properties of Piracetam as a nootropic agent in acute ischemic stroke, especially in patients with speech impairment (De Deyn, 1997; Zhang, 2016).

Piracetam is the pioneer of nootropics. Recently, this preparation has received renewed attention, given its role in the therapy of psychosomatic diseases. Piracetam is effective in preventing neurocirculatory dystonia of psychosomatic origin such as bronchial asthma, coronary heart disease (CHD), and arterial hypertension.

Among disadvantages of Piracetam therapy, including those of psychosomatic origin, are its excessive influence on the excitability of the central nervous system, which may manifest itself as increased nervousness of a patient. Therefore, it is advisable to prescribe the intake of this drug in the period before 3 p.m. Noteworthy, such side effect was observed in less than 5% of cases (Al-Kuraishy & Al-Gareeb, 2020).

Clinical trials of Citicoline performed in the United States in the treatment of acute and chronic cerebrovascular disorders demonstrated a slight positive effect of the substance, which may be associated with the time of drug administration (therapeutic window of 1-6 hours from the onset of the first symptoms of stroke), the dose, and the method of administration (Adibhatla & Hatcher, 2002). Other researchers argue that Citicoline is safe to use and can

favorably affect patients with acute ischemic stroke, and most favorable in less severe stroke in elderly patients who have not received recombinant tissue plasminogen activator. None of the neuroprotective agents has been effective in confirmatory clinical trials (Overgaard, 2014). Neuroprotective agents can promote reperfusion at the capillary level in the target tissue area and increase the period for effective recanalization by preserving brain tissue and reducing the hemorrhage rate. However, such an effect is possible only in combination with mechanical thrombectomy. Neuroprotectants have been shown to constrain ischemic damage while the patient is in the acute period of stroke. Therefore, the potential usefulness of neuroprotection as an adjunctive agent before, during, and after mechanical thrombectomy has been emphasized (Babadjouni, 2017).

Recent clinical study by a group of scientists from Japan confirmed that combined treatment with Citicoline and docosahexaenoic acid may have synergic benefits for partial improvement of memory deficits after passing brain ischemia and prevent neuron cell death in the brain (Nakazaki, 2019). A multicenter Italian clinical trial established the effect of Citicoline in elderly people with mild vascular cognitive impairment. It was found that by increasing cell metabolism and activating phospholipid biosynthesis in brain neuronal membranes, Citicoline was quite effective and can be recommended for patients with mild vascular impairment. No adverse effects of the drug were reported in the study (Cotroneo, 2013; Porfiryeva, 2020). There are also numerous scientific articles describing the benefits of Piracetam as a neuroprotective agent. A group of Chinese researchers from Hangzhou studied the effect of Piracetam on the rehabilitation of speech activity in patients after stroke. Evaluation of speech at the end of the trials did not show a significant improvement in general aphasia but showed a marked improvement in writing. The effect of Piracetam on general language and writing tends to improve for a short period but

decreases with time (Zhang, 2016).

A randomized, multicenter, placebo-controlled PASS (Piracetam in Acute Stroke Study) showed no efficacy of Piracetam in acute ischemic stroke when administered within 12 hours from the onset of acute ischemic stroke. However, post-analysis suggested that Piracetam may be useful when administered within 7:00 after onset, especially in patients with moderate to severe stroke (De Deyn, 1997).

However, the Guidelines 2013 by the American Heart Association state that no neuroprotective pharmacological agents have demonstrated clinical efficacy in various clinical trials, and, therefore, are not currently recommended (Jauch, 2013).

This study aims to update the comparison of the therapeutic effects of Citicoline and Piracetam in combination with standard intensive therapy on brain cells of patients with acute ischemic stroke. These drugs are used not as the main treatment, but as an additional neuroprotective therapy.

The purpose of the work is to evaluate the effectiveness of Citicoline and Piracetam in combination with standard intensive therapy in patients with acute ischemic stroke.

The task of this study was to find out the evidence for the use of neuroprotective agents in cerebrovascular disease; to conduct a comparative analysis of Citicoline and Piracetam preparations; to identify the consequences and general effectiveness of treatment in improving the blood supply to the brain.

MATERIALS AND METHODS

Materials

The study used and analyzed medical histories and records of patients with acute cerebrovascular disease in hospitals of major cities in Russia and Ukraine.

For this purpose, the effect of neuroprotective drugs in patients with cerebrovascular disease was analyzed and compared, namely, the effect and benefits of prescribing Citicoline and Piracetam have

been studied.

The study enrolled a total of 1120 case histories of patients with acute cerebral circulation disorder (acute ischemic stroke) confirmed through computer tomography data between December 2019 and June 2020 in different hospitals in 8 cities of post-Soviet countries (4 cities in Russia and Ukraine). In 35 case histories, patients received drugs from other pharmacological groups as neuroprotective therapy, and, thus, were excluded from the study. Hence, the study involved a total of 1,085 patients. All patients were treated in the neurological departments of different hospitals in Russian and Ukrainian cities.

The authors declare that the work is written with due consideration of ethical standards. The study was conducted in accordance with the ethical principles approved by the Ethics Committee of Ukrainian Engineering and Pedagogical Academy (Protocol № 3 of 15.02.2021).

Methods

The study included 2 groups of patients: Group 1 received Citicoline as an additional neuroprotective therapy, and Group 2 received Piracetam. The dose of Citicolin was 1.0 g per 200 ml of physiological solution, and that of Piracetam – 4.5 g per 200 ml of physiological solution. Both drugs were administered intravenously by drop infusion, with subsequent reduction of the dose until the drug was completely withdrawn.

The first group of patients who received Citicoline as neuroprotective therapy included 680 patients (62.7%). The second group consisted of patients who received Piracetam and included 405 patients (37.3%).

The majority of the patients analyzed (65.9% or 715 persons) were women, and the rest of 370 patients (34.1%) were men. The average age of the patients was 57 years (42 to 74 years) (Table 1).

Table 1. Basic information about patients

Total number of case histories - 1,085	
City	Number of case histories analyzed
Moscow (Russia)	450 (41.47%)
Saint Petersburg (Russia)	211 (19.51%)
Kyiv (Ukraine)	122 (11.2%)
Kharkiv (Ukraine)	75 (6.9%)
Novosibirsk (Russia)	71 (6.54%)
Odessa (Ukraine)	60 (5.53%)
Yekaterinburg (Russia)	57 (5.25%)
Lviv (Ukraine)	39 (3.6%)
Gender	
Women	715 (65.9%)
Men	370 (34.1%)
Age	
Up to 45 years old	165 (15.3%)
45-55 years old	380 (35%)
55-65 years old	452 (41.6%)
Older than 65	88 (8.1%)

Study Design

Recent literature data were processed by meta-analysis.

The main criteria for evaluating the effectiveness of treatment with neuroprotective drugs included:

Degree of cerebral vasoconstriction;

Severity of the clinical disease course;

Risk of neurological complications.

The level of psychosomatic disorders (level of the anxiety-depressive syndrome) in patients from both groups at the beginning of the study and after its completion was calculated using the Hamilton scale (HDRS). The work was conducted with the patients' consent (written agreement on non-disclosure of information, observance of moral and ethical norms). This study included 44 patients, 23 from the Piracetam group and 21 from the Citicoline group. All patients were conscious. The daily dose of Piracetam and Citicoline corresponded to the minimum recommended dose for pronounced effects on the normalization of the central nervous system.

During the study, the Glasgow Coma Scale was used to assess the condition of patients with ischemic stroke after hospitalization. All patients were recommended to be continuously monitored by a neurologist after stroke treatment.

According to the Glasgow Coma Scale criteria, all patients were divided into three groups. The first group consisted of patients with a stun (12-13 points), amounting to 580 (53.45%). The second group included patients with soporus (9-11 points), amounting to 400 people (36.88%). The third group consisted of patients in a coma (3-8 points), amounting to 105 people (9.67%).

Statistical analysis

Statistical methods of calculation were applied for the data processing. The Microsoft Excel 2016

(USA, MicrosoftCorp) was used as a database. The data were further processed using Statistica v. 7.0 software (StatSoft Inc., USA). Student's t-test was used to compare data between samples (groups of patients) after testing for normality of distribution. Mean values and standard error of the mean were calculated as well. Differences were significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Analyzing the symptomatology of the disease, the following results were obtained: sudden weakness in the arm, leg, and half of the face (which is unilateral). Severe headache, dizziness, and movement coordination disorders were noted in all 1085 patients (100%) who were included in the study. Of these, speech problems (speaking, writing, and comprehension disorders) were observed in 226 patients (20.8%), and a sudden deterioration or absence of vision was noted in 54 patients (4.9%). Loss of consciousness was recorded in 105 patients (9.67%), of whom only four patients survived. Pain in the remaining 101 patients was fatal.

Of all 1085 patients (100%), 984 patients (90.7%) had positive dynamics of general psychoneurological symptoms against the background of the above therapy ($p \leq 0.05$ with the start of therapy). The condition of 101 (9.3%) patients worsened, which was caused by the severity of the disease and resulted in a lethal outcome in the long-term period due to complications of the underlying disease.

The first (main) group included 680 patients with sudden weakness in the arm, leg, and half of the face, severe headache, dizziness, and impaired movement coordination ($p \geq 0.05$ with the start of therapy). Of these, 130 patients had speech problems and 22 patients had impaired eyesight. Only 9 patients had speech impairment, 5 of whom were found to be fatal.

The second group included 405 patients with sudden weakness in the arm, leg, and half of the face,

severe headache, dizziness, and impaired movement coordination. Of these, 96 patients had speech problems, and 32 patients were with impaired eyesight. There were 96 comatose patients, and unfortunately, none of them survived, which was attributed to severe complications in the long-term period of the disease ($p \geq 0.05$ with the start of therapy).

Among the 680 patients (100%) who received Citicoline as a neuroprotective therapy, 625 (91.9%) patients showed improvement in general condition already after three days ($p \leq 0.01$ with the start of therapy). In 50 patients (7.3%), the improvement occurred on the 4th day (Figure 1) ($p \leq 0.05$ with the start of therapy). Another five patients (0.8%) died of pulmonary embolism as a complication of

the underlying disease on day 3 from the start of the therapy. Analyzing 405 patients (100%) that received Piracetam, the results were disappointing. The regression of neurological symptoms occurred on day 4 in 59 patients (14.6%), day 5 in 195 patients (48.2%), and day 6 in 55 patients (13.4%) (Figure 2) (all possible outcomes at $p \geq 0.05$ with the start of therapy). The lethal outcome was observed in 96 patients (23.8%), of whom 56 patients died of pulmonary embolism and another 40 died of myocardial infarction (all possible outcomes at $p \geq 0.05$ with the start of therapy).

In Group 1, the regression of neurological symptoms was noted at 3 ± 1 days compared to the control group, in which the recovery time was slightly longer (5 ± 1 days, $p \leq 0.05$).

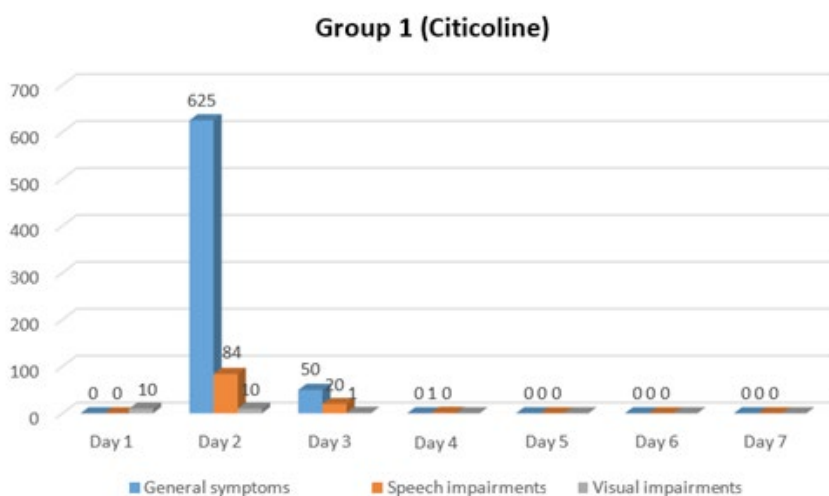


Figure 1. Improvement indices in the condition of patients depending on the use of Citicoline (number of patients)

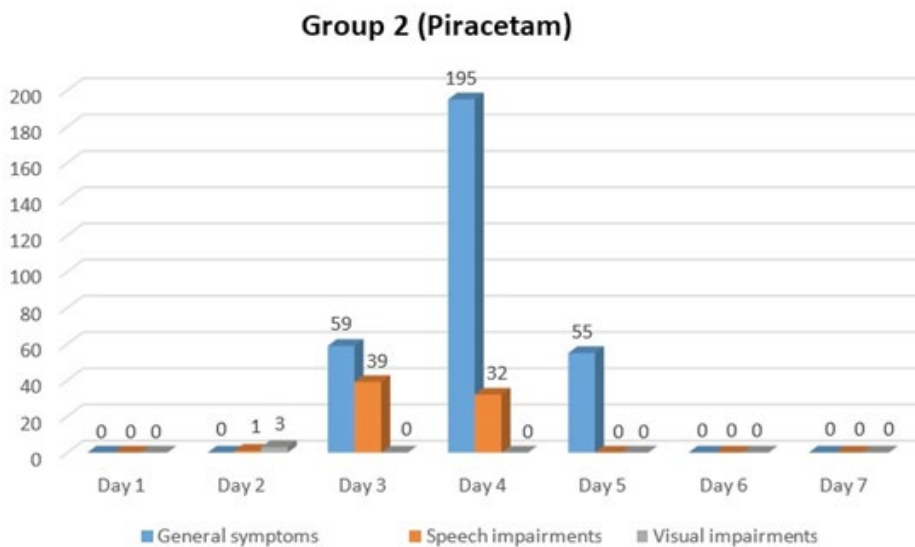


Figure 2. Improvement indices in the condition of patients depending on the use of Piracetam (number of patients)

Of all the patients analyzed, speech impairments were observed in 130 patients from the group that received Citicoline and 96 patients from the control group (Piracetam) ($p \leq 0.05$). As a result of the treatment, 105 patients in the main group, and 72 patients in the control group improved their speech ability ($p \leq 0.05$). Full recovery of the speech reactions was observed in 112 patients of the group, in which Citicoline was prescribed as neuroprotective therapy and in 76 patients of the Piracetam group during the long-term observation period (1.5–2 months) ($p \leq 0.05$). It is worth noting that patients in the first group improved speech after 2 ± 1 days, and the second group – after 3 ± 1 days ($p \geq 0.05$).

As a result of acute impairment of cerebral circulation, unilateral decrease or absence of eyesight was observed in 54 patients. After primary ophthalmological examination, all patients were diagnosed with the ocular ischemic syndrome. All patients were evenly divided into two groups, 22 patients from the Citicoline group and 32 from the Piracetam group (Piracetam). Treatment showed

improvement of visual function in 21 patients from the group, where Citicoline was administered as neuroprotective therapy. At that, the regression of visual symptoms was noted in 10 patients on the first day, 10 patients felt the improvement on the second day, and one patient – on the third day after the disease had begun. Quite different results were obtained in patients of the second group. Improvement of visual functions by 10% was noticed only in 3 patients on the second day from the start of the disease. This progress was explained by the mild general state of the patients and a rather high visual acuity (10-20%) compared to other patients where the acuity of vision was in the range from improper color perception to 10% of impairment. Also, the improvement of the visual function of the patients from the first group during the first days was insignificant (progress of $20 \pm 5\%$ from the initial one, $p \leq 0.05$). Analysis of distant eye consequences of the cerebrovascular disorder showed complete recovery of vision in 5 patients of Citicoline group 1.5–2 months after the loss of vision. Eight more patients had a $40 \pm 5\%$ increase of visual function during the same period ($p \leq 0.05$), and in

another eight patients, visual progress remained at the level of $20\pm 5\%$. In the group where Piracetam was used in the long-term period, full recovery of vision was not observed in any patient ($p \geq 0.05$). In three patients, the improvement was noted at $20\pm 5\%$ of the initial level. In the follow-up period, all 54 patients were excluded from observation.

The duration of hospital stay depending on the prescription of neuroprotective therapy has been analyzed. In the group of patients, to whom Citicoline was prescribed, the average hospitalization period lasted 7 ± 2 days, while in the control group, this figure was slightly longer, amounting to 9 ± 2 days ($p \leq 0.05$).

The use of Piracetam, on the other hand, produced a more pronounced psychosomatic effect in terms of decreasing the level of anxiety and depression. No pronounced changes were observed in patients treated for up to seven days, whereas patients who used Piracetam for more than seven days showed a 24% reduction in the level of anxiety and depression ($p \leq 0.05$) compared to the group of patients who took Citicoline.

No such effects were observed for Citicoline, which may be because Piracetam is the first of the nootropics with a complex effect, including the prevention of psychosomatic diseases, a protective effect, and normalization of the central nervous system.

The study found that the administration of Citicoline was more justified compared to Piracetam. After analyzing research works and articles by other scientists, both differences and convergence in results were found.

A recent study of Piracetam use in acute ischemic stroke indicated that the revised data did not provide conclusive evidence for the effect of using this drug in acute cerebrovascular accidents (Ricci, 2006).

Another study on the pharmacological treatment of aphasia after stroke with Piracetam, Piribedil (Pronoran), Bromocriptine, and Dextran 40 (Reopolyglucinum) in patients with acute cerebral circulation disorder did not reveal evidence that patients were more likely to experience improvement in speech performance at the end of the study after Piracetam treatment. Patients who received Piracetam as a neuroprotective therapy had slightly fewer adverse events, including death than those who received placebo. Such data raise some concerns that there may be an increased risk of death from Piracetam intake (Greener, 2001). The data in these two studies are consistent with the results of this work.

The study on neuroprotective properties of Citicoline found that this drug is non-toxic, which is confirmed by numerous preclinical data. Neuroprotective effect of Citicoline on the brain is based on the assumption that after injection or ingestion, this substance is sequentially hydrolyzed and dephosphorylated into cytidine and choline. These two metabolites then separately enter the brain tissue and are used to re-synthesize citicoline, which provides intracellular neuroprotection by supporting cellular phospholipid biosynthesis (Grieb, 2014).

Some data indicate the ability of Citicoline to normalize the patterns of neurotransmitter release. Under the conditions of cerebral hypoxia at ischemia, noradrenalin release may decrease, whereas dopamine release may increase. In several animal models, Citicoline has been shown to inhibit impaired neurotransmitter release in hypoxic states. Besides, administration of Citicoline to rats that were kept in a chronic hypoxic state decreased behavioral impairment and increased survival time. Additional studies have shown that Citicoline can increase vasodilation in animals with cerebral microcirculatory trauma, significantly increasing cerebral blood flow (Weiss, 1995).

The efficacy of Citicoline was also evaluated in a study that included 92 patients with chronic cerebrovascular disease. In this placebo-controlled study, 46 patients were randomized to each group. Patients received Citicoline (1000 mg/day intravenously) or placebo for two treatment cycles for four weeks, each with one week between cycles. The patient's response was assessed with several psychometric tests, measuring memory, behavior, attention, and emotional control. The results of the study showed that Citicoline significantly improved attention by reducing the number of incorrect reactions to nonverbal stimuli. Also, the continuous and progressive improvement was noted with Citicoline treatment on memory tests and assessments of emotion and behavior (Piccoli, 1994; Ignateva, 2020).

Hence, most of the processed materials confirm the results of this study and indicate a clear advantage of Citicoline over Piracetam applied to patients with an acute cerebrovascular accident, namely ischemic stroke. Patients who received Citicoline had faster regression of neurological symptoms, improvement of speech functions, and a significantly shorter period of inpatient treatment. In addition, the study obtained a positive effect of Citicoline as a neuroprotector in the improvement of visual symptoms by restoring the circulation of the optic nerve.

The results of this study did not identify any benefits of Piracetam, despite the large number of scientific articles stating the opposite. In the author's opinion, an improvement of general neurological symptoms in patients of the second group was noted due to basic therapy with anticoagulants, antiaggregants, and vasoactive drugs. As for the improvement of visual function, the use of Piracetam in a neuroprotective therapy was not justified. A slight improvement in visual acuity in 3 patients was associated with a mild course of the underlying disease.

Considering a large number of studies on the useful properties of Piracetam in patients with ischemic stroke with speech impairment and the data of this study allow stating that compared to other neuroprotective agents, Piracetam did not show any advantages. On the contrary, the recovery time of speech reactions was somewhat longer in the second group of patients. Consequently, the effectiveness of Piracetam for speech disorders is also not proven.

Therefore, the results of this study allow concluding that there are no proven neuroprotective properties of Piracetam.

CONCLUSIONS

The results of this study provided convincing evidence of the advantage of Citicoline over Piracetam. Nearly 92% of patients (625 people) indicated that their overall well-being had improved significantly by the third day. Citicoline was applied to all patients in the form of a neuroprotective agent. The effect of applying piracetam was noted later. Thus, in 405 patients, it manifested only on the 4th-5th day. Therefore, the use of citicoline also results in shorter hospitalization times. Furthermore, for citicoline, improvement in visual function was observed in 26 patients treated with citicoline. Similarly, in the piracetam group, this was reported in only three patients. This indicates the greater potential of citicoline as a complex action drug compared with piracetam, which has a limited effect at least on improving visual function.

Based on the evaluation of medical histories and medical records of patients from Russia and Ukraine, suffering from acute cerebrovascular accident, a neuroprotective therapy was prescribed. The first group of patients received Citicoline, and the second group – Piracetam. The analysis of literature and scientific works on the topic allowed stating the best effect for faster recovery after a stroke in Citicoline.

Citicolin is a new compound with a very broad spectrum of benefits for conditions associated with symptoms of neurological dysfunction. It works on several levels to support nervous health and optimal cognitive function. Citicoline has cholinergic and dopaminergic functions and supports the synthesis and incorporation of phospholipids into cell membranes and enhances antioxidant mechanisms in the body while suppressing the damaging effects of free radicals on nervous tissue. Citicoline should be considered a comprehensive therapeutic agent for maintaining brain health. Therefore, referring to these studies, Citicoline can be safely recommended as a neuroprotective therapy for patients with acute ischemic stroke.

As for Piracetam, the study did not identify any advantages of this drug over Citicoline in the treatment of the acute cerebrovascular disorder, and, thus, no evidence-based neuroprotective properties of Piracetam can be stated.

The use of Piracetam is justified in the prevention of psychosomatic disorders as this drug significantly reduces the frequency of anxiety-depressive syndrome by 24% ($p \leq 0.05$) starting from a period of more than seven days of the therapy.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Iryna Sokolova, Serafima Tazina and Oksana Zakharova contributed equally to the experimentation. Iryna Sokolova wrote and edited the article. Serafima Tazina designed and conducted the experiment. Oksana Zakharova studied scientific literature about the topic. All authors read and approved the final manuscript.

REFERENCES

- Adibhatla, R.M., Hatcher, J.F. (2002) Citicoline mechanisms and clinical efficacy in cerebral ischemia. *Journal of Neuroscience Research*, 70(2), 133-139.
- Al-Kuraishy, H.M., Al-Gareeb, A.I. (2020) Citicoline improves human vigilance and visual working memory: the role of neuronal activation and oxidative stress. *Basic and Clinical Neuroscience*, 11(4), 423-432.
- Babadjouni, R.M., Walcott, B.P., Liu, Q., Tenser, M.S., Amar, A.P., Mack, W.J. (2017) Neuroprotective delivery platforms as an adjunct to mechanical thrombectomy. *Neurosurgical Focus*, 42(4), E4.
- Belova, L.A. (2016) Stroke: treatment at the pre-hospital stage and in the conditions of a specialized hospital. *Remedium Volga Region*, 7(147), 17-22.
- Cotroneo, A.M., Castagna, A., Putignano, S., et al. (2013) Effectiveness and safety of citicoline in mild vascular cognitive impairment: the IDEALE study. *Clinical Interventions in Aging*, 8, 131-137.
- De Deyn, P.P., De Reuck, J., Deberdt, W., Vlietinck, R., Orgogozo, J.M. (1997) Treatment of acute ischemic stroke with piracetam. *Stroke*, 28(12), 2347-2352.
- Donmez, G., Outeiro, T.F. (2013). SIRT1 and SIRT2: emerging targets in neurodegeneration. *EMBO molecular medicine*, 5(3), 344-352.
- Flicker, L., Evans, J.G. (2004) Piracetam for dementia or cognitive impairment. *Cochrane Database of Systematic Reviews*, 1.
- Greener, J., Enderby, P., Whurr, R. (2001) Pharmacological treatment for aphasia following stroke. *Cochrane Database of Systematic Reviews*, 4, CD000424. doi: 10.1002/14651858.CD000424.
- Grieb, P. (2014) Neuroprotective properties of citicoline: facts, doubts and unresolved issues. *CNS Drugs*, 28(3), 185-193.
- Hacke, W., Kaste, M., Skyhoj Olsen, T., Orgogozo, J.M., Bogousslavsky, J. (2000) European stroke initiative (EUSI) recommendations for stroke management the European stroke initiative writing committee. *European Journal of Neurology*, 7(6), 607-623.

- Ignateva, E.V., Yartseva, I.V., Shprakh, Z.S., Prosalkova, I.R., Sasov, S.A., Orlova, O.V. (2020) Development and validation of dimeric macrocyclic tannin assay method in dosage forms. *Drug development & registration*, 9(4), 93-98.
- Jauch, E.C., Saver, J.L., Adams, Jr. H.P., et al. (2013) Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*, 44(3), 870-947.
- Nakazaki, E., Yabuki, Y., Izumi, H., et al. (2019) Combined citicoline and docosahexaenoic acid treatment improves cognitive dysfunction following transient brain ischemia. *Journal of Pharmacological Sciences*, 139(4), 319-324.
- Ortega, G., Jacas, C., Quintana, M., Ribo, M., Santamarina, E., Maisterra, O., Alvarez-Sabín, J. (2010) Citicoline treatment prevents neurocognitive decline after a first ischemic stroke. *Cerebrovascular Diseases*, 29(2), 268.
- Overgaard, K. (2014) The effects of citicoline on acute ischemic stroke: a review. *Journal of Stroke and Cerebrovascular Diseases*, 23(7), 1764-1769.
- Parfenov, V.A. (2012) Citicoline for ischemic stroke: ICTUS trial. *Neurology, Neuropsychiatry, Psychosomatics*, 4(4), 71-76.
- PASS II, Major ongoing stroke trials. (2001). *Stroke*, 32(6), 1449.
- Piccoli, F., Battistini, N., Carbonin, P., et al. (1994) CDP-choline in the treatment of chronic cerebrovascular pathologies. *Archives of Gerontology and Geriatric*, 18(3), 161-168.
- Polito, L., Kehoe, P.G., Davin, A., Benussi, L., Ghidoni, R., Binetti, G., Albani, D. (2013) The SIRT2 polymorphism rs10410544 and risk of Alzheimer's disease in two Caucasian case-control cohorts. *Alzheimer's & Dementia*, 9(4), 392-399.
- Porfiriyeva, N.N., Khutoryanskiy, V.V., Moustafine, R.I. (2020) A study of haloperidol release from polycomplex nanoparticles based on Eudragit® copolymers. *Drug Development & Registration*, 9(3), 45-50. (In Russ.).
- Ricci, S., Celani, M.G., Cantisani, T.A., & Righetti, E. (2006) Piracetam for acute ischaemic stroke. *Cochrane Database of Systematic Reviews*, 2, CD000419. doi: 10.1002/14651858.CD000419.pub3.
- Secades, J.J. (2011) Citicoline: pharmacological and clinical review, 2010 update. *Revista de Neurología*, 52(2), S1-S62.
- Shabanov, P.D. (2020) Clinical pharmacology of pyrroxane (proroxane). *Reviews on Clinical Pharmacology and Drug Therapy*, 18(4), 335-350.
- Sharayeva, A.T. (2018) Pharmacoepidemiological analysis of neuroprotectors use in acute disorders of cerebral circulation from the position of evidence-based medicine in the Kyrgyz Republic. *Bulletin of Scientific Education*, 49-001.
- Truelsen, T., Piechowski-Józwiak, B., Bonita, R., Mathers, C., Bogousslavsky, J., Boysen, G. (2006) Stroke incidence and prevalence in Europe: a review of available data. *European Journal of Neurology*, 13(6), 581-598.
- Weiss, G.B. (1995) Metabolism and actions of CDP-choline as an endogenous compound and administered exogenously as citicoline. *Life Sciences*, 56(9), 637-660.
- World Health Organization. (2018) Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016.
- Zhang, J., Wei, R., Chen, Z., Luo, B. (2016) Piracetam for aphasia in post-stroke patients: a systematic review and meta-analysis of randomized controlled trials. *CNS drugs*, 30(7), 575-587.

COVID-19: Mutated Strain, Treatment Options and Vaccine Development

Ayushi MAHAJAN* , Lakhvir KAUR**^o , Gurjeet SINGH*** ,
RK DHAWAN**** , Anureet KAUR*****

COVID-19: Mutated Strain, Treatment Options and Vaccine Development

SUMMARY

The ongoing outbreak of the COVID-19 is a significant threat to global health and the economy. This disease is a highly contagious pathogenic disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus has a high reproduction rate, due to that it is highly transmittable and has turned into a catastrophe. Scientists and researchers worldwide are exaggerating every possible approach to limit the spread of this malicious disease. An abrupt rise has been reported in the number of cases due to newly mutated strains like SARS-CoV-2 VUI 2020/12/01. To date, no specific drug is effective in the complete eradication of this dangerous disease but, some broad-spectrum antivirals such as Remdesivir and Lopinavir are being used in the management of this ailment. Also, every possible effort has been made in the development of vaccines for preventing the outbreak of this deadly virus. The BNT162b2 by Pfizer and m-RNA-1273 by Moderna have been recently launched into the market, which have shown undesirable effects in geriatrics leading to mortality. In this review, we have tried to highlight important aspects of the COVID-19 that will aid in global awareness and will help the researchers to investigate possible ways to eradicate this menace and design new moieties for its effectual management.

Key Words: COVID-19, SARS-CoV-2, Mutations, Spike protein, Pandemic, Vaccine.

COVID-19: Mutasyona Uğramış Tür, Tedavi Seçenekleri ve Aşı Geliştirme

ÖZ

Devam eden COVID-19 salgını, küresel sağlık ve ekonomi için önemli bir tehdittir. Bu hastalık, şiddetli akut solunum sendromu koronavirüs 2'nin (SARS-CoV-2) neden olduğu oldukça bulaşıcı bir patojenik hastalıktır. Virüs yüksek bir üreme oranına sahiptir, bu nedenle yüksek oranda bulaşabilir ve bir felakete dönüşmüştür. Dünya çapındaki bilim adamları ve araştırmacılar, bu kötücul hastalığın yayılmasını sınırlamak için mümkün olan her yaklaşımı fazlasıyla kullanıyorlar. SARS-CoV-2 VUI 2020/12/01 gibi yeni mutasyona uğramış suşlar nedeniyle vaka sayısında ani bir artış bildirilmiştir. Bugüne kadar, bu tehlikeli hastalığın tamamen ortadan kaldırılmasında spesifik bir ilaç etkili olmamıştır, ancak bu rahatsızlığın tedavisinde Remdesivir, Lopinavir gibi bazı geniş spektrumlu antiviraller kullanılmaktadır. Ayrıca, bu ölümcül virüsün ortaya çıkmasını önlemek için aşıların geliştirilmesinde mümkün olan her türlü çaba gösterilmiştir. Geriatriye ölüme yol açan istenmeyen etkiler gösteren Pfizer'ın BNT162b2'si ve Moderna'nın m-RNA-1273'ü, yakın zamanda piyasaya sürülmüştür. Bu derlemede, küresel farkındalığa yardımcı olacak ve araştırmacıların bu tehdidi ortadan kaldırmanın olası yollarını araştırmasına ve etkin yönetimi için yeni parçalar tasarlamasına yardımcı olacak COVID-19'un önemli yönlerini vurgulamaya çalıştık.

Anahtar kelimeler: COVID-19, SARS-CoV-2, Mutasyonlar, Spike protein, Pandemi, Aşı.

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* ORCID: 0000-0002-8666-4523, Department of Pharmaceutics, Khalsa College of Pharmacy, Amritsar, Punjab, India

** ORCID: 0000-0001-8091-2365, Department of Pharmaceutics, Khalsa College of Pharmacy, Amritsar, Punjab, India

*** ORCID: 0000-0003-4399-4693, Department of Pharmaceutics, Khalsa College of Pharmacy, Amritsar, Punjab, India

**** ORCID: 0000-0002-8587-6807, Department of Pharmacology, Khalsa College of Pharmacy, Amritsar, Punjab, India

***** ORCID: 0000-0002-2158-9569, Department of Pharmaceutics, Khalsa College of Pharmacy, Amritsar, Punjab, India

^o Corresponding Author: Dr. Lakhvir Kaur
e-mail: lakhvir86@gmail.com

INTRODUCTION

COVID-19 has become a global emergency that has drastically affected the health and the economy worldwide. The rampant spread of this life-threatening havoc has given sleepless nights to the masses and authorities. Humankind is at stake and scientists everywhere are scrambling to eradicate the root cause of this disease. COVID-19 is a deadly viral disease that has abducted many lives and the livelihood of human beings. The virus causing this disease is severe acute respiratory syndrome virus 2, i.e., SARS-CoV-2. The antigen was initiated from the seafood and wet animal market in Wuhan, Hubei, China, in December 2019, where bats, raccoon dogs, palm civets, snakes, and other animals are peddled (Bogoch et al., 2020; Lu et al., 2020). After this, numerous cases of pneumonia were observed with unfamiliar etiology. Later, reports from the deep sequencing analysis of the lower respiratory tract indicated the outbreak of a novel coronavirus and was named as COVID-19. Different states and territories have been infected with COVID-19 in the USA, China, Russia, Italy, Iran, Japan, India, etc., tolling about 76 million patients with more than 1.69 million deaths worldwide. The United States alone has confirmed at least 17.4 million cases in total, which is the highest count in the world. Looking at the current scenario, the World Health Organization [WHO] has declared it an emergency of international concern. This makes it an issue of high alert and hence builds urgency for public awareness. This article is an aid for controlling the present outbreak and future spread of COVID-19. In this review, complete detail about the viral mechanism, transmission, symptoms, mutated strains, diagnosis, various treatment options, and vaccine development landscape for coronavirus disease worldwide has been assimilated.

Phylogenetic Study

Coronavirus has been classified as a member of the Coronaviridae family, Orthocoronavirinae subfamily in the Nidovirales order (Zhu, 2019). The name coronavirus was derived from the crown-like spike proteins outlying the surface of the virus. The Coro-

navirus is enveloped, positive-sense, single-stranded RNA virus with size approximately equal to 20 nm. They are subdivided, based on genotypic and serological characters, into four genera: Alpha, Beta, Gamma, and Deltacoronavirus (Lefkowitz, 2018; Sexton, 2016; Su, 2017). Major species known to cause infections in humans include the highly pathogenic SARS-CoV and middle east respiratory syndrome [MERS] coronavirus and less rancorous species that include NL63, 229E, OC43, and HKU1. Like SARS-CoV and MERS-CoV, this newly emerged SARS-CoV-2 virus also belongs to the B lineage of the β -CoVs. The phylogenetic studies showed that the genome of this virus has an 80% resemblance to the SARS-CoV and 50% resemblance to the MERS-CoV (Lu et al., 2020; Ren et al., 2020).

Key Features of the Mechanism of Action of SARS-CoV -2

SARS-CoV-2 has a typical coronavirus structure, which encodes four structural proteins, i.e., nucleocapsid [N] protein, membrane [M] protein, spike [S] protein, and envelope [E] protein, and also several non-structural proteins. The process of viral entry and replication is depicted in Figure 1 (Bhoopathi et al., 2020).

The spike proteins outlying the virus's surface bind to angiotensin-converting enzyme 2 [ACE 2] receptors residing on the surface of the host cells, and fusion takes place between the viral and the cellular membranes (Belouzard et al., 2012). The protease enzymes transferrin and furin present in the host cell break the spike protein and release the viral RNA. The accomplishment of these steps leads to translation followed by RNA replication. The next step is the synthesis of structural viral proteins, M, S, and E, in the cytoplasm, embedded in the endoplasmic reticulum. Later, it is transferred to the endoplasmic reticulum-Golgi intermediate compartment, which serves as a site for coronavirus particle assembly (Song et al., 2004). At last, the formed vesicle is released through the process of exocytosis.

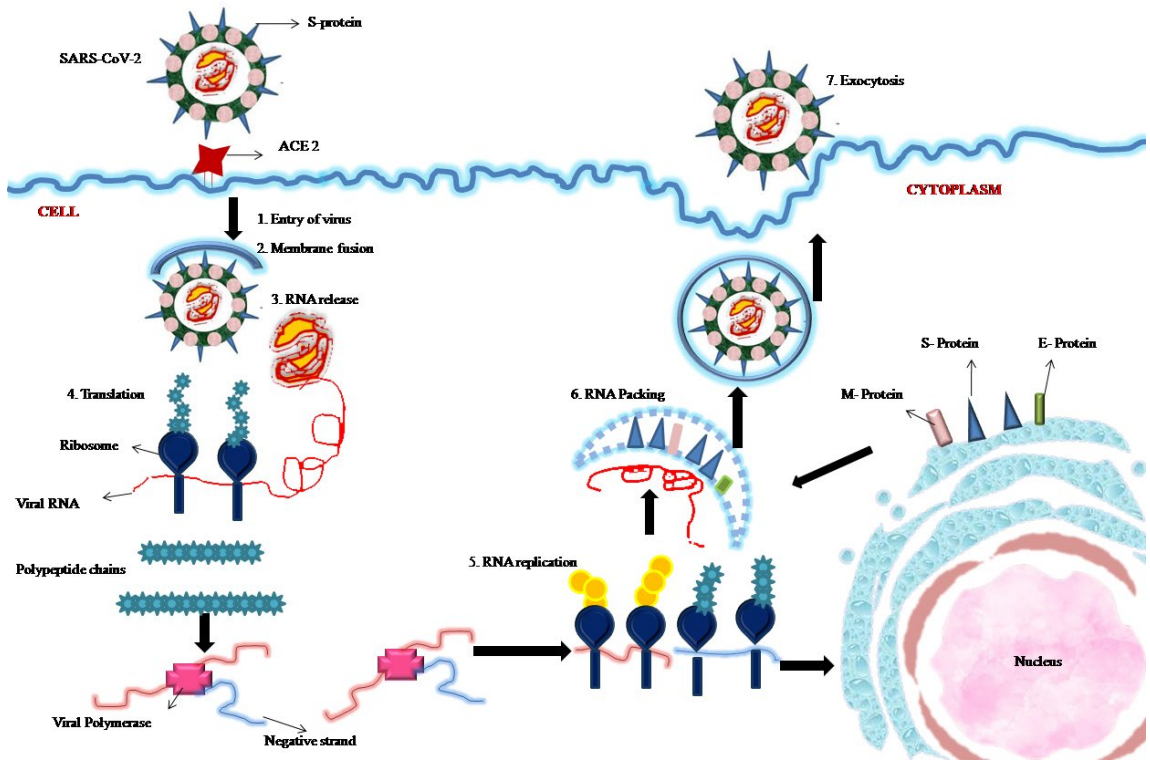


Figure 1. Mechanism of action of SARS-CoV-2

Primary Reservoir and Mode of Transmission of Coronavirus

As depicted in Figure 2, both SARS-CoV and MERS-CoV are zoonotic pathogens emerging from animals.

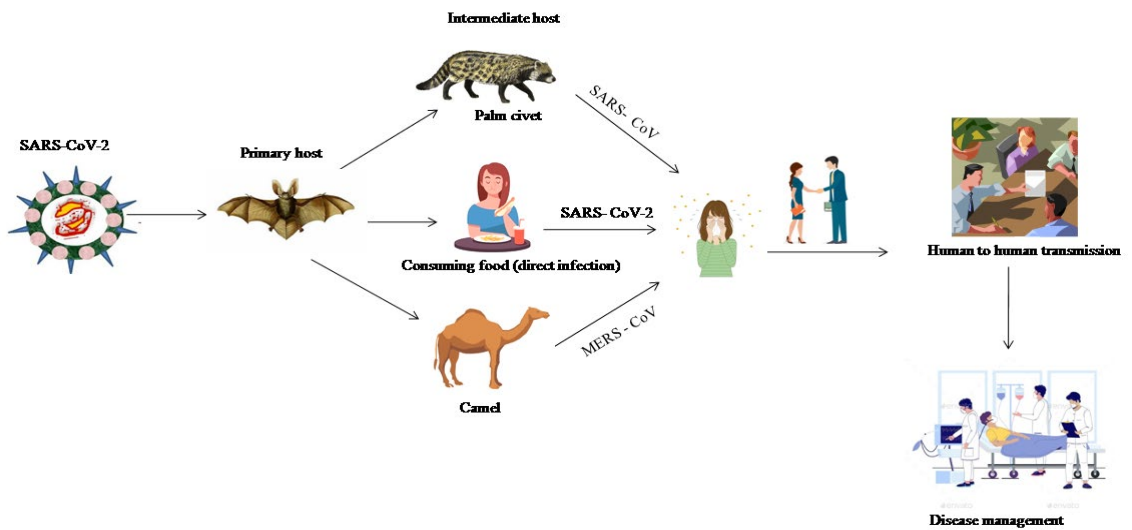


Figure 2. Primary reservoir and mode of transmission of coronavirus

They are considered to have been imparted possibly from bats to humans through some intervening mammalian hosts, known as secondary hosts such as raccoon dogs, palm civets, etc. (Kan et al., 2005; Bolles et al., 2011). An outbreak of SARS was reported in 2002 in China and rhinolophus bats were reported as the key reservoirs (Shi and Hu, 2008). MERS came into existence in 2012 in Saudi Arabia and reported the camel as the zoonotic source (Paden et al., 2018). This virus is transmitted from one person to another via direct contact or cough droplets. The reproduction number [R] of coronavirus was reported to be 2-3, which means that, on average, each infected person spreads the infection to an additional two to three persons, and this chain goes on (Liu et al., 2020). It mainly targets the epithelial cells of the lungs. The spike proteins present on the virus's surface attach to the ACE 2 receptors found in the lungs, heart, kidneys, and gastrointestinal tract, thus facilitating viral entry into target cells (Rabi et al., 2020). In a case study, nine COVID-19 positive pregnant women were included that underwent cesarean sections to evaluate the clinical peculiarities of COVID-19 in pregnancy and intrauterine vertical transmission. Amniotic fluid, cord blood, and neonatal throat swab samples were analyzed for the SARS-CoV-2 in the case of intrauterine transmission. The intrauterine transmission was not evident, but clinical presentations in the pregnant women were the same as that of infected adults (Chen et al., 2020).

The Newly Emerged Mutated Strains of SARS-CoV-2

During the global transmission, the SARS-CoV-2 has been mutated several times, leading to the emergence of a new strain of the virus called "SARS-CoV-2 VUI 202012/01". Since November 2020, a hike has been reported in the number of cases in London, accounting for around 60%. This novel strain has been developed through an array of multiple spike protein mutations, including 14 non-synonymous amino acid [AA] altering mutations, six synonymous non-AA al-

tering mutations, and three deletions. These are double deletion 69-70, deletion 144, the combination of N501Y, A570D, D614G in S protein, P681H, T716I, S982A, and D1118H (ECDC, 2020, December 21). The mutation N501Y is foremost and is located within the receptor-binding domain. It can stimulate the binding of S protein with ACE 2 receptors. It has been reported that this strain binds more tenaciously to the human ACE 2 receptor (CDC, 2020, December 29). Therefore, this strain can rapidly evade the host immune response by increasing binding sites and altering the surface structures recognized by antibodies (Volz et al., 2020). These unprecedented mutations make this variant 40-70% more rapidly transmissible than the other circulating strains of SARS-CoV-2 (Singh et al., 2021). A surge has been observed in the number of cases in South Africa due to the epiphany of another mutated SARS-CoV-2 strain called 501.V2. It is a matter of concern as the 501.V2 changes the shape of spike protein for better binding to the host cells, consequently rendering more contagious infection (Porterfield, 2021). This strain has shown two different mutations i.e., E484K and K417N (Ellyatt, 2021). Lately, scientists at Ohio State University have identified a new strain identical to the UK and South African strain. The variant is named COH.20G/501Y and is highly prevalent in Columbus. It has resulted from three mutations and is highly contagious (Wexner Medical Center, 2021).

Clinical Presentation and Diagnosis of the Disease

The spectrum of COVID-19 presentations varies from mild flu-like symptoms to life-threatening conditions like organ failure. This disease has shown potential comorbidities in young adults, unlike seasonal influenza and pneumonia. The symptoms may emerge 2 to 14 days after the infection. However, Chinese researchers claim the incubation period to be an average of 5.2 days (Li et al., 2020). The most common symptoms reported on the onset of COVID-19 were fever, cough, and fatigue, while other symptoms

include sputum production, dyspnoea, headache, hemoptysis, lymphopenia, and diarrhea (Huang et al., 2020). Older people, kids, and patients having severe health complications like lung diseases, heart diseases, diabetes, and cancer are more vulnerable to the infection as compared to adults. However, some detrimental anomalies may prove to be lethal such as RNAemia, acute respiratory distress syndrome, acute cardiac injury, and incidence of ground-glass opacities in subpleural regions of both lungs. In such cases, treatment may not be effective and neurological indications, ischemic and hemorrhagic strokes, and muscle injury may be ascertained (Mao et al., 2020).

The current methods of examination of COVID-19 include detection of the virus by techniques like a polymerase chain reaction or deep sequencing (Guo et al., 2020). Non-contrast chest computed tomography [CT] scans can also be taken into consideration for early diagnosis. Reverse transcription-polymerase chain reaction [RT-PCR] is the standard test for the detection of this virus. However, the efficiency of these methods depends on the presence of viral genome at the site of sample collection. Detection of antibodies, produced promptly after the infection, particularly immunoglobulin M, can be combined with polymerase chain reaction [PCR] to augment detection sensitivity and accuracy. RT-PCR is a genetically engineered in vitro technique used for the detection of the viral genome. Globally, various real-time RT-PCR protocols have been suggested for the diagnosis of COVID-19. These protocols differ in the genes they detect (Chu et al., 2020). However, the core issue with the real-time RT-PCR test is the induction of false-negative and false-positive results. It is stated that many suspected cases with typical clinical characteristics of COVID-19 and similar specific CT images were not diagnosed (Wang et al., 2020). Thus, a negative result does not eliminate the possibility of COVID-19 infection and should not be the only paradigm for treatment or patient management decisions. One of the reports from COVID-19 unveiled a patient with fever, cough, and coarse breathing

sounds of both lungs and his sputum showed positive RT-PCR results that confirmed the COVID-19 infection (Lei et al., 2020). The combination of real-time RT-PCR and clinical features aids in managing the SARS-CoV-2 outbreak (Xi et al., 2020). The results from real-time RT-PCR using primers in different genes can be affected by the variation of viral RNA sequences. Gene variation and rapid transformation of this novel coronavirus may result in false-negative results. To avoid false-negative results, multiple target gene amplification can be used.

Another method to detect COVID-19 is a serological test, also called the antibody test. It detects the existence of the virus by identifying the presence of specific proteins on the surface of the virus. Antibody response to SARS-CoV-2 infection usually develops after 7-14 days (Wolfel et al., 2020; Zhao et al., 2020). Lately, serological enzyme-linked immunosorbent assays were designed using recombinant antigens derived from the spike protein of SARS-CoV-2, which encouraged robust and scalable determination of seroconversion. This facilitates the screening of individuals who may experience evidence of past infection (Amanat et al., 2020). Due to an alarming spike in the number of cases every day, proficient testing kits are required.

Prevention and Management of the Disease

Prevention and management of the disease are the two essential tools in the fight against COVID-19. Collaborative measures of public and government are needed to eradicate this disease from its root. Cleanliness and sanitation are essential measures that are highly recommended. Domestic disinfectants may control the growth of the virus on the surfaces. The use of sanitizers and soaps for washing hands is highly recommended. People should wear a mask in public places and should maintain social distancing. Isolation becomes a necessary step as this virus is highly infectious and can spread very fast. Isolation alludes to the segregation of infected individuals from healthy ones. All the suspected patients in the containment

zones are hospitalized and kept in isolation under keen observation until they are tested negative. Patients who are tested positive for COVID-19 are hospitalized until two of their samples are tested negative.

Treatment Options

There is no specific treatment strategy that has shown complete efficacy in the treatment of this calamity but still, some treatment options have been

tried that can be used in the management of this disease.

Allopathy

COVID-19 is a highly contagious virus that spreads from person to person. There are not many treatment options for this disease because there are no specific antiviral drugs or vaccines effective against COVID-19 infection.

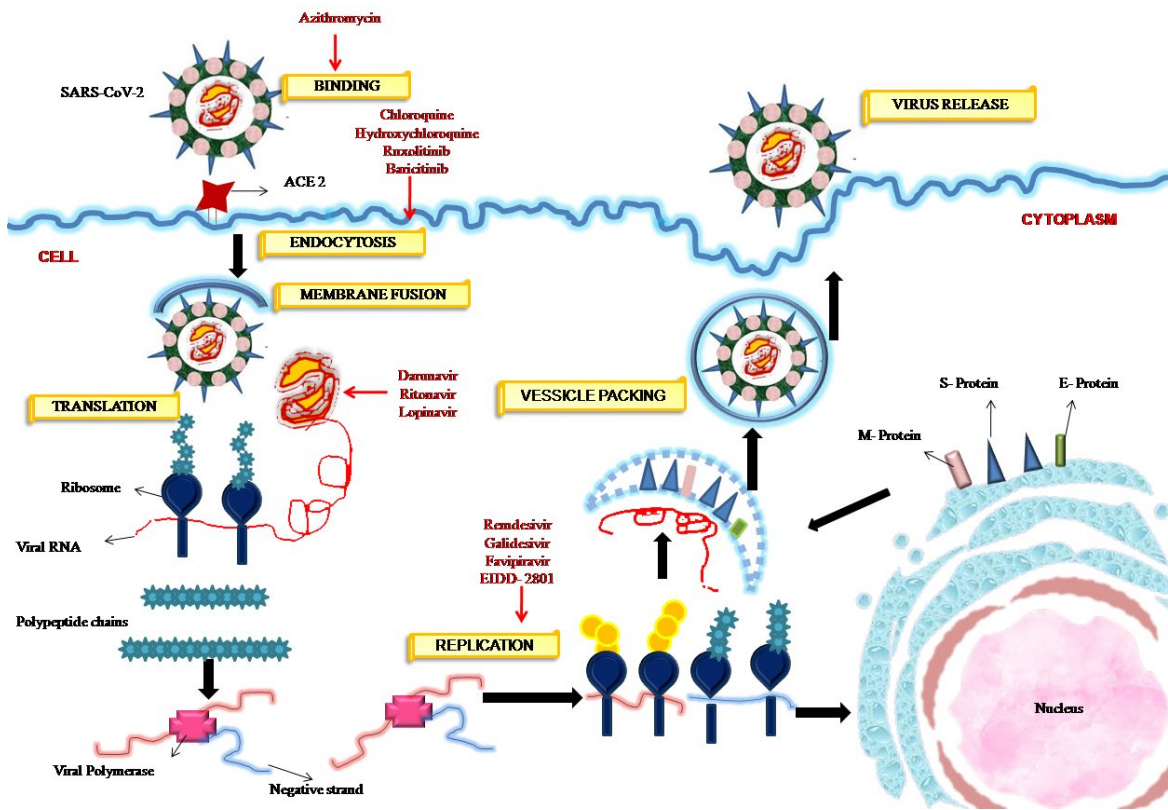


Figure 3. Mechanism of action of various allopathic drugs in the treatment of COVID-19.

One of the approaches to treat this dreadful disease is the use of broad-spectrum antiviral drugs like nucleoside analogs and HIV-protease inhibitors (Lu et al., 2020). The treatment regime that has been used showed that 75 patients were administered currently available antiviral drugs. The therapy included oral administration of 75 mg Oseltamivir, 500 mg Lopinavir, 500 mg Ritonavir twice a day and the intravenous administration of 0.25 g Ganciclovir for 3–14 days. Previously used drugs in the treatment of SARS-CoV

and MERS-CoV, such as a combination of Lopinavir and Ritonavir are also found to be effective (Chu et al., 2004; Momattin et al., 2019). Another report showed that a combination of anti-malarial drugs i.e., Chloroquine and antiviral drug Remdesivir are also effective. Concomitant therapy of Hydrochloroquine and Azithromycin decreased viral burden (Rajaiah et al., 2020). A study conducted at Bichat-Claude Bernard University Hospital, Paris, France, on the compassionate use of Remdesivir in treating COVID-19

reported lucrative results. Complications were reported in patients with advanced disease stages during infusion of Remdesivir (Dubert et al., 2020). EIDD-2801 have shown high therapeutic potential against seasonal viral infections and can be used as another potential drug in the treatment of COVID-19 (Toots et al., 2019). Lately, few patients infected with COVID-19 were treated with combined therapy of 200 mg Lopinavir and 50 mg Ritonavir twice a day and a combination of Oseltamivir and Chloroquine at Sawai Man Singh Hospital, Jaipur, India, and were then tested negative for the COVID-19 infection (Chattopadhyay, 2020). A study reported the effects of Favipiravir vs Lopinavir/ Ritonavir against COVID-19 in a non-randomized clinical trial. In this trial, 35 patients were treated with Favipiravir, and 45 patients were treated with Lopinavir/ Ritonavir for 14 days. It was inferred that Favipiravir was more effective than Lopinavir/ Ritonavir, with a lower incidence of viral load and higher rates of improvement in chest imaging (Cai et al., 2020). The most acceptable way to treat this disease is vaccination. Therefore, vaccines are being processed, and some are at the stage of clinical trials. Recently, a randomized, controlled clinical trial in the United Kingdom has found that a commonly used steroid, Dexamethasone effectively saved the lives of people who were seriously ill with COVID-19. Dexamethasone has been shown to cut deaths by about one-third in patients on ventilators because of coronavirus infection. Immunomodulatory drugs such as Tocilizumab can be used as another management strategy in treating COVID-19 patients with the risk of cytokine storms (Luo et al., 2020). JAK inhibitors like Ruxolitinib and Baricitinib demonstrate both anti-inflammatory and antiviral effects and can be used as a treatment option (Mehta et al., 2020). Figure 3 illustrates the mechanism of action of various allopathic drugs (Ojha et al., 2021).

Unani

Unani medicines or plant-based medicines are non-toxic with lesser side effects. The Unani system

is a traditional medicine system that is being explored for providing preventive, supportive, and rehabilitative care to patients. Different plants such as *Glycyrrhiza glabra*, *Allium cepa*, *Allium sativum*, *Curcuma longa*, *Ocimum sanctum*, *Ocimum tenuiflorum*, *Daucus maritimus*, etc. are effective against viral infections. The decoction of these drugs along with lemon juice and honey was found to be effective against cough and cold (Bano et al., 2013; Fatima et al., 2016; Ghoke et al., 2018; Hashemipour et al., 2014; Konowalchuk and Speirs, 1978; Miladi et al., 2012; Omer et al., 2014; Praditya et al., 2019). An *in vitro* activity of the plant *Glycyrrhiza glabra* was determined and it showed activity against several viruses including SARS-related coronavirus, HIV-1, and respiratory syncytial virus (Fiore et al., 2008). This plant is also effective against various viruses like Influenza A virus [IAV], Human immune virus [HIV], and SARS-associated coronavirus (Anagha et al., 2014). On January 29, 2020, the Government of India released a recommendation based on Indian traditional medicine practices Ayurveda, Homeopathy, and Unani, New Delhi. The advisory includes the ways of preventive management and described a list of some Unani medicines.

Homeopathy

Homeopathy is another efficient therapeutic method in the management and treatment of various diseases. Homeopathic treatment is advantageous as medicine has not to wait until the pathology's cause is found, unlike other treatment options. Therefore, the treatment can be immediate and beneficial for the patient (Kalliantas et al., 2020). According to the Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy [AYUSH] ministry of India, homeopathic medicine Arsenicum album-30 can be administered in the fasted state daily for three days as a prophylactic medicine against the infection. It is highly diluted arsenic trioxide and acts as homeopathic prophylaxis. It targets HT29 cells and human macrophages and thus, reduces NF- κ B hyperactivity i.e., decreased expression of reporter gene GFP in transfecting HT29 cells and TNF- α release in macrophages (Bellavite,

2015). As it is not clinically documented that Arsenic album-30 medicine is an effective medicine, it has been criticized by many researchers as pseudoscience. In a clinical trial data from Homeopathy Research Institute, Hong Kong represented 18 people in 6 groups with homeopathic medication. Group one had a female of age 62 who was treated with Bryoniaalb 30C. The other groups had age groups from 18 to 49 and were successfully treated with Bryoniaalb 30C, Gelsemium 30C, Arsenicumalb 30C, and Eupatorium perforatum 30C. All these drugs showed promising effects in the treatment of COVID-19 (Haque et al., 2020).

Plasma from convalescent patients

Convalescent plasma [CP] is a provisional approach for treatment until hyperimmune globulin, drug therapies, and vaccines are available. It has been successfully employed during other coronaviruses outbreaks. Therefore, it is established as the first option in the current situation. The treatment with CP is acquired by using extracorporeal therapy. The plasma of the infected patients is transfused with the plasma obtained from the survivors with the prior infection that contains antibodies against the causative agent of the disease. These antibodies provide passive immunity to the patient by identifying and neutralizing the viral entities (Burnouf and Seghatchian, 2014). A study depicted SARS-CoV-2 specific antibody titers ranging between 1.800 and 16.200, and neutralizing antibodies [Nabs] titers between 80 and 480 in the plasma obtained from the recovered patients of COVID-19, which was infused into the infected patient that reduced the viral load (Shen et al., 2020).

Immunity development

Strong immunity is a crucial weapon in the fight against COVID-19. Both innate and adaptive immune systems are triggered by the invasion of SARS-CoV. The expression of interferon type 1 [IFN-1] is inhibited, followed by inhibition of phosphorylation of signal transducer and activator of transcription 1 [STAT-1] (de Wit et al, 2016). The third defensive response is the immune system enervation through ex-

cessive and prolonged IFN-1 production by plasmacytoid dendritic cells [pDCs]. This further leads to an entry of neutrophils, inflammatory monocytes, and macrophages which results in inflammation of the lungs (Prompetchara et al, 2020). A high death rate is observed in older people, probably due to weak immunity, which leads to faster progress of COVID-19 (Li et al., 2005). Intake of vitamin C-rich food such as citrus fruits is recommended. Giloy [*Tinosporacordifolia*], Ashwagandha [*Withaniasomnifera*], and Tulsi [*Ocimum sanctum*] are some of the plants which boost up the immunity and can avenge against COVID-19 by hindering the action of protease M^{pro} or 3Cl^{pro}. The primary chemical constituents of Giloy e.g., Berberine, β -Sitosterol, Coline, Tetrahydropalmatine, and Octacosanol, can be used against SARS-CoV-2. These compounds target the main protease enzyme that is crucial for virus replication (Chowdhury, 2020). Two compounds, namely Withanoside V and Somniferine, can significantly bind to SARS-CoV-2 M^{pro} and can inhibit it. Dihydrodieuginol B and Tulsinol A, B, C, D, E, F, G are the main constituents of Tulsi that have potentially inhibit the protease enzyme (Shree et al., 2020). It is also advisable to take zinc and iodine supplements. Therefore, it is essential to boost our immune system, and hence, the use of immune system boosters should be practiced.

Vaccine research and development

The COVID-19 outbreak is a stark reminder of the ongoing challenge of vaccine development. Robust actions are needed in research and development [R&D] of vaccines to eradicate this disease. The spike protein of the SARS-CoV-2 is the primary target of the vaccine because this can produce neutralizing antibodies that can directly block the virus from infecting healthy cells. According to the global COVID-19 vaccine R&D landscape, about 115 vaccine candidates are there. Out of which, 78 are confirmed, and 37 are unconfirmed. Out of 78 approved candidates, 73 are active and are currently at exploratory or pre-clinical stages. Some of the candidates like mRNA-1273 from Moderna, Ad5-nCoV from CanSino Biologicals, INO-4800 from Inovio, and LV-SMENP-DC and pathogen-specific aAPC from Shenzhen Ge-

no-Immune Medical Institute are at the clinical stage of vaccine development (Thanh et al., 2020).

A striking feature of the vaccine development landscape for COVID-19 is the multiple strategies that have been employed to generate SARS-CoV-2 vaccines, including DNA- and RNA-based vaccines, viral vector vaccines, inactivated virus vaccines, live-attenuated virus vaccines, and recombinant protein vaccines. Recently, the Russian government announced that the country has developed and approved the world's first SARS-CoV-2 vaccine. Gamaleya Research Institute of Epidemiology and Microbiology, Moscow developed the vaccine against COVID-19 named Sputnik V. Sputnik V uses adenoviruses like Ad5 and Ad26 viral vector to deliver the gene for the SARS-CoV-2 spike protein. These are administered in two shots, i.e, 'loaded' Ad26 vector is distributed in the first dose, and the 'loaded' Ad5 vector follows in a second dose after 21 days. This double-vector approach of vaccine administration is quite advantageous as the first dose develops antibodies against the Ad26 serotype. The second dose uses an Ad5 serotype as a vector to enhance the immune response of the body (Dobrovidova, 2020).

A similar approach has been used by the University of Oxford and AstraZeneca, but with a different adenovirus vector which is a chimp vector-like Ch-AdOx. These adenovirus vectors can cause severe infections such as acute respiratory infections, fever,

and diarrhea in humans. The primary issue regarding this vaccine is that it has not yet undergone phase III clinical trials. The lack of accurate data about phase I and II trials exacerbates the cynicism about the safety and efficacy of the vaccine (Caddy, 2020). The m-RNA based BNT162b2 was found to be 95% effective. A comparative study, where four vaccines naming Moderna, AstraZeneca/Oxford, Pfizer/BioNTech, and SputnikV were evaluated and compared for their efficacy and safety. The Moderna, AstraZeneca/Oxford, and Pfizer/BioNTech vaccines seemed effective in preventing COVID-19. No evidence for the ability of the Sputnik V vaccine is published yet. All four vaccines were safe with minor side effects such as headache, joint pain, fatigue, and fever (De and Joseph, 2020). Recently, severe anaphylaxis has been reported in patients who have received the Pfizer and Moderna [mRNA vaccine] vaccines. A report stated the mortality of 29 frail aged persons with serious health problems inoculated with the BNT162b2. Common adverse effects like nausea, diarrhea, and fever were observed (Torjesen, 2021). These vaccines, therefore, may lead to severe consequences and are too risky for the elderly. Covaxin is an inactivated vaccine developed by Bharat Biotech. It is found to be effective against the UK variant of SARS-CoV-2 (Sakpal et al., 2021). A list of vaccine candidates approved and vaccines still under development are mentioned in Table 1 and Table 2, respectively (Regulatory affairs professional society, 2021, January 14).

Table 1. List of approved vaccines.

S.No	Candidate	Type of vaccine	Sponsor
1	BNT162b2	mRNA-based vaccine	Pfizer; BioNTech
2	Covaxin	Inactivated vaccine	Bharat Biotech; National Institute of Virology
3	m-RNA-1273	mRNA-based vaccine	Moderna
4	Corona Vac	Inactivated vaccine (formalin with album adjuvant)	Sinovac
5	BBIBP-Cor V	Inactivated vaccine	Beijing Institute of Biological Products; China National Pharmaceutical Group (Sinopharm)
6	EpiVacCorona	Peptide vaccine	Federal Budgetary Research Institution State Research Center of Virology and Biotechnology
7	Sputnik V	Non-replicating viral vector	Gamaleya Research Institute, Acellena Contract Drug Research, and Development

Table 2. List of vaccines under clinical trials.

S.No	Candidate	Mechanism	Trial phase	Sponsor
1	AZD1222	Replication-deficient viral vector vaccine	Phase 3	The University of Oxford; AstraZeneca; IQVIA; Serum Institute of India
2	Ad5-nCoV (Convidicea)	Recombinant vaccine	Phase 3	CanSino Biologics
3	JNJ-78436735	Non-replicating viral vector	Phase 3	Johnson&Johnson
4	NVX-CoV2373	Nanoparticle vaccine	Phase 3	Novavax
5	INO-4800	DNA vaccine (plasmid)	Phase 2/3	Inovio Pharmaceuticals
6	Bacillus Calmette Guerin (BCG) vaccine	Live-attenuated vaccine	Phase 2/3	The University of Melbourne and Murdoch Children's Research Institute; Radboud University Medical Center; Faustman Lab at Massachusetts General Hospital
7	VIR-7831	Plant-based adjuvant vaccine	Phase 2/3	Medicago; GSK; Dynavax
8	ZyCoV-D	DNA vaccine (plasmid)	Phase 2	Zydus Cadila
9	AG0301-COVID19	DNA vaccine	Phase ½	AnGes, Inc.

Future Perspectives

WHO has declared COVID-19 as a pandemic as it is spreading at a frenetic pace and has affected millions of people globally. Comprehensive measures are needed to repress the outbreak of COVID-19 and to obstruct the person-to-person transmission. To prevent the spread of this disease, social distancing should be practiced. Preventive measures and health care guidelines for the public and health care workers have been reported by WHO to manage this pernicious disease. This virus is rapidly mutating and emerging into a more infectious pathogen. COVID-19 is culminating in a massive transformation in every sector of life. The worst-case scenario is that numerous industries and governments have succumbed to its effects, due to which a multitude of people have lost their livelihoods. In the best-case scenario, this crisis has also presented an opportunity for more flexible and prudent technology use. Also, this pandemic has led to a tremendous patient workload that makes healthcare providers prone to burnout and depression. Therefore, telemedicine has lingered as a cost controlling and high convenience system. Being a pandemic, COVID-19 is not easy to eradicate, so extensive research is mandatory to combat this deadly disease.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

The idea of the manuscript (Kaur L., Singh G), supervision (Kaur L., Singh G., Dhawan R.K), literature search (Mahajan A), article writing (Mahajan A., Kaur L), critical review (Kaur A., Kaur L., Mahajan A).

REFERENCES

Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T., Chromikova, V., McMahan, M., ...Krammer, F. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nature Medicine*, 26(7), 1033-1036. doi: 10.1038/s41591-020-0913-5.

Anagha, K., Manasi, D., Priya, L., Meera, M. (2014). Scope of Glycyrrhiza glabra (Yashtimadhu) as an antiviral agent: a review. *International Journal of Current Microbiology and Applied Sciences*, 3(12), 657-665. Retrieved from <https://www.ijcmas.com/vol-3-12/Korhalkar%20Anagha,%20et%20al.pdf>

Bano, N., Ahmed, A., Tanveer, M., Khan, GM., Ansari, M.T. (2017). Pharmacological Evaluation of Ocimum sanctum. *Journal of Bioequivalence & Bioavailability*, 9(3), 387-392. doi:10.4172/jbb.1000330

Bellavite P. (2015). Homeopathy and integrative medicine: keeping an open mind. *Journal of Medicine and the Person*, 13(1), 1–6. doi: 10.1007/s12682-014-0198-x

- Belouzard, S., Millet, J. K., Licitra, B. N., Whittaker, G. R. (2012). Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses*, 4(6), 1011–1033. doi: 10.3390/v4061011
- Boopathi, S., Poma, A. B., Kolandaivel, P. (2020). Novel 2019 coronavirus structure, mechanism of action, antiviral drug promises, and rule out against its treatment. *Journal of Biomolecular Structure & Dynamics*, 1–10. doi: 10.1080/07391102.2020.1758788
- Bogoch, I. I., Watts, A., Thomas-Bachli, A., Huber, C., Kraemer, M., Khan, K. (2020). Pneumonia of unknown etiology in Wuhan, China: Potential for international spread via commercial air travel. *Journal of Travel Medicine*, 27(2), 1-3. doi: 10.1093/jtm/taaa008
- Bolles, M., Donaldson, E., Baric, R. (2011). SARS-CoV and emergent coronaviruses: viral determinants of interspecies transmission. *Current Opinion in Virology*, 1(6), 624–634. doi: 10.1016/j.coviro.2011.10.012
- Burnouf, T., & Seghatchian, J. (2014). Ebola virus convalescent blood products: Where we are now and where we may need to go. *Transfusion and Apheresis Science*, 51(2), 120–125. doi: 10.1016/j.transci.2014.10.003
- Caddy, S. (2020). Russian SARS-CoV-2 vaccine. *British Medical Journal*, 370, m3270. doi: 10.1136/bmj.m3270
- Cai, Q., Yang, M., Liu, D., Chen, J., Shu, D., Xia, J., ... Liu, L. (2020). Experimental treatment with Favipiravir for COVID-19: An open-label control study. *Engineering*, 6(10), 1192–1198. doi: 10.1016/j.eng.2020.03.007
- Centers for disease control and prevention (CDC). (2020, December 29). *Interim: Implications of the Emerging SARS CoV-2 Variant VOC 202012/01*. Retrieved from <https://www.cdc.gov/coronavirus/2019ncov/more/scientific-brief-emerging-variant.html>
- Chang, J. S., Wang, K. C., Yeh, C. F., Shieh, D. E., & Chiang, L. C. (2013). Fresh ginger (*Zingiber officinale*) has antiviral activity against human respiratory syncytial virus in human respiratory tract cell lines. *Journal of Ethnopharmacology*, 145(1), 146–151. doi: 10.1016/j.jep.2012.10.043
- Chattopadhyay, A. (2020). *Doctors cure three coronavirus patients with combination of drugs used to treat swine flu, malaria, HIV*. Retrieved from <https://thelogicalindian.com/news/covid-19-jai-pur-hiv-drugs-coronavirus-outbreak-20183>
- Chen, H., Guo, J., Wang, C., Luo, F., Yu, X., Zhang, W., ... Zhang, Y. (2020). Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: retrospective review of medical records. *Lancet*, 395(10226), 809–815. doi: 10.1016/S0140-6736(20)30360-3
- Chowdhury, P. (2020). In silico investigation of phytoconstituents from Indian medicinal herb, ‘*Tinospora cordifolia* (giloy)’ against SARS-CoV-2 (COVID-19) by molecular dynamics approach. *Journal of Biomolecular Structure & Dynamics*, 1–18. doi:10.1080/07391102.2020.1803968
- Chu, C. M., Cheng, V. C., Hung, I. F., Wong, M. M., Chan, K. H., Chan, K. S., ... Yuen, K.Y. (2004). Role of lopinavir/ritonavir in the treatment of SARS: Initial virological and clinical findings. *Thorax*, 59(3), 252–256. doi: 10.1136/thorax.2003.012658
- Chu, D. K. W., Pan, Y., Cheng, S. M. S., Hui, K. P. Y., Krishnan, P., Liu, Y., ... Poon, L. L. M. (2020). Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clinical Chemistry*, 66(4), 549–555. doi: 10.1093/clinchem/hvaa029
- de Wit, E., van Doremalen, N., Falzarano, D., & Munster, V. J. (2016). SARS and MERS: Recent insights into emerging coronaviruses. *Nature Reviews Microbiology*, 14(8), 523–534. doi:10.1038/nrmicro.2016.81
- De, Soto., Joseph, A. (2020). “Evaluation of the Moderna, Pfizer/Biontech, Astrazeneca/Oxford and Sputnik V Vaccines for COVID-19.” *OSF Preprints*. doi:10.31219/osf.io/e4rqu
- Dobrovidova, O. (2020). *Russia’s Sputnik Vaccine Stunt Could Cast a Long Shadow*. Retrieved from <https://science.thewire.in/health/russia-sputnik-covid-19-vaccine/>
- Dubert, M., Visseaux, B., Isernia, V., Bouadma, L., Deconinck, L., Patrier, J., ... Lescure, F. X. (2020). Case report study of the first five COVID-19 patients treated with remdesivir in France. *International journal of infectious diseases*, 98, 290–293. doi: 10.1016/j.ijid.2020.06.093

- Ellyatt, H. (2021). *Covid variant found in South Africa is worrying experts: Here's what we know so far*. Retrieved from <https://www.cnn.com/2021/01/06/south-africa-covid-strain-a-guide-to-what-you-need-to-know.html>
- European Centre for Disease Prevention and Control (ECDC). (2020, December 21). *Rapid increase of a SARS-CoV-2 variant with multiple spike protein mutations observed in the United Kingdom*. Retrieved from <https://www.ecdc.europa.eu/sites/default/files/documents/SARS-CoV-2-variant-multiple-spike-protein-mutations-United-Kingdom.pdf>
- Fatima, M., Zaidi, N. U., Amraiz, D., Afzal, F. (2016). In vitro antiviral activity of *Cinnamomum cassia* and its nanoparticles against H7N3 influenza A virus. *Journal of Microbiology and Biotechnology*, 26(1), 151–159. doi: 10.4014/jmb.1508.08024
- Fiore, C., Eisenhut, M., Krausse, R., Ragazzi, E., Pelati, D., Armanini, D., Bielenberg, J. (2008). Antiviral effects of Glycyrrhiza species. *Phytotherapy Research*, 22(2), 141–148. doi: 10.1002/ptr.2295
- Ghoke, S. S., Sood, R., Kumar, N., Pateriya, A. K., Bhatia, S., Mishra, A., ... Singh, V. P. (2018). Evaluation of antiviral activity of *Ocimum sanctum* and *Acacia arabica* leaves extracts against H9N2 virus using embryonated chicken egg model. *BMC Complementary and Alternative Medicine*, 18(1), 174. doi: 10.1186/s12906-018-2238-1
- Guo, L., Ren, L., Yang, S., Xiao, M., Chang, D., Yang, F., ... Wang, J. (2020). Profiling early humoral response to diagnose novel coronavirus disease (COVID-19). *Clinical Infectious Diseases*, 71(15), 778–785. doi: 10.1093/cid/ciaa310
- Hashemipour, M.A., Tavakolineghad, Z., Arabzadeh, S.A., Iranmanesh, Z., Nassab, S.A. (2014). Antiviral activities of honey, royal jelly, and acyclovir against HSV 1. *Wounds*, 26(2), 47-54. Retrieved from <https://www.woundsresearch.com/article/antiviral-activities-honey-royal-jelly-andacyclovir-against-hsv-1>
- Haque, M.I., Shafin, A., Mahmud, M.D. (2020). Combined homeopathy and allopathy treatment for COVID-19: a review. *Bangladesh Journal of Infectious Diseases*, 7, 38-45. doi: 10.3329/bjid.v7i00.50161
- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., ... Cao, B. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*, 395(10223), 497–506. doi: 10.1016/S01406736(20)30183-5
- Kalliantas, D., Kallianta, M., Karagianni, C. S. (2020). Homeopathy combat against coronavirus disease (COVID-19). *Journal of Public Health*, 1–4. doi: 10.1007/s10389020-01305-z
- Kan, B., Wang, M., Jing, H., Xu, H., Jiang, X., Yan, M., ... Xu, J. (2005). Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. *Journal of Virology*, 79 (18), 11892–11900. doi: 10.1128/JVI.79.18.11892-11900.2005
- Konowalchuk, J., & Speirs, J. I. (1978). Antiviral effect of commercial juices and beverages. *Applied and Environmental Microbiology*, 35(6), 1219–1220. doi: 10.1128/AEM.35.6.1219-1220.1978
- Lefkowitz, E. J., Dempsey, D. M., Hendrickson, R. C., Orton, R. J., Siddell, S. G., Smith, D. B. (2018). Virus taxonomy: The database of the International Committee on Taxonomy of Viruses (ICTV). *Nucleic Acids Research*, 46(1), 708–717. doi: 10.1093/nar/gkx932
- Lei, J., Li, J., Li, X., Qi, X. (2020). CT Imaging of the 2019 Novel Coronavirus (2019-nCoV) Pneumonia. *Radiology*, 295(1), 18. doi: 10.1148/radiol.2020200236
- Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., ... Feng, Z. (2020). Early transmission dynamics in Wuhan, China, of novel coronavirus infected pneumonia. *The New England Journal of Medicine*, 382(13), 1199- 1207. doi:10.1056/NEJMoa2001316
- Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., ... Tan, X. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Research*, 67(1), 18- 23. doi: 10.1016/j.antiviral.2005.02.007
- Liu, Y., Gayle, A. A., Wilder-Smith, A., Rocklöv, J. (2020). The reproductive number of COVID-19 is higher compared to SARS coronavirus. *Journal of Travel Medicine*, 27(2), 1-4. doi: 10.1093/jtm/taaa021

- Lu, H., Stratton, C. W., Tang, Y. W. (2020). Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. *Journal of Medical Virology*, 92(4), 401-402. doi: 10.1002/jmv.25678
- Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., ... Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: Implications for virus origins and receptor binding. *Lancet*, 395(10224), 565-574. doi:10.1016/S01406736(20)30251-8
- Luo, P., Liu, Y., Qiu, L., Liu, X., Liu, D., Li, J. (2020). Tocilizumab treatment in COVID-19: A single center experience. *Journal of Medical Virology*, 92(7), 814-818. doi: 10.1002/jmv.25801
- Mao, L., Jin, H., Wang, M., Hu, Y., Chen, S., He, Q., ... Hu, B. (2020). Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. *JAMA Neurology*, 77(6), 683-690. doi: 10.1001/jamaneurol.2020.1127
- Mehta, P., Ciurtin, C., Scully, M., Levi, M., & Chambers, R. C. (2020). JAK inhibitors in COVID-19: the need for vigilance regarding increased inherent thrombotic risk. *The European Respiratory Journal*, 56(3), 2001919. doi: 10.1183/13993003.01919
- Miladi, S., Abid, N., Debarnôt, C., Damak, M., Canard, B., Aouni, M., & Selmi, B. (2012). In vitro antiviral activities of extracts derived from *Daucus maritimus* seeds. *Natural Product Research*, 26(11), 1027-1032. doi: 10.1080/14786419.2010.550263
- Momattin, H., Al-Ali, A. Y., Al-Tawfiq, J. A. (2019). A systematic review of therapeutic agents for the treatment of the middle east respiratory syndrome coronavirus (MERS CoV). *Travel Medicine and Infectious Disease*, 30, 9-18. doi:10.1016/j.tmaid.2019.06.012
- Ojha, P.K., Kar, S., Krishna, J.G., Roy, K., Leszczynski, J. (2021). Therapeutics for COVID 19: From computation to practices where we are, where we are heading to. *Molecular Diversity*, 25, 625-659. doi: 10.1007/s11030-020-10134-x
- Omer, M. O., Almalki, W. H., Shahid, I., Khuram, S., Altaf, I., Imran, S. (2014). Comparative study to evaluate the antiviral efficacy of Glycyrrhiza glabra extract and ribavirin against the Newcastle disease virus. *Pharmacognosy Research*, 6(1), 6-11. doi: 10.4103/0974 8490.122911
- Paden, C. R., Yusof, M., Al Hammadi, Z. M., Queen, K., Tao, Y., Eltahir, Y. M., ... Al Muhairi, S. (2018). Zoonotic origin and transmission of Middle East respiratory syndrome coronavirus in the UAE. *Zoonoses and Public Health*, 65(3), 322-333. doi: 10.1111/zph.12435.
- Porterfield, C. (2021). *South African Coronavirus Strain Is 50% More Infectious, Scientists Say*. Retrieved from <https://www.Porterfield.com/sites/carlieporterfield/2021/01/18/south-african-coronavirusstrain-is-50-more-infectious-scientists-say/?sh=33d08ccb4475>
- Praditya, D., Kirchhoff, L., Brüning, J., Rachmawati, H., Steinmann, J., Steinmann, E. (2019). Anti-infective properties of the golden spice curcumin. *Frontiers in microbiology*, 10, 912. doi: 10.3389/fmicb.2019.00912
- Promptchara, E., Ketloy, C., Palaga, T. (2020). Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. *Asian Pacific Journal of Allergy and Immunology*, 38(1), 1-9. doi: 10.12932/AP-200220-0772
- Rabi, F. A., Al Zoubi, M. S., Kasasbeh, G. A., Salameh, D. M., Al-Nasser, A. D. (2020). SARS-CoV-2 and coronavirus disease 2019: what we know so far. *Pathogens*, 9(3), 231. doi: 10.3390/pathogens9030231
- Rajaiah, R., Abhilasha, K.V., Shekar, M.A., Vogel, S.N., Vishwanath, B.S. (2020). Evaluation of mechanisms of action of re-purposed drugs for treatment of COVID-19. *Cellular Immunology*, 358, 104240. doi: 10.1016/j.cellimm.2020.104240
- Regulatory affairs professional society. (2021, January 14). *COVID-19 Vaccine Tracker*, <https://www.raps.org/news-and-articles/news-articles/2020/3/covid-19-vaccine-tracker>
- Ren, L. L., Wang, Y. M., Wu, Z. Q., Xiang, Z. C., Guo, L., Xu, T., ... Wang, J. W. (2020). Identification of a novel coronavirus causing severe pneumonia in human: A descriptive study. *Chinese Medical Journal*, 133(9), 1015-1024. doi: 10.1097/CM9.0000000000000722
- Sapkal, G. N., Yadav, P. D., Ella, R., Deshpande, G. R., Sahay, R. R., Gupta, N., ... Bhargava, B. (2021). Neutralization of UK-variant VUI-202012/01 with COVAXIN vaccinated human serum. *BioRxiv*. doi:10.1101/2021.01.26.426986

- Sexton, N. R., Smith, E. C., Blanc, H., Vignuzzi, M., Peersen, O. B., Denison, M. R. (2016). Homology-based identification of a mutation in the coronavirus RNA-dependent RNA polymerase that confers resistance to multiple mutagens. *Journal of Virology*, 90(16), 7415–7428. doi: 10.1128/JVI.00080-16
- Shen, C., Wang, Z., Zhao, F., Yang, Y., Li, J., Yuan, J., ... Liu, L. (2020). Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *Journal of the Medical Association*, 323(16), 1582–1589. doi: 10.1001/jama.2020.4783
- Shi, Z., & Hu, Z. (2008). A review of studies on animal reservoirs of the SARS coronavirus. *Virus Research*, 133(1), 74–87. doi: 10.1016/j.virus-res.2007.03.012
- Shree, P., Mishra, P., Selvaraj, C., Singh, S. K., Chaube, R., Garg, N., Tripathi, Y. B. (2020). Targeting COVID-19 (SARS-CoV-2) main protease through active phytochemicals of ayurvedic medicinal plants – *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Giloy), and *Ocimum sanctum* (Tulsi) - a molecular docking study. *Journal of Biomolecular Structure & Dynamics*, 1–14. doi:10.1080/07391102.2020.1810778
- Singh, J., Ehtesham, N.Z., Rahman, S.A., Hasnain, S.E. (2021). Structure-function investigation of a new VUI-202012/01 SARS-CoV-2 variant. *BioRx-iv*. doi: 10.1101/2021.01.01.425028
- Song, H. C., Seo, M. Y., Stadler, K., Yoo, B. J., Choo, Q. L., Coates, S. R., ... Han, J. H. (2004). Synthesis and characterization of a native, oligomeric form of recombinant severe acute respiratory syndrome coronavirus spike glycoprotein. *Journal of Virology*, 78(19), 10328–10335. doi: 10.1128/JVI.78.19.10328-10335.2004
- Su, S., Wong, G., Shi, W., Liu, J., Lai, A., Zhou, J., ... Gao, G. F. (2016). Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. *Trends in Microbiology*, 24(6), 490–502. doi: 10.1016/j.tim.2016.03.003
- Thanh Le, T., Andreadakis, Z., Kumar, A., Gomez Roman, R., Tollefsen, S., ... Mayhew, S. (2020). The COVID-19 vaccine development landscape. *Nature Reviews Drug Discovery*, 19(5), 305–306. doi: 10.1038/d41573-020-00073-5
- Toots, M., Yoon, J. J., Cox, R. M., Hart, M., Sticher, Z. M., Makhsous, N., ... Plemper, R. K. (2019). Characterization of orally efficacious influenza drug with high resistance barrier in ferrets and human airway epithelia. *Science Translational Medicine*, 11(515), eaax5866. doi: 10.1126/scitranslmed.aax5866
- Torjesen, I. (2021). COVID-19: Doctors in Norway told to assess severely frail patients for vaccination. *British Medical Journal*, 372(8276), n167. doi: 10.1136/bmj.n167
- Volz, E., Hill, V., McCrone, J. T., Price, A., Jorgensen, D., O’Toole, Á., ... Connor, T. R. (2021). Evaluating the effects of SARS-CoV-2 spike mutation D614G on transmissibility and pathogenicity. *Cell*, 184(1), 64–75. doi: 10.1016/j.cell.2020.11.020
- Wang, Y., Kang, H., Liu, X., & Tong, Z. (2020). Combination of RT-qPCR testing and clinical features for diagnosis of COVID-19 facilitates management of SARS-CoV-2 outbreak. *Journal of Medical Virology*, 92(6), 538–539. doi: 10.1002/jmv.25721
- Wexner Medical Center. (2021). *Researchers Discover New Variant of COVID-19 Virus in Columbus, Ohio*. Retrieved from <https://wexnermedical.osu.edu/mediaroom/pressreleaselisting/new-sars-cov2-variant>.
- Wolfel, R., Corman, V. M., Guggemos, W., Seilmaier, M., Zange, S., Muller, M. A., ... Wendtner, C. (2020). Virological assessment of hospitalized patients with COVID 2019. *Nature*, 581(7809), 465–469. doi: 10.1038/s41586-020-2196-x
- Xi, M., Wei, Q., Qihua, F., & Ming, G. (2020). Understanding the influence factors in viral nucleic acid test of 2019 novel coronavirus (2019-ncov). *Chinese Journal of Laboratory Medicine*, 43, E002-E002. Retrieved from <https://covid19.elsevierpure.com/en/publications/understanding-the-influence-factors-in-viral-nucleic-acid-test-of>
- Zhao, J., Yuan, Q., Wang, H., Liu, W., Liao, X., Su, Y., ... Zhang, Z. (2020). Antibody responses to sars-cov-2 in patients with novel coronavirus disease 2019. *Clinical Infectious Diseases*, 71(16), 2027–2034. doi: 10.1093/cid/ciaa344
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., ... Tan, W. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *The New England Journal of Medicine*, 382(8), 727–733. doi: 10.1056/NEJMoa2001017

Çözünürlüğü Düşük Olan Etken Maddeler İçin Farmasötik Yaklaşımlar ve Aprepitantın Çözünürlüğü

Hakan NAZLI* , Burcu MESUT** , Yıldız ÖZSOY***,°

Pharmaceutical Approaches for Low Solubility Agents and Solubility of Aprepitant

SUMMARY

Advances in technology have broken new ground in the area of new active pharmaceutical ingredients discovery. Although the number of newly discovered active ingredients increases, only a few of them manage to survive for further development. Even if some of the discovered active ingredients have appropriate pharmacological activity, they are eliminated in the early stages of drug development due to their undesired physicochemical properties. Most of the time low solubility leads to bioavailability problems. Increasing the solubility and hence bioavailability of an active pharmaceutical ingredient is an integral part of pharmaceutical technology and development. In the first part of this review, information about the methods that can be used to increase the solubility is given. In the second part, studies aiming to increase the solubility of aprepitant, a low-solubility active ingredient, are discussed.

Key Words: Solubility, Bioavailability, Solubility Enhancement Techniques, Particle Size Reduction, Solid Dispersions, Aprepitant

Çözünürlüğü Düşük Olan Etken Maddeler İçin Farmasötik Yaklaşımlar ve Aprepitantın Çözünürlüğü

ÖZ

Gelişen teknoloji yeni etken maddelerin tasarlanması ve keşfedilmesi konularında çağ atlanmasını sağlamıştır. Her ne kadar yeni bulunan etken madde sayısı artsa da bunların çok az bir kısmı geliştirilmeye değer olarak seçenekler arasında kalmayı başarabilmektedir. Keşfedilen bazı etken maddeler uygun farmakolojik aktiviteye sahip olsa dahi, uygun olmayan fizikokimyasal özellikleri nedeniyle ilaç geliştirme basamaklarının daha ilk aşamalarında elenmektedir. Geliştirilmeye devam edildiklerinde ise ilerleyen aşamalarda düşük biyoyararlanım sorunları ile karşılaşmaktadır. Çoğu zaman, görülen biyoyararlanım sorunlarının temelindeki neden etken maddenin çözünürlüğünün düşük olmasıdır. Bu özellikteki etken maddelerin çözünürlüklerinin ve dolayısıyla biyoyararlanımlarının artırılması farmasötik teknolojideki çalışma alanlarının önemli bir bölümünü kapsamaktadır. Bu derleme çalışmasının ilk bölümünde çözünürlüğü düşük etken maddelerin çözünürlüğünü arttırmada kullanılacak yöntemlerle ilgili bilgiler verilmiş, ikinci kısımda ise çözünürlüğü düşük bir etken madde olan aprepitantın çözünürlüğünü artırma hedefli çalışmalar ele alınmıştır.

Anahtar kelimeler: Çözünürlük, Biyoyararlanım, Çözünürlük Artırma Teknikleri, Partikül Boyutu Küçültme, Katı Dispersiyonlar, Aprepitant

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* ORCID: 0000-0001-5763-1450, Trakya Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 22030, Merkez, Edirne, Türkiye

** ORCID: 0000-0003-2838-1688, İstanbul Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 34116, Beyazıt, İstanbul, Türkiye

*** ORCID: 0000-0002-9110-3704, İstanbul Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 34116, Beyazıt, İstanbul, Türkiye

° Corresponding Author: Yıldız ÖZSOY

Tel: +90 0212 440 00 00-13498; e-mail: yozsoy@istanbul.edu.tr,

GİRİŞ

Günümüzde ilaç uygulamasının en basit, hızlı ve tercih edilen şekli ilaçların oral yoldan verilmesidir. Hasta açısından düşünüldüğünde bir ilacın kolayca yutularak alınması oldukça rahattır. Diğer yollar ile kıyaslandığında oral uygulamada hastanın tedaviye olan uyuncu daha yüksek olduğundan tedavinin başarı şansı artmaktadır (Jaskirat, Manpreet, & Harikumar, 2013). Oral dozaj formlarının diğerlerine göre çok sayıda üstünlüğü bulunmakla birlikte etken maddelerin gastrointestinal sistem sıvılarındaki çözünürlüğünün düşük olması oral uygulamadaki en büyük engellerdendir (Kurmi, 2016). Farmasötik kimyada ve yüksek hızlı ilaç tarama teknolojilerindeki gelişmeler, etken maddelerin keşfi sürecinde çığır açmış, uygun farmakolojik aktiviteye sahip çok sayıda molekül geliştirilmiştir. Ancak bu moleküllerin çoğu yüksek lipofiliklik, düşük su çözünürlüğü ve yüksek moleküler ağırlık gibi istenmeyen çeşitli fizikokimyasal özelliklere sahiptir (Bajaj, 2012). Son yıllarda, terapötik etkisi yüksek, fakat su çözünürlüğü düşük olan etken maddelerin toplam ilaçların yaklaşık % 25-40'ı olduğu tahmin edilmektedir. Zayıf çözünürlük, gastrointestinal sistemde absorpsiyonu düşürür ve sonuç olarak bu ilaçların klinik kullanımlarını sınırlandırır (Bikiaris, 2011). Dolayısıyla bunların ilaç olarak kullanılabilmesi için uygun formülasyonlarının hazırlanması ilaç endüstrisi için oldukça zorlu bir süreç olmaktadır (Kurmi, 2016).

Çözünürlük belirli bir miktardaki çözücüde çözünen maksimum madde miktarı olarak tanımlanır. Sayısal olarak belirli bir sıcaklıktaki doygun çözeltinin konsantrasyonunun bulunmasıyla belirlenir. Tablo 1'de çözünürlükle ilgili çeşitli tanımlamalar verilmiştir (Verma, 2011).

Kesin bir limit olarak kabul edilmemekle birlikte çözünürlük %1'in (10mg/mL) altına indikçe etken maddenin farmakolojik etkisini göstermesi güçleşmektedir (P. Sharma, 2012). 37 °C'de pH 1-7 aralığında su çözünürlüğü 10mg/ml'nin altında olan ilaçlar potansiyel biyoyararlanım problemleri gösterirler (Semalty, 2014). Sudaki düşük çözünürlük, temel olarak yüksek lipofiliklik ve yavaş çözünmeye yol açan güçlü moleküller arası kuvvetler olmak üzere iki önemli parametreye bağlıdır (Seo, 2003).

İlaç çözünürlüğünün farmasötik teknoloji açısından önemi, ilacın ancak çözülmüş halde emilebilmesinin mümkün olmasındadır. Çözünürlük basamağı ilacın oral biyoyararlanımı açısından en önemli basamaklardan biridir (S. K. Patil, 2011). Ayrıca su çözünürlüğü düşük olan ilaçların farmakokinetik parametreleri genellikle birey içi ve bireyler arası büyük değişkenlik gösterir. Bu durum ilaç geliştirmede Faz I çalışmalarının tasarlanmasını ve yürütülmesini çok zorlaştırır. Doz-yanıt eğrilerinin değerlendirilmesi, doz önerisi ve optimizasyonunun yapılması zorlaşır. Suda çözünmeyen ilaçlar genellikle gıda-ilac etkileşimi gibi ilaç etkileşimleri için de daha yüksek eğilime sahiptir. Tüm bu durumlar terapötik penceresi dar olan ilaçlar için daha da önemli bir problem haline gelir (S. Jain, 2012).

Tablo 1. Çözünürlük Tanımlaması (EP7.0, 2010; USP30-NF25, 2007)

Tanım	1 birim çözünen için gereken çözücü
Çok çözünür	<1
Serbestçe çözünür	1-10
Çözünür	10-30
Eser miktarda çözünür	30-100
Az çözünür	100-1.000
Çok az çözünür	1.000-10.000
Çözünmez	>10.000

Çözünme olayı, çözünen madde içindeki iyonlar arası veya moleküller arası bağların kırılmasını, çözücü içinde boşluk sağlamak için çözücü moleküllerinin ayrılmasını ve çözücü ile çözünen molekül veya iyon arasındaki etkileşimi içerir (A. N. Patil, 2017).

İlaçlar çözünürlüklerine ve membran geçirgenliklerine göre Biyofarmasötik Sınıflandırma Sistemi (BCS) adı altında gruplara ayrılmıştır. Bu sınıflandırma sistemi dört alt sınıftan oluşmaktadır. Sınıfların özellikleri Tablo 2'de gösterilmiştir. Özellikle sınıf II ve sınıf IV'te bulunan ilaçların gastrointestinal sis-

temdeki çözünürlüklerinin ve çözünme hızlarının artırılması biyoyararlanımlarının artmasını sağlayabilmektedir (Kawabata, 2011).

Tablo 2. BCS sınıflandırma sistemi (Kawabata, 2011)

BCS Sınıfı	Çözünürlük	Geçirgenlik
I	Yüksek	Yüksek
II	Düşük	Yüksek
III	Yüksek	Düşük
IV	Düşük	Düşük

BCS'ye göre ilacın kullanımdaki en yüksek dozu, pH 1-7,5 aralığında 250mL suda çözünebiliyorsa ilaç yüksek çözünürlüklü, ilacın emilimi verilen dozun %90'ından büyük ise de ilacın yüksek geçirgenliğe sahip olduğu kabul edilir. Ayrıca ilacın etiket dozunun %85'inden fazlası 30 dakika içinde çözünüyorsa ilacın hızla çözüldüğü kabul edilir (Kawabata et al., 2011). Çözünme hızı kavramı Noyes-Whitney eşitliği (1) ile açıklanabilir (D. Horter & Dressman, 2001).

$$\frac{dC}{dt} = \frac{A \cdot D}{h} x (C_s - C) \quad (1)$$

dC/dt = çözünme hızı,

A =yüzey alanı, D =çözünen maddenin difüzyon sabiti, h =difüzyon yüzeyi katmanının kalınlığı,

C_s =çözünürlük, C =herhangi bir t anındaki konsantrasyon

Denklemden görüldüğü gibi çözünürlük (C_s), halihazırda çözülmüş ilaç konsantrasyonu (C), difüzyon tabakasının kalınlığı (h), çözünmenin gerçekleştiği yüzey alanı (A) ve difüzyon katsayısı (D) çözünme hızı için kritik faktörlerdir (D. Horter ve Dressman, 2001). Çözünme olayı sink koşulda gerçekleştiğinde C_s değeri C 'ye göre çok daha büyük olacaktır. İlaç moleküllerinin sudaki difüzyon katsayısı göreceli olarak yüksektir ve difüzyon katmanının kalınlığını in vivo ortamda değiştirmek mümkün değildir. Bu nedenle formülasyon stratejileri çözünürlüğü veya yüzey alanını artırmaya odaklıdır (S. Jain, 2015). Çözünme hızı genellikle bir ilacın emiliminde hız kısıtlayıcı olduğundan ilacın ıslanabilme özelliğinin ve yüzey alanının artışı biyoyararlanımın iyileşmesi sağlar (Jas-

kirat, 2013). İlaçların çözünürlüğü (C_s), partikül boyutu, sıcaklık, basınç, çözücü ve çözünen maddenin kimyasal yapısı, molekül boyutu, molekülün polaritesi, polimorfizm gibi özelliklerden etkilenir (Kurmi, 2016; A. N. Patil, 2017; S. K. Patil, 2011).

1. Çözünürlüğü artırmada kullanılan yaklaşımlar

Sudaki çözünürlüğü zayıf olan ilaçların çözünürlüğünü ve çözünme hızını iyileştirmek için farklı yaklaşımlar bulunmaktadır. Bu yaklaşımlar genellikle yüzey alanını artırmak için partikül boyutunun küçültülmesi, yüzey etken maddelerin kullanılması, suda çözünür komplekslerin hazırlanması, etken maddenin kristal yapısının değiştirilmesi, ön ilaç veya ilacın tuz formları gibi etken maddede kimyasal modifikasyonlar yapılması gibi yaklaşımları içerir (Bikaris, 2011).

İlaçların çözünürlüğünü arttırmada kullanılan teknikler Şekil 1'de özetlenmiştir.

İlaçların çözünürlüğünü arttırmada kullanılan tekniklere ait bilgiler aşağıda özetlenmiştir.

1.1. Ön İlaç Oluşturma

Vücutta bazı kimyasal veya biyolojik dönüşümler geçirerek farmakolojik olarak aktif bileşiğe dönüşen ilaçlar ön ilaç olarak tanımlanmaktadır. Ön ilaçların kendisi genellikle farmakolojik olarak inaktif bileşiklerdir (Hussain & Rytting, 1974). Bu yöntem çözünürlüğü, metabolik stabiliteyi, geçirgenliği artırmak ve toksisiteyi azaltmak için yaygın olarak kullanılmaktadır. Ön ilaç hazırlama stratejisi, ya polar kısım ilavesiyle (fosfat veya ester grupları gibi) ya da aktif ilacın kristal yapısındaki değişikliklerle gerçekleştirilir (S. Jain et al., 2015). Örneğin valgansiklovir ve valasiklovir sırasıyla gansiklovir ve asiklovirin valin esterleridir. Fosaprepitant aprepitantin fosfatlanmış türevi olup intravenöz (I.V.) olarak kullanım için geliştirilmiştir. Ön ilaç yaklaşımı kullanılarak ana ilaçların çözünürlüklerinde ve dolayısıyla biyoyararlanımlarında artış elde edilmiştir (S. Jain et al., 2015; Lasseter et al., 2007).

Kimyasal modifikasyon	Çözücüde değişiklikler	Fiziksel modifikasyon	Diğer
<ul style="list-style-type: none">• Ön-ilaç• Tuz oluşturma• Ko-kristalizasyon (Birlikte kristallendirme)	<ul style="list-style-type: none">• pH ayarlaması• Kosolvan kullanımı• Miselizasyon• Hidrotropi	<ul style="list-style-type: none">• Çözünür polimorfların kullanımı• Poröz yapıya yükleme• Partikül boyutu küçültme (Mikronizasyon, Nanostüpsiyon)• Fiziksel kompleksler (siklodekstrin, dendrimer)• Katı dağılımlar (amorflar)	<ul style="list-style-type: none">• Farklı nanoteknolojik yaklaşımlar (konjugatlar vb.)• Miseller• Mikro-emülsiyonlar• Nano-emülsiyonlar• Lipozomlar• Amfifilik polimerler• Lipid Taşıyıcılar (SLN)

Şekil 1. Çözünürlüğü Artırmada Kullanılan Teknikler

1.2. Tuz Oluşturma

Asidik veya bazik yapıli ilaçların çözünürlüğünü artırmada kullanılabilecek en basit ve etkili yöntemlerdendir. Asidik veya bazik ilaçların tuzları, genel olarak, karşılık gelen asit veya baz formlarından daha yüksek çözünürlüğe sahiptir. Dolayısıyla ilaç molekülü tuzuna çevrilerek çözünürlüğü artırılabilir. Örneğin fenobarbital – fenobarbital sodyum (S. K. Patil, 2011; Zaheer, 2011). 1995'ten 2006'ya kadar Birleşik Devletler Gıda ve İlaç Dairesi (FDA) tarafından onaylanan yaklaşık 300 yeni kimyasal oluşumdan 120'si tuz formunda bulunmaktadır (Tao, Zhao, Wu, & Zhou, 2009).

1.3. Ko-kristalizasyon

Ko-kristaller iki veya daha fazla sayıda kristal yapının kovalent olmayan bağlarla bir arada tutulmasıyla elde edilir (Korotkova & Kratochvíl, 2014). Bunlar aynı zamanda moleküler kompleksler olarak da isimlendirilebilir (S. K. Patil, 2011). Ko-kristallerin solvatlardan temel farkı yapıyı oluşturan moleküllerin fiziksel halidir. Solvatlarda bu bileşenlerden biri sıvı biri katı haldeyken ko-kristallerde her ikisi de katı halde bulunur (A. V. Yadav, 2009). Bu yöntem özellikle nötral bileşiklerde tuz oluşumuna alternatif olarak kullanılabilecek bir yöntemdir. Ayrıca sınırlı sayıdaki tuz oluşturuç moleküle kıyasla genel olarak güvenli olarak kabul edilen (GRAS) listesinde, hidrojen bağı

oluşturma işlevine sahip ko-kristal oluşturuç olarak kullanabilen çok sayıda molekül mevcuttur (Sugandha, 2014). Asit-amit, asit-piridin, amit-piridin gibi çeşitli fonksiyonel grupların etkileşimi ko-kristalizasyon için büyük bir fırsat sunar (Li, 2015).

Ko-kristaller çözücü uçurulması, beraber öğütme gibi çeşitli yöntemlerle hazırlanır (S. K. Patil, 2011; Savjani, 2012). Etken maddelerin çözünürlüğünün yanında akışkanlık, yığın yoğunluğu, sıkıştırılabilirlik, ufalanabilirlik, erime noktası, higroskopik özellikleri gibi diğer fizikokimyasal özelliklerini de ko-kristalizasyon ile değiştirmek mümkündür (Korotkova & Kratochvíl, 2014). Sakarin, nikotinamid ve asetik asit ko-kristalizasyon ajanı olarak kullanılabilen maddelere örnek olarak verilebilir (Zaheer, 2011).

1.4. pH Ayarlaması

Su çözünürlüğü düşük bir ilaç molekülü asidik (proton verebilen) veya bazik (proton alabilen) kısımlara sahipse ortam pH'sının değişmesiyle çözünürlük artırılabilir. Bu yöntem kolayca uygulanabilir ancak pH değişiminde (örneğin gastrointestinal sistem boyunca veya pH'sı farklı ortamlarla seyrelme durumunda) etken maddenin çökme ihtimali vardır. Ayrıca fizyolojik olmayan uç pH değerlerinde lokal veya sistemik toksisite riski, ilacın çözünür olduğu pH değerindeki görülebilecek stabilite problemleri de göz önünde bulundurulmalıdır (Vemula, 2010).

1.5. Kosolvan Kullanımı

Suda düşük çözünürlüğe sahip olan ilaçların çözünürlükleri su ile karışabilen başka çözücüler ortama eklendiğinde genellikle artış gösterir. Bu çözücüler kosolvanlar veya yardımcı çözücüler olarak adlandırılır. Farklı polietilen glikol çeşitleri, propilen glikol, gliserin ve etanol en sık kullanılan kosolvanlardır (Zaheer, 2011). Farklı kosolvanlar birlikte kullanıldıklarında sinerjistik bir etki elde edilmesi de mümkündür (Seedher ve Agarwal, 2009). İlaç endüstrisinde sık başvurulan, basit bir yöntem olmakla birlikte önemli bir dezavantajı vardır. Çözücü ortamın seyrelmesiyle çözme kapasitesinde hızla azalma görülür. Bunun sonucunda etken madde hızla ve kontrolsüzce çöker. Suda düşük çözünürlüklü bir çok ilaç için tek başına bu teknik yeterli olmadığından, çoğu zaman diğer çözünürlük artırıcı yöntemlerle beraber kullanılır (Vemula, 2010).

1.6. Misel Aracılı Çözündürme (Miselizasyon)

Yüzey etken maddeler polar ve nonpolar bölgeler içeren, farmasötik teknolojide çok önemli bir yere sahip olan moleküllerdir. Polar grubun yüküne göre anyonik, katyonik, zwitteryonik veya non-iyonik olarak sınıflandırılırlar (S. K. Patil, 2011). Eklendikleri çözücülerin yüzey gerilimlerini düşürürler. Düşük yüzey gerilimine sahip çözeltilerde partiküllerin yüzey ıslanma özellikleri artacağından çözünürlükte de artış görülür (Hammond, 2007).

Sulu ortamlarda yüzey etken maddeler misel adı verilen, iç tarafı apolar, dış tarafı polar olan iki bölgeyi sistemler oluştururlar. Sürfaktanların konsantrasyonları kritik misel konsantrasyonunun üzerine çıktığında (bu değer çoğu surfaktan için %0,05-0,10 arasındadır) çözeltideki hidrofobik ilaç molekülleri miseller içerisinde hapsedilir. Su çözünürlüğü düşük olan ilaçların çözünürlüğünde artış görülmesine sebep olan bu olay miselizasyon veya misel aracılı çözündürme olarak isimlendirilir (Vemula, 2010).

Misel aracılı çözündürme, çözünürlük artışı sağlamada bilinen en eski ve etkili tekniklerinden biridir. Kritik misel konsantrasyonu düşük, biyolojik sistemle uyumlu ve yüksek çözündürme gücüne sahip noniyono-

nik yüzey etken maddelerin ortaya çıkışı ile bu teknik daha da önem kazanmıştır. (Tayade ve Modi, 2007).

1.7. Hidrotropi

Hidrotropi ortama büyük miktarlarda ikinci bir çözünen madde eklenmesinin asıl çözülmek istenen maddenin çözünürlüğünde artış sağlaması prensibine dayanan bir çözündürme işlemidir. Hidrotrop kavramı ilk kez 1916 yılında Neuberg tarafından, yüksek konsantrasyonlarda, suda az çözünen maddelerin su çözünürlüğünü önemli ölçüde artıran anyonik organik tuzları belirtmek için kullanılmıştır (Nidhi, 2011). Çözünürlüğün artmasındaki temel mekanizma çözünen maddenin hidrotropik ajanlarla etkileşimidir (Vemula, 2010).

Hidrotrop maddeler minimum hidrotrop konsantrasyon üzerinde çözelti içerisinde yeni mikro çevreler oluşturur. Hidrotropik maddeler diğer çözünürlük artırıcı maddelerden farklı olarak özel geometrik özelliklere sahiptir. Örneğin orto, meta ve para izomerleri olan maddelerin hidrotropik özellikleri farklılık gösterir. Bu durum hidrotropiyi tuzla (salt-in) veya miseller aracılığıyla çözündürmeden farklı kılar (Kumar, 2014). Hidrotrop maddeler hem hidrofilik hem de hidrofobik bölgelere sahip olmakla birlikte misel oluşturamayacak kadar küçüktür (Kumar, 2014). Hidrotropik çözeltiler, yüzey etken madde çözeltileri gibi kolloidal özellikler göstermez ve hidrotropik ajan ile çözünen arasında zayıf bir etkileşim vardır. Bazı tipik hidrotroplar hidroksibenzenler, hidroksibenzoatlar ve benzensülfonatlardır. Bu yöntemin miselizasyon, kosolvan kullanımı, tuzla çözündürme yöntemleriyle kıyaslandığında en büyük avantajı pH'dan bağımsız olması, yüksek seçiciliğe sahip olması ve herhangi bir emülsifikasyona gerek duymamasıdır (Nidhi, 2011).

1.8. Farklı Yapıdaki Kristal (Polimorf) Kullanılması

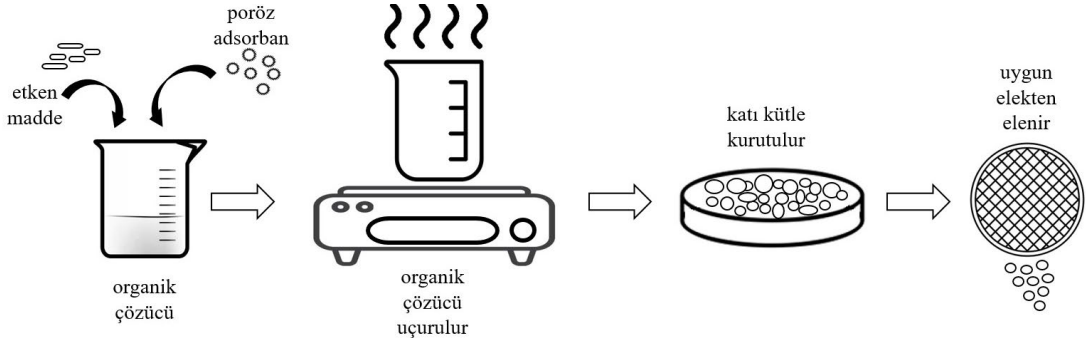
Polimorfizm, bir element veya bileşiğin birden fazla kristal formda kristallenme kabiliyetidir. Farklı ilaç polimorfları kimyasal olarak aynıdır, ancak çözünürlük, erime noktası, yoğunluk, doku, stabilite gibi özelliklerinde farklılıklar görülür, yani farklı fiziko-kimyasal özellikler sergiler (Savjani, 2012). Amorf

katıların entalpi, entropi ve serbest enerjisi, karşılık gelen kristal yapıya göre daha yüksektir. Bu yüksek serbest enerji, çözünürlük ve çözünme hızının artması ile sonuçlanır (Murdande, Pikal, Shanker, & Bogner, 2010). Genel olarak, bir ilacın susuz kristal formu, hidratlarından daha fazla çözünürlüğe sahiptir. Bunun nedeni, hidratların halihazırda suyla etkileşim halinde olmaları ve bu nedenle, su ile daha fazla etkileşim için anhidratlara (termodinamik olarak daha yüksek enerjili) kıyasla kristal parçalanması için daha az enerjiye sahip olmalarıdır. Metastabil kristal formlar da stabil polimorflara göre daha yüksek enerjiye ve dolayısıyla daha yüksek çözünürlüğe sahiptir. Bu nedenle farklı katı ilaç formlarının çözünme sıralaması amorf > metastabil polimorf > stabil polimorf şeklindedir (S. K. Patil, 2011). Öte yandan, amorf ka-

tılar, kristallere göre fiziksel olarak kararsızdır. Amorf ilaçların kristallenmeye karşı stabilitesi, farmasötik geliştirme için kritiktir çünkü kristalizasyon amorf katıların avantajlarını ortadan kaldırır (Qian, 2010).

1.9. Poröz Yapılara Yükleme

Bu yöntemde etken madde çözeltisinin çözücüsü uçurularak etken maddenin partikül boyutunun küçülmüş bir halde adsorban üzerinde çökmesi amaçlanır. Kolloidal silikon dioksit, laktoz, mannitol, çeşitli nişasta türevleri, mikrokristal selüloz, çeşitli süper dağıtıcılar (primojel, Ac-Di-Sol, Kollidon CL, sodyum nişasta glikolat) adsorban olarak kullanılabilir. Aynı zamanda bu yöntemle tozun akışını da iyileştirmek mümkün olabilmektedir (S. Jain, 2012). Bu yöntemin basamakları Şekil 2'de gösterilmiştir.

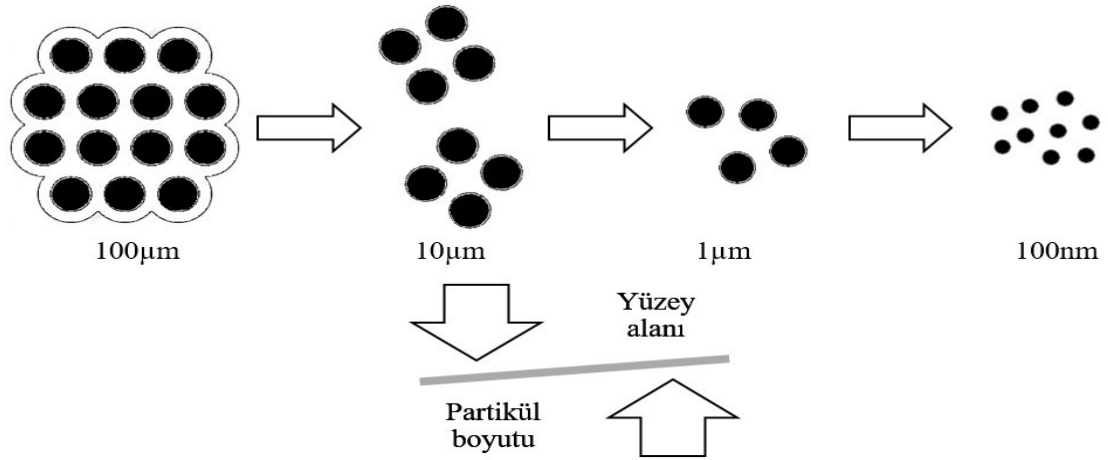


Şekil 2. Poröz yapılarla yüklemde işlem basamakları

1.10. Partikül Boyutunun Küçültülmesi

Biyoyararlanım özünde ilaç partikül boyutu ile doğrudan ilgilidir. Partikül boyutunun küçültülmesi Şekil 3'te gösterildiği gibi toplam yüzey alanını artıracığından ilacın çözünme hızını artırır. Partiküllerin geleneksel yöntemlerle küçültülmesi için çeşitli değirmenler (jet değirmenler, rotor-stator kolloid değirmenleri) mevcuttur (Vemula, 2010). Bu yöntemlerle ancak 2-5µm seviyelerine inilebilmekte olup bu seviyeler çözünürlüğün artırılabilmesi için yeterli değildir (Branham, 2012). İlaç moleküllerinin nano boyuta getirilmesinde çöktürme teknikleri, bilyalı (zirkonyum, cam veya polistirenden yapılmış) veya yüksek hızlı değirmenlerde öğütme, yüksek basınçlı homojenizasyon gibi yöntemler kullanılabilir (Savjani, 2012).

Günümüzde nanosüspansiyon/nanokristal hazırlanmasında iki farklı yaklaşım bulunmaktadır. Bunlar aşağıdan yukarı (bottom-up) ve yukarıdan aşağı (top-down) yaklaşımlarıdır. Aşağıdan yukarı yöntemlerde nanokristaller etken maddenin uygun bir çözücü içindeki çözeltisinden kontrollü bir şekilde çöktürülmesi/kristallendirilmesiyle elde edilir. Genellikle stabilizatör olarak surfaktanlar (elektrostatik stabilizasyon) ve polimerler (sterik stabilizasyon) kullanılır. Yukarıdan aşağı yaklaşımda ise yüksek hızlı, bilyalı değirmenler (NanoCrystals®), su içinde yüksek basınçlı homojenizasyon (DissoCubes®), susuz ortamda yüksek basınçlı homojenizasyon (Nanopure®), çöktürme ve yüksek basınçlı homojenizasyon kombinasyonu (NANOEDGE™) gibi yöntemler kullanılır (Bajaj, 2012; Chauhan, 2012).



Şekil 3. Partikül boyutu ve toplam yüzey alanının değişimi

Partikül boyutunun küçültülmesiyle ilgili kullanılan yöntemlerde genellikle yardımcı madde/ etken madde oranı düşüktür. Nanokristal yapılarının stabilizasyonu için kullanılan yüzey etken madde oranları genellikle yüksek olmadığından formülasyonlar iyi tolere edilirler. Maddelerin kristal formlarının amorf formlarına göre daha stabil olması yöntemin bir diğer avantajıdır. Çözünürlüğü artırmada diğer yöntemlerden sonuç alınmadığı durumlarda kullanılabilen kurtarıcı bir yöntemdir. Bununla birlikte ayrı ayrı küçük parçacıklar aglomerasyona yatkın olabileceklerinden işlem parametreleri sıkıca kontrol altında tutulmalıdır (Vemula, 2010). Yukarıdan aşağıya tekniği ile üretilen formülasyonlar, Ostwald olgunlaşması (Ostwald ripening) nedeniyle geniş bir partikül boyutu dağılımına ve düşük stabiliteye eğilimlidir (X. Zhang, 2018). Bazı durumlarda öğütme işlemi günler sürebilmekte ve bu durumda yüksek enerji sarfiyatından dolayı ilaç maliyeti yükselmektedir. Ayrıca öğütme işlemi sırasındaki yabancı madde kontaminasyonu da (bilya ve öğütücü kaynaklı) göz önünde bulundurulması gereken diğer bir faktördür (Sadeghi., 2016).

Partikül boyutunun küçültülmesinde kullanılan bir diğer yöntem ultrasonik kristalizasyondur. Bu me-

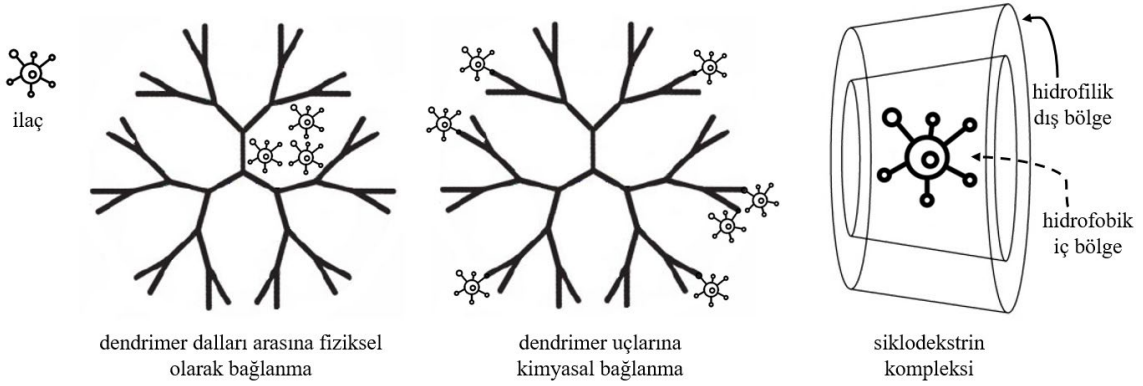
totla etken madde çözeltisi içerisinde etken maddenin çözünmediği bir çözücü eklenir ve kristalizasyonu tetiklemek için 20-100kHz arasında ultrasonik dalga kullanılır. Ultrasonik dalganın kullanılması çekirdeklenmeyi teşvik etmesinin yanında oluşan partiküllerin boyutunun belirli bir aralıkta kalmasını sağlar. Böylece çok daha dar bir aralıkta partiküller elde edilmiş olur (S. K. Patil, 2011).

Partikül boyutunun azaltılması süperkritik sıvıların kullanımı ile de gerçekleştirilebilir. Süperkritik sıvılar kritik sıcaklığının (T_c) ve basıncının (P_c) üzerinde bulunan sıvı ve gaz arasındaki geçiş sistemleridir. Kritik noktada, sıvının fiziksel özellikleri ve maddelerin çözünürlüğü büyük ölçüde değişir. (Lu, 1990). İlaç partikülleri süperkritik sıvı içinde çözüldükten sonra, oldukça küçük partikül boyutlarında yeniden kristalenebilirler. Bu amaçla süperkritik çözücü genişlemesi (RESS), gaz anti-solvan ile kristalizasyon (GAS) gibi yöntemler kullanılabilir (Bagade, 2014). Süperkritik CO_2 en yaygın kullanılan süperkritik çözücüdür. Yöntem güvenilir ve çevre dostudur. Süperkritik çözücülerin sunduğu esneklik ve hassasiyet, ilaç partiküllerinin oldukça dar bir aralık içerisinde, mikron altı seviyelere kadar (5nm-2µm) indirilebilmesine imkan tanır (Vemula, 2010).

1.11. Kompleks Oluşturma

Kompleks oluşumu genellikle yeni bir tür oluşturmak için bir substrat ve ligandın geri dönüşümlü olarak birleşimleri olarak tanımlanır. Kimyasal bağın türüne göre, kompleksler genel olarak koordinasyon kompleksleri ve moleküler kompleksler olmak üzere iki gruba ayrılır. Koordinasyon kompleksleri, bir çift

elektronun etkileşen bir molekülden diğerine aktarıldığı koordinasyon bağları tarafından oluşturulurken metal-iyon koordinasyon kompleksleri buna örnek verilebilir (Semalty, 2014). Moleküler kompleksleşmenin ardında hidrojen bağları, van der Waals kuvvetleri, hidrofobik etkileşimler gibi kuvvetler yatar. Bunlara örnek olarak siklodekstrin veya dendrimer kompleksleri (Şekil 4) verilebilir.



Şekil 4. Dendrimer ve siklodekstrin kompleksleri

1.11.a. Siklodekstrinler

Bazı mikroorganizma ve bitkilerde, nişastayı siklodekstrinler adı verilen siklik ürünlere indirgeyen enzimler bulunur. Siklodekstrinler lipofilik bir merkezi boşluk ve hidrofilik bir dış yüzeyden oluşan siklik oligosakkaritlerdir (Şekil 4). Bu özelliklerden dolayı siklodekstrinler, hem çözelti halinde hem de katı halde inklüzyon kompleksleri oluşturabilir (Rawat ve Jain, 2004). Inklüzyon kompleksleri, siklodekstrinlerin hidrofobik iç boşlukları ile ilaç molekülleri arasında kovalent olmayan etkileşimler kurulmasıyla oluşur. İlaç geliştiricileri siklodekstrin ve türevlerini çözünürlüğü, biyoyararlanımı ve stabiliteyi artırmak, ilacın kötü tadını maskeleyerek ve ilaca bağlı görülen yan etki ve toksisiteyi azaltmak amacıyla kullanmaktadırlar (Lee ve Lee, 1995).

Farmasötik teknolojide kullanıma uygun siklodekstrinler (α , β , γ -siklodekstrin), farklı çaplarda halkalı yapılar oluşturmak için 1,4 konfigürasyonunda bağlanmış 6, 7 veya 8 dektroz molekülü içerirler. Oluşan bu halkanın içi lipofilik bir çekirdeğe ve dışı ise hidrofilik bir kabuğa sahiptir (Vemula et

al., 2010). Siklodekstrin ilaç kompleksleri Higuchi ve Connors'ın tanımladığı faz çözünürlük diyagramlarına göre açıklanabilir. Bu diyagramlar ilaç çözünürlüğünün artan siklodekstrin konsantrasyonu ile nasıl değiştiğini gösterir. A_L tipi diyagramlar, çözünmüş ilaç konsantrasyonları ile sulu bir ortama eklenen siklodekstrin miktarları arasındaki doğrusal ilişkileri temsil eder. Artan siklodekstrin konsantrasyonu ile doğrusallıktan yukarı (pozitif) yönlü sapmalar A_p tipi diyagramları, doğrusallıktan aşağı (negatif) yönlü sapmalar ise A_N tipi diyagramları oluşturur (Sakham, 2018).

Siklodekstrinlerin oluşturduğu çoğu kompleks genellikle A_L tipidir. Komplekslerin dilüe olması kolosan veya yüzey etkin madde içeren sistemlerde görülebileceği gibi etken maddenin çökmesine sebep olmaz. A_p tipi kompleksler için bu durum geçerli değildir (Vemula, 2010).

Tüm bu özelliklerine rağmen yüksek maliyetleri ve istenen çözünürlüğün elde edilebilmesi için genellikle yüksek miktarlarda kullanılmaları sebebiyle yaygın şekilde kullanılamazlar. Ayrıca bazı siklodeks-

trinlerin belirgin renal toksisiteye neden oldukları da raporlanmıştır (Chaudhari, 2007).

1.11.b Dendrimerler

Dendrimerler, merkezi bir çekirdeğin etrafında ileri derecede dallanma gösteren, reaktif, üç boyutlu makromoleküllerdir. 1980'lerin ortalarında piyasaya sürülmelerinden bu yana, bu yeni polimerik malzeme sınıfı, benzersiz yapıları ve özelliklerinden dolayı büyük ilgi görmüştür (Otto & de Villiers, 2018). Dendrimerler, doğrusal veya dallanmış klasik polimerlerden, daha belirgin tanımlanmış olmaları (örneğin molekül ağırlıkları aralık değil tek bir değer olarak ifade edilir), yüksek derecede moleküler tekdüzeliğe sahip olmaları ve ayrıca çok sayıda işlevsel uç gruplarına sahip olmaları nedeniyle ayrılır (D'Emanuele & Attwood, 2005). Suda az çözünen ilaçların çözünürlüğünü arttırmak için dendrimerlerin başarıyla kullanıldığı çok sayıda çalışma mevcuttur. Dendrimerler ilaçların çözünürlüğünü fiziksel kompleksler veya kovalent konjugasyonlar oluşturarak artırabilirler. Dendrimerler ilaçlarla tekli sistemler oluşturduklarından genellikle çözünürlükte seyreltmeye bağlı değişiklik görülmez (Choudhary, 2017).

1.12. Katı Dispersiyonlar

Katı dispersiyonlar ilk olarak 1961 yılında Sekiguchi ve arkadaşları tarafından geliştirilen ve suda az çözünen ilaçların oral biyoyararlanımını artırmak için kullanılan bir yöntemdir. Bunlar, suda az çözünen ilaçların hidrofilik taşıyıcılar içindeki moleküler karışımları olarak tanımlanabilir. Kullanılan taşıyıcının özelliklerine bağlı olarak ilaç salım profilinin değiştirilmesi mümkündür (Vasconcelos, 2007). Katı dispersiyonlar ötektik karışımlar, katı veya camsı çözeltiler gibi alt sınıflara ayrılır (Janssens ve Van den Mooter, 2009).

Katı dispersiyonlarda taşıyıcılar yapılarına göre üç ayrı bölümde incelenebilir:

1. nesil kristal taşıyıcılar (üre, sükröz, laktoz, manitol, ksilitol gibi çeşitli şekerler, sitrik ve süksinik asit gibi organik asitler)

2. nesil polimerik taşıyıcılar (PVP, PEG, polimetakrilatlar (Eudragit), çeşitli selüloz türevleri (HPMC, EC, HPC), siklodekstrinler)
3. nesil surfaktanlar, polimer karışımları ve surfaktan-polimer karışımları (poloksamerler, Tween 80, Gelucire 44/14) (Jaskirat, 2013; Verma, 2011)

Katı dispersiyonların ilacın çözünme hızında ve biyoyararlanımında artış sağlaması yüksek oranda poroziteye sahip olmaları (özellikle çözücü uçurulması ile hazırlanmış olanlar), ilaç moleküllerinin ıslanabilirliğini artırmaları, ilacın partikül boyutunu azaltmaları ve buna bağlı olarak çözünme yüzey alanını artırmaları ile ilişkilendirilmektedir. Ek olarak katı dispersiyonlarda ilacın amorf halde bulunması nedeniyle kristal yapının bozulması için gereken enerji ve zamanın gerekmemesi, çözünme hızının artmasıyla sonuçlanır (Kaur, 2012; V. B. Yadav ve Yadav, 2009).

Katı dispersiyonların çeşitli avantajları vardır. Özellikle ilaçların vücut sıvılarındaki çözünürlüklerini ve biyoyararlanımlarını artırır. Geleneksel partikül boyutu küçültme yöntemlerine göre daha etkilidir. Geleneksel yöntemlerle küçültülen partiküller 2-5µm aralığında kısıtlı olup aglomerasyon göstermeye meyillidir. Katı dispersiyonlardaki taşıyıcılar aglomerasyonu büyük ölçüde önler. Sıvı ilaçlar bu yöntemle katı forma dönüştürülebilir. (Vo, Park, & Lee, 2013). Fakat tüm bu avantajlarına rağmen ticari ürün olarak piyasaya çıkan katı dispersiyon sayısı kısıtlıdır. Bunun en büyük nedenlerinden biri stabilite problemleridir. Zamanla formülasyondaki etken maddenin kristal yapısındaki değişikliklere bağlı olarak çözünme hızında düşüşler görülebilmektedir. Nem ve sıcaklık değişimlerine karşı hassastır. Ayrıca bazı katı dağılımlarda yapışma problemlerinin görülmesi üretimlerini güçleştirebilir. Ölçek büyütme sırasında da çeşitli sorunlar çıkartabilirler (Argade, 2013).

Katı dispersiyonların hazırlanması füzyon (eritme) metodu, çözücü bazlı metodlar ve yeni metodlar olmak üzere üç ana başlıkta incelenebilir. Füzyon metodları fiziksel karıştırma, eritme, sıcak eriyik eks-

trüzyonu gibi alt başlıklara ayrılabilir. Çözücü bazı metodlarda çözücü uçurma, püskürterek kurutma, liyofilizasyon gibi yöntemler sayılabilir. Elektrosponing ve süperkritik çözücü bazı metodlar ise daha yeni katı dispersiyon üretim yöntemleridir (Baghel, 2016; Boghra, 2011; Chaturvedi ve Verma, 2012).

1.13. Lipit Bazlı Formülasyonlar

Düşük su çözünürlüğüne sahip ilaçların oral biyo-

yararlanımlarının artırılması için yağ bazlı formülasyonların kullanımı son yıllarda oldukça ilgi çekmektedir. Hazırlanma kolaylıkları, berraklıkları, filtrelenilme özellikleri ve çözücü kapasitelerinin yüksek olması nedeniyle potansiyel ilaç taşıyıcı sistemler arasında önemli bir yere sahiptir (Patel, Kukadiya, Mashru, Surti, & Mandal, 2010). Lipid bazlı ilaç sistemler Tablo 3'te sınıflandırılmıştır.

Tablo 3. Lipid Formülasyonların Sınıflandırılması (Čerpnjak, Zvonar, Gašperlin, & Vrečer, 2013)

Tip	İçerik	Özellik	Avantaj	Dezavantaj
Tip I	Gliseritler (%100)	Suda dağılmaz, sindirilmesi gereklidir.	GRAS statüsünde, kapsül kabuğuyla geçimli formülasyonlar.	İlaç yağda çok çözünmüyorsa çoğu zaman çözme kapasitesi yetersizdir.
Tip II	Gliseritler (%40-80) Lipofilik surfaktanlar (%20-60)	Suda çözünen yardımcı madde içermez. (SEDDS)	Çözücü kapasitesini kaybetme olasılığı çok düşüktür.	Bulanık s/y tipi emülsiyon. (0,25-2µm)
Tip IIIA	Gliseritler (%40-80) Lipofilik surfaktanlar (%20-40) Hidrofilik surfaktanlar (%20-40)	Suda çözünen ve çözünmeyen yardımcı maddeler içerir. (SEDDS veya SMEDDS)	Berrak veya neredeyse berrak dağılımlar, ilaç emilimi için sindirim gerekmez.	Formülasyonun çözücü kapasitesini kaybetme olasılığı vardır.
Tip IIIB	Gliseritler (< %20) Hidrofilik surfaktanlar (%20-50) Kosolvanlar (%20-50)	Sadece suda çözünen yardımcı maddeler içeren, düşük oranda yağ içeren formüller. (SMEDDS)	Berrak dağılımlar, ilaç emilimi için formülasyonun sindirilmesi gerekmez.	Formülasyonun çözücü kapasitesini kaybetme olasılığı yüksektir.
Tip IV	Lipofilik surfaktanlar (%0-20) Hidrofilik surfaktanlar (%20-80) Kosolvanlar (%0-80)	Yağ içermeyen sadece yüzey etken madde ve yardımcı yüzey etken madde içeren formülasyonlar.	Birçok ilaç için yüksek çözücü kapasitesine sahip, misel tipi çözelti oluşturur.	Formülasyon, çözücü kapasitesini kaybeder, sindirilmeden GIS'den geçebilir.

Kendiliğinden emülsifiye olabilen sistemlerde ilaçlar yağlar, kısmi gliseritler, yüzey aktif maddeler, yardımcı yüzey aktif maddeler ve kosolvanlar gibi yardımcı maddelerin uygun oranlardaki karışımlarında çözülür. Kendiliğinden emülsifiye (SEDDS) ve kendiliğinden mikro/nano emülsifiye (SMEDDS veya SNEDDS) olan sistemler olmak üzere ikiye ayrılabilir (Agrawal, 2012).

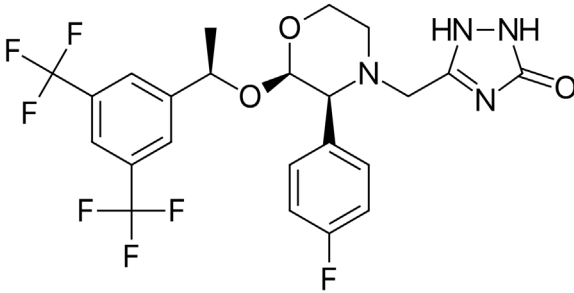
SMEDDS izotropik, transparan ve kinetik olarak kararlı çözeltilerdir. Suyla veya gastrointestinal kanal sıvılarıyla karıştıktlarında Y/S mikro yapıli emülsiyonları oluştururlar. Bu tip ilaç sistemlerinin tek avantajları çözünürlüğü artırmak değildir. İlaçlar küçük damlacıkların içerisinde zaten çözülmüş halde bulunduğundan ve geniş bir yüzey alanı sağladıklarından salım ve emilim özelliklerini de iyileştirirler.

Ayrıca lenfatik taşıma mekanizmaları da SMEDDS formülasyonlarında ilaç emilimine katkı sağlar (Qureshi, 2015). Hazırlanması özel teknolojilere göre de daha kolaydır (Mesut, 2020).

Kendiliğinden emülsifiye sistemler biyoyaralanımın artmasının yanında bireyler arası emilim değışkenliğinin ve besin etkisi nedeniyle görülen birey içi değışkenliklerin azalmasını da sağlar. GIS'deki enzimatik etkiye karşı koruma sağlayacağından proteinik maddelerin verilebilmesine olanak tanır. Üretimleri basit olup kolaylıkla ölçek büyütme yapılabilir (Agrawal, 2012). Ancak yüksek miktarda yüzey etken madde kullanımı özellikle kronik hastalıkların tedavisinde bu tip formülasyonların kullanımını kısıtlayabilir (Vemula, 2010).

2. Çözünürlüğü düşük bir etken madde: Aprepitant

Aprepitant (Emend®) yeni bir ilaç sınıfı olan nörokinin NK₁ reseptör antagonistlerinden ticari olarak üretilen ilk ilaçtır. Diğer ajanlarla kombinasyon halinde, yetişkinlerde yüksek düzeyde emetojenik kemoterapi ile ilişkili akut ve gecikmiş kemoterapiye bağlı bulantı ve kusmanın önlenmesinde endikedir (Dando ve Perry, 2004). Aprepitant beyaz/beyazımsı renkte, kristal yapılı, higroskopik olmayan bir maddedir. pKa'sı 9,7 erime derecesi 254 °C'dir (Ren, Zhou, Wei, Li, & Chen, 2014). Kimyasal isimlendirilmesi 3-[[[(2R,3S)-2-[(1R)-1-[3,5-bis(triflorometil)fenil]etoksi]-3-(4-florofenil)morfolin-4-il]metil]-4,5-dihidro-1H-1,2,4-triazol-5-on olan bileşiğin molekül ağırlığı 534g/mol'dür. pH 7'de ölçülen logP'si 4,8 olduğundan göreceli olarak yüksek bir lipofillığe sahiptir (Yeo, An, Park, Kim, & Lee, 2020). Bileşiğin kimyasal yapısı Şekil 5'te gösterilmektedir.



Şekil 5. Aprepitantın kimyasal yapısı

Aprepitant literatürde bazı kaynaklarda BCS II (Angi et al., 2014; Penumetcha et al., 2016) bazı kaynaklarda ise BCS IV (Kalvakuntla, 2016; Nanaki, 2019; R. Sharma, 2016) olarak sınıflandırılmıştır. pH 2-10 arasında 3-7µg/mL gibi oldukça düşük bir çözünürlüğe sahip zayıf bazik özellikte bir ilaçtır (Yeo, 2020). Yani suda pratik olarak çözünmez. Etanol ve izopropil asetat içinde eser miktarda, asetonitril içinde ise az miktarda çözünür. Bu nedenle bileşik formüle edilmemiş olarak uygulandığında, oral biyoyararlanımı oldukça sınırlıdır (Angi, 2014).

Aprepitantın Çözünürlüğünün Artırılmasına Yönelik Yapılan Çalışmalar

Düşük çözünürlüğe sahip bileşikler besinlerle alındığında, midede kalma süresinin artmasıyla

daha yüksek emilim gösterme eğilimindedir. Örneğin 100mg aprepitant içeren klasik dozaj formu, tokluk şartlarında uygulandığında açlık şartlarına göre insanlarda emilimin 3 kat arttığı gösterilmiştir (Merisko-Liversidge ve Liversidge, 2011). Bu yöntem aprepitant gibi düşük çözünürlüklü ilaçların oral biyoyararlanımını iyileştirmek için etkili bir yaklaşım olsa da, özellikle bulantı ve kusmanın önlenmesi gibi endikasyonlarda kullanılacak ilaçlar için iyi bir seçenek değildir (Shono, 2010).

Aprepitantın çözünürlüğünü artırmak için ilk olarak çeşitli tuz formları hazırlanmış, ancak tüm tuzların kimyasal stabilitelerinin zayıf olduğu gösterilmiştir. Aprepitantın geleneksel formülasyonları kullanılarak sistemik ve etkili bir tedavi uygulamak zordur. Bu nedenle Merck&Co. firması çözünürlük hızını artırmak için partikül boyutu küçültme yöntemi kullanmış ve Emend®'in biyoyararlanımını yaklaşık % 65'e yükseltebilmiştir (Ren, 2014). Emend®'in üretiminde partiküllerinin mikron altı boyuta öğütüldüğü ve polimer veya yüzey aktif madde ile stabilize edildiği NanoCrystal® teknolojisi kullanılmıştır (Merisko-Liversidge ve Liversidge, 2011; Shono, 2010). İlacın tam olarak emilememesi terapötik etkiye ulaşmak için daha yüksek dozda ilaç kullanmayı gerektireceğinden ilaca bağlı yan etkilerin de artmasına neden olur. Aprepitantın çözünürlüğünü ve çözünme hızını artırma çabaları, geliştirilmeye açık biyoyararlanım oranı (% 65) ve ilacın yüksek üretim maliyeti nedeniyle hiçbir zaman durdurulmamıştır (Ren, 2014).

Yapılan literatür taramasında hazırlanmış olan formülasyonların çoğunlukla aprepitantın katı dispersiyonları (6 adet) olduğu görülmüştür. İkinci sırada ise aprepitantın çeşitli yöntemlerle partikül boyutunun küçültülerek nanokristallerinin (5 adet) hazırlandığı formülasyonlar bulunmaktadır. Bunların dışında emülsiyon (1 adet), mikroemülsiyon (2 adet), siklodekstrin kompleksleri (2 adet), ağızda dağılan film (1 adet), katı çözelti (1 adet), surfaktan bazlı formülasyon (1 adet) gibi çalışmalar literatürde kayıtlı bulunmaktadır. Bu derleme kapsamında incelenmiş olan literatürler Tablo 4'te özetlenmiştir.

Tablo 4. Aprepitantin Çözünürlüğünün Artırılmasına Yönelik Çalışmalar

Formülasyon Tipi	Formülasyon Bileşenleri	Çözünürlük ve Biyoyararlanım Özellikleri	Referans
Siklodekstrin kompleksi	Aprepitant 125 mg β-siklodekstrin 260 mg Avicel PH200 40 mg SLS 40 mg Aerosil 5 mg	Yapılan dissolüsyon çalışmalarında referans ürün ile benzer bir profil elde edilmiştir. İn vivo çalışmalar 18 sağlıklı gönüllü insanda yapılmış, referans ürünle (Emend®) kıyaslandığında biyoyararlanımın istatistiksel açıdan benzer olduğu bildirilmiştir.	(Ridhurkar, 2013)
Siklodekstrin kompleksi	Aprepitant 40 mg sülfobutil eter-β-siklodekstrin 336mg Mikrokristal selüloz 45 mg SLS 20 mg	Test edilen tüm dissolüsyon ortamlarında referans üründen daha hızlı bir çözünme hızı görüldüğü bildirilmiştir. Beagle cinsi köpeklerde yapılan in vivo çalışmalarda formülasyon biyoyararlanımının referans ürünle neredeyse aynı olduğu gösterilmiştir.	(Ren, 2014)
Nanokristal	<u>Etanol içi konsantrasyonlar</u> Aprepitant 14 mg/mL Soluplus® 70 mg/mL SLS 2,8 mg/mL Solvan : Antisolvan (Etanol : Distile Su) 1 : 20	Tokluk şartlarındaki barsak ortamında referans üründen yaklaşık 2 kat daha yüksek bir çözünürlük değeri elde edilmiştir. Açlık şartlarında elde edilen çözünürlük değeri referans ürünle benzer olmuştur. Beagle cinsi köpeklerde yapılan in vivo çalışmalar bu formülasyonun biyoyararlanımının referans ürünle oldukça benzer olduğunu göstermiştir.	(Angi, 2014)
Nanokristal	Nano aprepitant (224,5nm) Mikronize aprepitant (20µm) Mikronize aprepitant (1µm)	20µm boyutundaki kristallerle kıyaslandığında nanokristallerin su çözünürlüğünde 110,4 kat artış görüldüğü bildirilmiştir. Açlık koşullarında yapılan in vivo çalışmalarda nanokristallerin aprepitant biyoyararlanımını 20µm'lik kristallere göre 2,05 kat 1µm'lik kristallere göre ise 1,43 kat artırdığı bildirilmiştir.	(X.-Y. Zhang, 2014)
Katı dispersiyon	Aprepitant Vitamin E TPGS HPMC PEG 4000 SLS Magnezyum stearat	Elde edilen bu dozaj formunun in vitro dissolüsyon çalışmalarında çözünme hızını artırdığı gösterilmiştir.	(Chandra-sekhara Rao, 2014)
Nanokristal	Aprepitant 50mg/10mL Tween 80 %1 Poloksamer 188 %3 Mannitol	Bilyalı değirmen + yüksek basınçlı homojenizasyon işlemlerinden geçirildikten sonra elde edilen 120nm boyutunda (PDI 0,268) nanokristaller ile yapılan çözünürlük çalışmasına göre formülasyonun aprepitantin su çözünürlüğünü 40,2 kat artırdığı, ayrıca yapılan dissolüsyon çalışmasında 45 dakika içinde aprepitantin %99'unun salındığı bildirilmiştir.	(Attari, 2016)
Taşıyıcıya adsorbe ettirilmiş mikro-emülsiyon	Aprepitant 80 mg/mL Capmul MCM C10 300 µL Tween 80 555 µL Transcutol 145 µL Aerosil 200 1 g	Damlacık boyutu 148nm (PDI 0,21) olan formülasyon geliştirilip Aerosil 200 ile toz forma çevrilmiştir. Dissolüsyon çalışmaları ve tavşanlarda gerçekleştirilen in vivo çalışmalarda ilacın çözünme hızında ve biyoyararlanımında 1,5 ila 2 kat artış olduğu bildirilmiştir.	(Kamboj, 2015)
Katı dispersiyon	Aprepitant:Soluplus 1:5 Aseton	Hazırlanan katı dispersiyonların çözünme hızını yaklaşık olarak 9 kat artırdığı bildirilmiştir. İn vivo çalışmalarda formülasyonun biyoyararlanımı toz aprepitanta göre 2,4 kat artırdığı gösterilmiştir. Bu çalışmada ayrıca katı dispersiyonların Emend® ile istatistiksel olarak benzer biyoyararlanıma sahip olduğu sonucuna varılmıştır.	(Liu, 2015)
Taşıyıcıya adsorbe ettirilmiş mikro-emülsiyon	Capmul MCM C8 %30 Tween 80 %50 Transcutol %20 Modifiye magnezyum silikat (1,2g/mL)	Damlacık boyutu 127nm (PDI 0,24) olan formülasyonda, aprepitantin mide vasatında 10 dakika içerisinde %80'inin çözüldüğü gösterilmiştir. Ayrıca tavşanlarda gerçekleştirilen in vivo biyoyararlanım çalışmasında bu formülasyonun biyoyararlanımının istatistiksel açıdan anlamlı olarak (yaklaşık %10) artış sağladığı bildirilmiştir.	(Kamboj ve Rana, 2016)

Katı dispersiyon	Aprepitant:Soluplus 1:4	pH 6,5 barsak vasatında yapılan dissolüsyon çalışmasında, 30 dakika sonunda formülasyonun çözünme hızının aprepitantla kıyaslandığında 6-7 kat artış görüldüğü bildirilmiştir.	(Penumetcha, 2016)
Ağızda dağılan film	Pullulan %30 Demirhindi pektini %70 + %0,1 sıvı glukoz	Hazırlanan ağızda dağılan film formülasyonunun anlamlı ölçüde daha hızlı çözünme gösterdiği bildirilmiştir. Ek olarak, formülasyon referans ürünle (Aprecap®) kıyaslandığında tavşanlardaki biyoyararlanımı anlamlı ölçüde (%15) artırdığı gösterilmiştir.	(R. Sharma, 2016)
Nanokristal	Aprepitant 125 mg Tween 80 %1 Poloksamer 188 %3	Liyofilizasyon + yüksek basınçlı homojenizasyon tekniklerini kullanarak hazırlanan nanokristaller (35,78nm PDI 0,26) ile yapılan dissolüsyon testlerinde, çalışılan ortamdan bağımsız olarak 45. dakikada çözünen aprepitant miktarında 2 kat artış görüldüğü bildirilmiştir.	(Kalvakuntla, 2016)
Katı çözelti	Aprepitant Aseton-%5 SLS çözeltisi-2- pirolidon Poloksamer 407 Aerosil 300 Mikrokrystal selüloz Krosprovidon SLS	Akışkan yatakta hazırlanan bu aprepitant formülasyonunun 0,1N HCl ortamında orjinal ilaç olan Emend®den daha hızlı salım yaptığı gösterilmiştir.	(Barmpalexis, 2018)
Nanokristal	Aprepitant Süperkritik CO ₂ Mentol	Süperkritik çözücüler kullanılarak üretilen aprepitant nanokristalleri (23nm) ile işlem görmemiş kristaller (25,6µm) çözünme hızları açısından pH 6,8 fosfat tamponunda kıyaslanmıştır. Nanokristallerin çözünme hızını 8,2 kat artırdığı bildirilmiştir.	(Sodefian, 2018)
Katı dispersiyon	Aprepitant Polietilenoksit Cloisite-Na kili	Hazırlanmış tüm formülasyonların toz aprepitanta göre daha hızlı çözüldükleri raporlanmıştır.	(Pappa, 2018)
Emülsiyon	Aprepitant Kolesterol hemisüksinat Lipoid E80 Gliserin Sodyum oleat Sükroz Orta zincirli trigliserit (MCT)	Yüksek basınçlı homojenizatör kullanılarak aprepitant-kolesterol hemisüksinat iyon çifti kompleksi ile intravenöz kullanıma uygun nanoemülsiyon (100nm) hazırlanmıştır. Bu formülasyonun, sıçanlarda biyoyararlanımı ve dolaşımında kalma süresini, hem aprepitant çözeltisine göre hem de klasik emülsiyona göre artırdığı gösterilmiştir.	(X. Y. Zhang, 2020)
Katı dispersiyon	Aprepitant Soluplus Poloksamer 188	Farklı oranlarda soluplus ve poloksamer 188 kullanılarak aprepitantın katı dispersiyonlarını hazırlanmıştır. Hazırlanan tüm katı dispersiyonların aprepitant çözünme hızını belirgin şekilde artırdığı gösterilmiştir.	(Nanaki, 2019)
Katı dispersiyon	Aprepitant 100 mg Fosfotidilkolin 100 mg Neusilin 200 mg Kroskarmelloz sodyum 50 mg	Hazırlanan fosfotidilkolin bazlı katı dispersiyonun, suda toz aprepitanttan 40 kat daha fazla çözünebildiği gösterilmiştir. Ayrıca dissolüsyon testinde bu formülasyonun çözünme hızının saf aprepitanttan yaklaşık 6,5 kat yüksek olduğu gösterilmiştir.	(Yeo, 2020)
Yüzey etken madde bazlı formülasyon	Jelatin Oleik asit sodyum tuzu Aprepitant	Jelatin ve oleik asit sodyum tuzundan hareketle yeni bir çözündürme ajanı sentezlenmiş, bu maddenin %3'lük çözeltisinde aprepitant çözünürlüğünün yaklaşık 55 kat arttığı bildirilmiştir.	(Kim, 2020)

SONUÇ

Çözünürlüğü düşük olan etken maddelerin çözünürlüğünü iyileştirip, biyoyararlanımını artırmak için literatürde kayıtlı, geçerliliği kanıtlanmış birçok yöntem bulunmaktadır. Ancak bu yöntemlerin düşük çözünürlüklü her etken maddeye uygulanabilmesi mümkün değildir. Uygun yöntemin belirlenmesinde maddenin fizikokimyasal özellikleri oldukça önemli rol oynamaktadır.

Aprepitant kanser kemoterapisine bağlı olarak görülen bulantı ve kusmanın önlenmesinde kullanılan, diğer antiemetiklerden farklı bir etki mekanizmasına sahip bir etken maddedir. Avrupa Tıbbi Onkoloji Derneği (ESMO) ve Amerikan Klinik Onkoloji Derneği (ASCO) gibi önde gelen kuruluşların kılavuzlarında, kullanımını çoğu zaman birinci sıra ilaç olarak önerilen, klinikte vazgeçilmez bir ilaçtır. Emiliminin önündeki temel engelin düşük çözünme hızı olduğu düşünüldüğünde çözünme hızını artıran formülasyonların ge-

liştirilmeye çalışılması ilk seçenektir. Aprepitant için yapılan literatür taramasında üretilen formülasyonların çoğunlukla katı dispersiyon şeklinde hazırlanmış oldukları görülmüştür. Bunu nanokristal formülasyonları izlemektedir. Her iki yöntem de çeşitli dezavantajlara sahip olup özellikle kanser hastalarının hayat kalitesini önemli ölçüde iyileştiren bu antiemetik ilacın oral biyoyararlanımını iyileştirmek için daha fazla çalışmaya ihtiyaç duyulmaktadır. Böylelikle klinik açısından önemli bu ilacın, gelecekte daha etkili, güvenli ve yan etkisi azaltılmış formülasyonlarının kullanılabilmesi mümkün olabilecektir.

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KAYNAKLAR

- Agrawal, S., Giri, T. K., Tripathi, D. K., Ajaz, A., Alexander, A. (2012). A Review on Novel Therapeutic Strategies for the Enhancement of Solubility for Hydrofobic Drugs through Lipid and Surfactant Based Self Micro Emulsifying Drug Delivery System: A Novel Approach. *American Journal of Drug Discovery and Development*, 2(4), 143-183. <https://doi.org/10.3923/ajdd.2012.143.183>
- Angi, R., Solymosi, T., Otvos, Z., Ordasi, B., Glavinas, H., Filipcsei, G., ... Darvas, F. (2014). Novel continuous flow technology for the development of a nanostructured Aprepitant formulation with improved pharmacokinetic properties. *European Journal of Pharmaceutics and Biopharmaceutics*, 86(3), 361-368. <https://doi.org/10.1016/j.ejpb.2013.10.004>
- Argade, P. S., Magar, D. D., Saudagar, R. B. (2013). Solid Dispersion: Solubility Enhancement Technique for poorly water soluble Drugs. *Journal of Advanced Pharmacy Education & Research*, 3(4), 427-439. Retrieved from <https://japer.in/en>
- Attari, Z., Kalvakuntla, S., Reddy, M. S., Deshpande, M., Rao, C. M., Koteswara, K. B. (2016). Formulation and characterisation of nanosuspensions of BCS class II and IV drugs by combinative method. *Journal of Experimental Nanoscience*, 11(4), 276-288. <https://doi.org/10.1080/17458080.2015.1055841>
- Bagade, O. M., Kad, D. R., Bhargude, D. N., Bhosale, D. R., & Kahane, S. K. (2014). Consequences and Impose of Solubility Enhancement of Poorly Water Soluble Drugs. *Research Journal of Pharmacy and Technology*, 7(5), 598-607. <https://doi.org/10.5958/0974-360X>
- Baghel, S., Cathcart, H., O'Reilly, N. J. (2016). Polymeric Amorphous Solid Dispersions: A Review of Amorphization, Crystallization, Stabilization, Solid-State Characterization, and Aqueous Solubilization of Biopharmaceutical Classification System Class II Drugs. *Journal of Pharmaceutical Sciences*, 105(9), 2527-2544. <https://doi.org/10.1016/j.xphs.2015.10.008>
- Bajaj, A., Rao, M. R., Pardeshi, A., Sali, D. (2012). Nanocrystallization by evaporative antisolvent technique for solubility and bioavailability enhancement of telmisartan. *AAPS PharmSciTech*, 13(4), 1331-1340. <https://doi.org/10.1208/s12249-012-9860-x>
- Barmapalexis, P., Grypioti, A., Eleftheriadis, G. K., Fatouros, D. G. (2018). Development of a New Aprepitant Liquefied Formulation with the Aid of Artificial Neural Networks and Genetic Programming. *AAPS PharmSciTech*, 19(2), 741-752. <https://doi.org/10.1208/s12249-017-0893-z>

- Bikiaris, D. N. (2011). Solid dispersions, Part II: new strategies in manufacturing methods for dissolution rate enhancement of poorly water-soluble drugs. *Expert Opinion on Drug Delivery*, 8(12), 1663-1680. <https://doi.org/10.1517/17425247.2011.618182>
- Boghra, R. J., Kothawade, P. C., Belgamwar, V. S., Nerkar, P. P., Tekade, A. R., Surana, S. J. (2011). Solubility, Dissolution Rate and Bioavailability Enhancement of Irbesartan by Solid Dispersion Technique. *Chemical and Pharmaceutical Bulletin*, 59(4), 438-441. <https://doi.org/10.1248/cpb.59.438>
- Branham, M. L., Moyo, T., Govender, T. (2012). Preparation and solid-state characterization of ball milled saquinavir mesylate for solubility enhancement. *Eur J Pharm Biopharm*, 80(1), 194-202. <https://doi.org/10.1016/j.ejpb.2011.08.005>
- Čerpnjak, K., Zvonar, A., Gašperlin, M., Vrečer, F. (2013). Lipid-based systems as a promising approach for enhancing the bioavailability of poorly water-soluble drugs. *Acta Pharmaceutica*, 63(4), 427-445. <https://doi.org/10.2478/acph-2013-0040>
- Chandrasekhara Rao, B., Vidyadhara, S., Sasidhar, R. L. C., Chowdary, Y. A. (2014). Dissolution enhancement of poorly soluble drug aprepitant by hot melt extrusion method using hydrophilic polymer: A solid dispersion technique. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 5(3), 1469-1485. Retrieved from <https://www.rjpbcs.com/>
- Chaturvedi, A. K., Verma, A. (2012). Solubility Enhancement Of Poorly Water Soluble Drugs By Solid Dispersion. *Journal of Pharmaceutical Sciences and Research*, 3(1), 26-34. [https://doi.org/10.13040/IJPSR.0975-8232.3\(1\).26-34](https://doi.org/10.13040/IJPSR.0975-8232.3(1).26-34)
- Chaudhari, P., Sharma, P., Barhate, N., Kulkarni, P., Chetan, M. (2007). Solubility enhancement of hydrophobic drugs using synergistically interacting cyclodextrins and cosolvent. *Current Science*, 92(11), 1586-1591. Retrieved from <https://www.jstor.org/stable/24097723?seq=1>
- Chauhan, N. N., Patel, N. V., Suthar, S. J., Patel, J. K., Patel, M. P. (2012). Micronization of BCS Class-II Drugs by Various Approaches for Solubility Enhancement – A Review. *Research Journal of Pharmacy and Technology*, 5(8), 999-1005. Retrieved from <https://rjptonline.org/AbstractView.aspx?PID=2012-5-8-1>
- Choudhary, S., Gupta, L., Rani, S., Dave, K., Gupta, U. (2017). Impact of Dendrimers on Solubility of Hydrophobic Drug Molecules. *Front Pharmacol*, 8, 261. <https://doi.org/10.3389/fphar.2017.00261>
- D'Emanuele, A., & Attwood, D. (2005). Dendrimer-drug interactions. *Adv Drug Deliv Rev*, 57(15), 2147-2162. <https://doi.org/10.1016/j.addr.2005.09.012>
- D. Horter, & Dressman, J. B. (2001). Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Advanced Drug Delivery Reviews*, 46, 75-87. [https://doi.org/10.1016/s0169-409x\(00\)00130-7](https://doi.org/10.1016/s0169-409x(00)00130-7)
- Dando, T. M., & Perry, C. M. (2004). Aprepitant - A review of its use in the prevention of chemotherapy-induced nausea and vomiting. *Drugs*, 64(7), 777-794. <https://doi.org/10.2165/00003495-200464070-00013>
- European Pharmacopoeia 7.0. (2010).
- Hammond, R. B., Pencheva, K., Roberts, K. J., Auffret, T. (2007). Quantifying solubility enhancement due to particle size reduction and crystal habit modification: case study of acetyl salicylic acid. *J Pharm Sci*, 96(8), 1967-1973. <https://doi.org/10.1002/jps.20869>

- Hussain, A., & Rytting, J. H. (1974). Prodrug Approach to Enhancement of Rate of Dissolution of Allopurinol. *Journal of Pharmaceutical Sciences*, 63(5), 798-799. <https://doi.org/10.1002/jps.2600630535>
- Jain, S., Patel, N., Lin, S. (2015). Solubility and dissolution enhancement strategies: current understanding and recent trends. *Drug Dev Ind Pharm*, 41(6), 875-887. <https://doi.org/10.3109/03639045.2014.971027>
- Jain, S., Sandhu, P., Gurjar, M., Malvi, R. (2012). Solubility Enhancement By Solvent Deposition Technique: An Overview. *Asian Journal of Pharmaceutical and Clinical Research*, 5(4), 15-19. Retrieved from <https://innovareacademics.in/journals/index.php/ajpcr>
- Janssens, S., & Van den Mooter, G. (2009). Review: physical chemistry of solid dispersions. *J Pharm Pharmacol*, 61(12), 1571-1586. <https://doi.org/10.1211/jpp/61.12.0001>
- Jaskirat, S., Manpreet, W., Harikumar, S. L. (2013). Solubility Enhancement By Solid Dispersion Method: A Review. *Journal of Drug Delivery & Therapeutics*, 3(5), 148-155. <https://doi.org/10.22270/jddt.v3i5.632>
- Kalvakuntla, S., Deshpande, M., Attari, Z., Kunnatur, B. K. (2016). Preparation and Characterization of Nanosuspension of Aprepitant by H96 Process. *Advanced Pharmaceutical Bulletin*, 6(1), 83-90. <https://doi.org/10.15171/apb.2016.013>
- Kamboj, S., & Rana, V. (2016). Formulation optimization of aprepitant microemulsion-loaded silicated corn fiber gum particles for enhanced bioavailability. *Drug Development and Industrial Pharmacy*, 42(8), 1267-1282. <https://doi.org/10.3109/03639045.2015.1122611>
- Kamboj, S., Sharma, R., Singh, K., Rana, V. (2015). Aprepitant loaded solid preconcentrated microemulsion for enhanced bioavailability: A comparison with micronized Aprepitant. *European Journal of Pharmaceutical Sciences*, 78, 90-102. <https://doi.org/10.1016/j.ejps.2015.07.008>
- Kaur, J., Aggarwal, G., Singh, G., Rana, A. C. (2012). Improvement Of Drug Solubility Using Solid Dispersion. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(2), 47-53. Retrieved from <https://innovareacademics.in/journals/index.php/ijpps>
- Kawabata, Y., Wada, K., Nakatani, M., Yamada, S., Onoue, S. (2011). Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. *Int J Pharm*, 420(1), 1-10. <https://doi.org/10.1016/j.ijpharm.2011.08.032>
- Kim, D., Park, C., Meghani, N. M., Tran, T. T. D., Tran, P. H. L., Park, J. B., Lee, B. J. (2020). Utilization of a fattigation platform gelatin-oleic acid sodium salt conjugate as a novel solubilizing adjuvant for poorly water-soluble drugs via self-assembly and nanonization. *Int J Pharm*, 575, 118892. <https://doi.org/10.1016/j.ijpharm.2019.118892>
- Korotkova, E. I., Kratochvíl, B. (2014). Pharmaceutical Cocrystals. *Procedia Chemistry*, 10, 473-476. <https://doi.org/10.1016/j.proche.2014.10.079>
- Kumar, V. S., Raja, C., Jayakumar, C. (2014). A Review On Solubility Enhancement Using Hydrotropic Phenomena. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(6), 1-7. Retrieved from <https://innovareacademics.in/journals/index.php/ijpps>
- Kurmi, R., Mishra, D. K., Jain, D. K. (2016). Solid dispersion: A novel means of solubility enhancement. *Journal of Critical Reviews*, 3(1), 1-8. Retrieved from <http://www.jcreview.com/>
- Lasseter, K. C., Gambale, J., Fin, B., Bergman, A., Constanzer, M., Dru, J., . . . Murphy, M. G. (2007). Tolerability of fosaprepitant and bioequivalency to aprepitant in healthy subjects. *Journal of Clinical Pharmacology*, 47(7), 834-840. <https://doi.org/10.1177/0091270007301800>

- Lee, B. J., & Lee, J. R. (1995). Enhancement of Solubility and Dissolution Rate of Poorly Water-soluble Naproxen by Complexation with 2-Hydroxypropyl β -D-Dextrin. *Archives of Pharmacal Research*, 18(1), 22-26. <https://doi.org/10.1007/BF02976502>
- Li, A.-Y., Xu, L.-L., Chen, J.-M., Lu, T.-B. (2015). Solubility and Dissolution Rate Enhancement of Triamterene by a Cocrystallization Method. *Crystal Growth & Design*, 15(8), 3785-3791. <https://doi.org/10.1021/acs.cgd.5b00439>
- Liu, J. W., Zou, M. J., Piao, H. Y., Liu, Y., Tang, B., Gao, Y., ... Cheng, G. (2015). Characterization and Pharmacokinetic Study of Aprepitant Solid Dispersions with Soluplus. *Molecules*, 20(6), 11345-11356. <https://doi.org/10.3390/molecules200611345>
- Lu, B. C.-Y., Zhang, D., Sheng, W. (1990). Solubility enhancement in supercritical solvents. *Pure and Applied Chemistry*, 62(12), 2277-2285. <https://doi.org/10.1351/pac19906212277>
- Merisko-Liversidge, E., & Liversidge, G. G. (2011). Nanosizing for oral and parenteral drug delivery: a perspective on formulating poorly-water soluble compounds using wet media milling technology. *Adv Drug Deliv Rev*, 63(6), 427-440. <https://doi.org/10.1016/j.addr.2010.12.007>
- Mesut, B., Şahin, Ş. H. T., Özsoy, Y. (2020). Budesonide-Loaded Self-Nanoemulsion Formulations Optimisation and Characterisation Studies. *Latin American Journal of Pharmacy*, 39(7), 1368-1378. Retrieved from <http://www.latamjpharm.org/>
- Murdande, S. B., Pikal, M. J., Shanker, R. M., Bogner, R. H. (2010). Solubility advantage of amorphous pharmaceuticals: II. Application of quantitative thermodynamic relationships for prediction of solubility enhancement in structurally diverse insoluble pharmaceuticals. *Pharm Res*, 27(12), 2704-2714. <https://doi.org/10.1007/s11095-010-0269-5>
- Nanaki, S., Eleftheriou, R. M., Barmpalexis, P., Kostoglou, M., Karavas, E., Bikiaris, D. (2019). Evaluation of Dissolution Enhancement of Aprepitant Drug in Ternary Pharmaceutical Solid Dispersions with Soluplus® and Poloxamer 188 Prepared by Melt Mixing. *Sci*, 1(1). <https://doi.org/10.3390/sci1010011.v1>
- Nidhi, K., Indrajeet, S., Khushboo, M., Gauri, K., Sen, D. J. (2011). Hydrotropy: A Promising Tool For Solubility Enhancement: A Review. *International Journal of Drug Development & Research*, 3(2), 26-33. Retrieved from <https://www.ijddr.in/>
- Otto, D. P., & de Villiers, M. M. (2018). Poly(amidoamine) Dendrimers as a Pharmaceutical Excipient. Are We There yet? *J Pharm Sci*, 107(1), 75-83. <https://doi.org/10.1016/j.xphs.2017.10.011>
- Pappa, C., Nanaki, S., Giliopoulos, D., Triantafyllidis, K., Kostoglou, M., Avgeropoulos, A., Bikiaris, D. (2018). Nanostructured Composites of Sodium Montmorillonite Clay and PEO Used in Dissolution Improvement of Aprepitant Drug by Melt Mixing. *Applied Sciences*, 8(5). <https://doi.org/10.3390/app8050786>
- Patel, V., Kukadiya, H., Mashru, R., Surti, N., Mandal, S. (2010). Development of Microemulsion for Solubility Enhancement of Clopidogrel. *Iranian Journal of Pharmaceutical Research*, 9(4), 327-334. Retrieved from <http://ijpr.sbmu.ac.ir/>
- Patil, A. N., Shinkar, D. M., Saudagar, R. B. (2017). Review Article: Solubility Enhancement By Solid Dispersion. *International Journal of Current Pharmaceutical Research*, 9(3), 15-18. <https://doi.org/10.22159/ijcpr.2017v9i3.19583>
- Patil, S. K., Wagh, K. S., Parik, V. B., Akarte, A. M., Baviskar, D. T. (2011). Strategies For Solubility Enhancement Of Poorly Soluble Drugs. *International Journal of Pharmaceutical Sciences Review and Research*, 8(2), 74-80.

- Penumetcha, S. S., Gutta, L. N., Dhanala, H., Yamili, S., Challa, S., Rudraraju, S., ... Rudraraju, V. (2016). Hot melt extruded Aprepitant-Soluplus solid dispersion: preformulation considerations, stability and in vitro study. *Drug Development and Industrial Pharmacy*, 42(10), 1609-1620. <https://doi.org/10.3109/03639045.2016.1160105>
- Qian, F., Huang, J., & Hussain, M. A. (2010). Drug-Polymer Solubility and Miscibility: Stability Consideration and Practical Challenges in Amorphous Solid Dispersion Development. *Journal of Pharmaceutical Sciences*, 99(7), 2941-2947. <https://doi.org/10.1002/jps.22074>
- Qureshi, M. J., Mallikarjun, C., Kian, W. G. (2015). Enhancement of solubility and therapeutic potential of poorly soluble lovastatin by SMEDDS formulation adsorbed on directly compressed spray dried magnesium aluminometasilicate liquid loadable tablets: A study in diet induced hyperlipidemic rabbits. *Asian Journal of Pharmaceutical Sciences*, 10(1), 40-56. <https://doi.org/10.1016/j.ajps.2014.08.003>
- Rawat, S., & Jain, S. K. (2004). Solubility enhancement of celecoxib using β -cyclodextrin inclusion complexes. *European Journal of Pharmaceutics and Biopharmaceutics*, 57(2), 263-267. <https://doi.org/10.1016/j.ejpb.2003.10.020>
- Ren, L. L., Zhou, Y., Wei, P., Li, M., Chen, G. G. (2014). Preparation and Pharmacokinetic Study of Aprepitant-Sulfobutyl Ether-beta-Cyclodextrin Complex. *AAPS PharmSciTech*, 15(1), 121-130. <https://doi.org/10.1208/s12249-013-0044-0>
- Ridhurkar, D. N., Ansari, K. A., Kumar, D., Kaul, N. S., Krishnamurthy, T., Dhawan, S., Pillai, R. (2013). Inclusion complex of aprepitant with cyclodextrin: evaluation of physico-chemical and pharmacokinetic properties. *Drug Development and Industrial Pharmacy*, 39(11), 1783-1792. <https://doi.org/10.3109/03639045.2012.737331>
- Sadeghi, F., Ashofteh, M., Homayouni, A., Abbaspour, M., Nokhodchi, A., Garekani, H. A. (2016). Antisolvent precipitation technique: A very promising approach to crystallize curcumin in presence of polyvinyl pyrrolidone for solubility and dissolution enhancement. *Colloids and Surfaces B: Biointerfaces*, 147, 258-264. <https://doi.org/10.1016/j.colsurfb.2016.08.004>
- Saokham, P., Muankaew, C., Jansook, P., Loftsson, T. (2018). Solubility of Cyclodextrins and Drug/Cyclodextrin Complexes. *Molecules*, 23(5). <https://doi.org/10.3390/molecules23051161>
- Savjani, K. T., Gajjar, A. K., Savjani, J. K. (2012). Drug solubility: importance and enhancement techniques. *ISRN Pharm*, 2012, 195727. <https://doi.org/10.5402/2012/195727>
- Seedher, N., & Agarwal, P. (2009). Various solvent systems for solubility enhancement of enrofloxacin. *Indian Journal of Pharmaceutical Sciences*, 71(1). <https://doi.org/10.4103/0250-474x.51958>
- Semalty, A. (2014). Cyclodextrin and phospholipid complexation in solubility and dissolution enhancement: a critical and meta-analysis. *Expert Opin Drug Deliv*, 11(8), 1255-1272. <https://doi.org/10.1517/17425247.2014.916271>
- Seo, A. (2003). The preparation of agglomerates containing solid dispersions of diazepam by melt agglomeration in a high shear mixer. *International Journal of Pharmaceutics*, 259(1-2), 161-171. [https://doi.org/10.1016/s0378-5173\(03\)00228-x](https://doi.org/10.1016/s0378-5173(03)00228-x)
- Sharma, P., Kapoor, A., Bhargav, S. (2012). A Review on: Solubility Enhancement by Implementing Solid Dispersion Technique for Poorly Water Soluble Drug. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 3(1), 847. Retrieved from <https://www.rjpbcs.com/>

- Sharma, R., Kamboj, S., Singh, G., Rana, V. (2016). Development of aprepitant loaded orally disintegrating films for enhanced pharmacokinetic performance. *European Journal of Pharmaceutical Sciences*, 84, 55-69. <https://doi.org/10.1016/j.ejps.2016.01.006>
- Shono, Y., Jantratid, E., Kesisoglou, F., Reppas, C., Dressman, J. B. (2010). Forecasting in vivo oral absorption and food effect of micronized and nanosized aprepitant formulations in humans. *Eur J Pharm Biopharm*, 76(1), 95-104. <https://doi.org/10.1016/j.ejpb.2010.05.009>
- Sodeifian, G., Sajadian, S. A., Daneshyan, S. (2018). Preparation of Aprepitant nanoparticles (efficient drug for coping with the effects of cancer treatment) by rapid expansion of supercritical solution with solid cosolvent (RESS-SC). *Journal of Supercritical Fluids*, 140, 72-84. <https://doi.org/10.1016/j.supflu.2018.06.009>
- Sugandha, K., Kaity, S., Mukherjee, S., Isaac, J., Ghosh, A. (2014). Solubility Enhancement of Ezetimibe by a Cocrystal Engineering Technique. *Crystal Growth & Design*, 14(9), 4475-4486. <https://doi.org/10.1021/cg500560w>
- Tao, T., Zhao, Y., Wu, J., Zhou, B. (2009). Preparation and evaluation of itraconazole dihydrochloride for the solubility and dissolution rate enhancement. *Int J Pharm*, 367(1-2), 109-114. <https://doi.org/10.1016/j.ijpharm.2008.09.034>
- Tayade, P., & Modi, A. (2007). A comparative solubility enhancement profile of valdecoxib with different solubilization approaches. *Indian Journal of Pharmaceutical Sciences*, 69(2). <https://doi.org/10.4103/0250-474x.33156>
- U.S. Pharmacopeia - National Formulary (USP 30 - NF 25). (2007). (Vol. 1).
- Vasconcelos, T., Sarmiento, B., Costa, P. (2007). Solid dispersions as strategy to improve oral bioavailability of poor water soluble drugs. *Drug Discov Today*, 12(23-24), 1068-1075. <https://doi.org/10.1016/j.drudis.2007.09.005>
- Vemula, V. R., Lagishetty, V., Lingala, S. (2010). Solubility Enhancement Techniques. *International Journal of Pharmaceutical Sciences Review and Research*, 5(1), 41-51.
- Verma, S., Rawat, A., Kaul, M., Saini, S. (2011). Solid Dispersion: A Strategy For Solubility Enhancement. *International Journal Of Pharmacy&Technology*, 3(2), 1062-1099. Retrieved from <https://www.ijptonline.com/>
- Vo, C. L., Park, C., Lee, B. J. (2013). Current trends and future perspectives of solid dispersions containing poorly water-soluble drugs. *Eur J Pharm Biopharm*, 85(3 Pt B), 799-813. <https://doi.org/10.1016/j.ejpb.2013.09.007>
- Yadav, A. V., Shete, A. S., Dabke, A. P., Kulkarni, P. V., Sakhare, S. S. (2009). Co-crystals: a novel approach to modify physicochemical properties of active pharmaceutical ingredients. *Indian J Pharm Sci*, 71(4), 359-370. <https://doi.org/10.4103/0250-474X.57283>
- Yadav, V. B., & Yadav, A. V. (2009). Enhancement of solubility and dissolution rate of Fenofibrate by melt granulation technique. *International Journal of PharmTech Research*, 1(2), 256-263.
- Yeo, S., An, J., Park, C., Kim, D., Lee, J. (2020). Design and Characterization of Phosphatidylcholine-Based Solid Dispersions of Aprepitant for Enhanced Solubility and Dissolution. *Pharmaceutics*, 12(5), 407-427. <https://doi.org/10.3390/pharmaceutics12050407>
- Zaheer, A., Naveen, M., Santosh, M. K., Imran, K. (2011). Solubility Enhancement Of Poorly Water Soluble Drugs: A Review. *International Journal Of Pharmacy&Technology*, 3(1), 807-823. Retrieved from <https://www.ijptonline.com/>
- Zhang, X.-Y., Li, Q., Sun, J.-X., Qu, C.-H., Zheng, A.-P. (2014). Influences of Nanometer Effects on the Characters of Water-Insoluble Drug Aprepitant in Vivo and in Vitro. *Chinese Pharmaceutical Journal*, 49(14), 1226-1232. <https://doi.org/10.11669/cpj.2014.14.011>

- Zhang, X., Xing, H., Zhao, Y., Ma, Z. (2018). Pharmaceutical Dispersion Techniques for Dissolution and Bioavailability Enhancement of Poorly Water-Soluble Drugs. *Pharmaceutics*, 10(3). <https://doi.org/10.3390/pharmaceutics10030074>
- Zhang, X. Y., Wei, Y., Cao, Z. J., Xu, Y., Lu, C., Zhao, M. Q., . . . Tang, X. (2020). Aprepitant Intravenous Emulsion Based on Ion Pairing/Phospholipid Complex for Improving Physical and Chemical Stability During Thermal Sterilization. *AAPS PharmSciTech*, 21(3). <https://doi.org/10.1208/s12249-019-1605-7>

An Overview on Floating Drug Delivery Systems (FDDS); Conventional and New Approaches for Preparation and *In Vitro* –*In Vivo* Evaluation

Fatemeh SHARIAT RAZAVI* , Maryam KOUCHAK **° ,
Fatemeh FEIZOLESLAM*** , Maryam VEYSI ****

An Overview on Floating Drug Delivery Systems (FDDS); Conventional and New Approaches for Preparation and *In Vitro* –*In Vivo* Evaluation

SUMMARY

Floating drug delivery systems (FDDS) are oral dosage forms that are able to float on the contents of the stomach and remain in the stomach for a long time. They offer an opportunity to prevail over the short gastric residence time of the usual dosage forms of the drug and play an important role in slowly delivering drug substances to the upper part of the gastrointestinal tract over a continuous period. Two methods have been proposed for the development of FDDS, including non-effervescent and effervescent systems. The present review briefly explains various technologies and their mechanism to design FDDS along with *in vitro* - *in vivo* tests for evaluation of them. In addition, new approaches to their preparation have been introduced.

Key Words: Floating drug delivery systems, Gastro retentive, Effervescent, Non-effervescent, Novel floating drug delivery systems.

Yüzer İlaç Salım Sistemlerine (FDDS) Genel Bir Bakış; Hazırlık ve *İn Vitro* - *İn Vivo* Değerlendirmede Geleneksel ve Yeni Yaklaşımlar

ÖZ

Yüzen ilaç taşıyıcı sistemler (FDDS) mide içeriği üzerinde yüzebilen ve midede uzun süre kalabilen oral dozaj formlarıdır. İlacın geleneksel dozaj formlarının kısa midede kalma süresine üstün gelme fırsatı sunarlar ve ilaç maddelerinin sürekli bir süre boyunca gastrointestinal sistemin üst kısmına yavaşça verilmesinde önemli bir rol oynarlar. FDDS'nin geliştirilmesi için efervesan olmayan ve efervesan sistemler dahil olmak üzere iki yöntem önerilmiştir. Bu inceleme, kısaca çeşitli teknolojileri ve bunların FDDS tasarım mekanizmalarını, bunların değerlendirilmesi için *in vitro* - *in vivo* testlerle birlikte açıklamaktadır. Ayrıca bunların hazırlanmasına yönelik yeni yaklaşımlar tanıtılmaktadır.

Anahtar Kelimeler: Yüzer ilaç Salım sistemleri, Gastro retentif, Efervesan, Efervesan olmayan, Yeni yüzen ilaç salım sistemleri.

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* ORCID: 0000-0002-5324-8267, Nanotechnology Research Center, Department of Pharmaceutics, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

** ORCID: 0000-0002-1399-7335, Nanotechnology Research Center, Department of Pharmaceutics, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

*** ORCID: 0000-0002-2558-9777, Department of Pharmaceutics, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

**** ORCID: 0000-0003-1358-6963, Department of Pharmaceutics, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

INTRODUCTION

To maximize the therapeutic effectiveness and reduce the side effects of drugs, multiple delivery systems are applied. Oral administration of medicinal drugs is currently the most effective route of administration owing to its various advantages, such as low treatment cost, high patient compliance, dosage form flexibility, and ease of administration (Shivakumar, Gowda, & Kumar, 2004). One of the problems of oral delivery systems is the short drug residence at the site of absorption. Gastro retentive drug delivery systems (GRDDS) can prolong drug residence to several hours in the gastric area. GRDDS have valuable characteristics, including high therapeutic efficacy and bioavailability for narrow absorption window drugs and solubility improvement of less soluble drugs in environments with high pH. Different methods, including floating drug delivery systems (FDDS), have been introduced for improving the gastric residence

of drugs (Garg & Gupta, 2008). This review aims to describe different techniques used in developing floating dosage forms, identify their mechanisms of action and introduce *in vitro* and *in vivo* evaluation methods for them.

Floating drug delivery systems (FDDS)

Davis first introduced FDDS in 1968. These systems are known to have lower densities than the gastric fluid, remaining buoyant for a long time in the stomach. They are recognized as an important means of achieving adequate gastric retention and drug bioavailability (Badoni, Ojha, Gnanarajan, & Kothiyal, 2012). In addition, they are appropriate systems for the delivery of drugs, which have a narrow absorption window in the upper small intestine or stomach (Singh & Kim, 2000). In view of the buoyancy mechanisms, non-effervescent systems and effervescent systems, have been applied to develop FDDS (Figure 1).

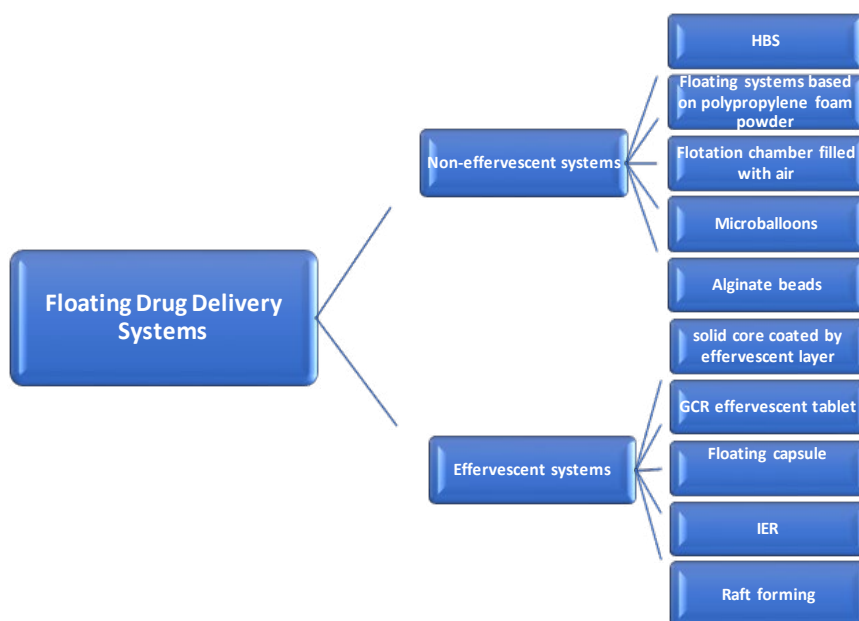


Figure 1. Classification of Floating Drug Delivery Systems

Non-effervescent systems

Such systems are generally composed of a highly swellable hydrocolloid in a matrix-forming polymer such as polycarbonate, polystyrene, polymethacrylate, or polyacrylate. Typically a polysaccharide or

a cellulosic compound is used as the swellable part. Upon contact with gastric fluids, the hydrocolloid is hydrated and forms a low-density gel network that entraps the air and can be floated on stomach fluid. The release of the drug is directly controlled by these colloidal gels. Hydrophilic drugs are mainly released

by diffusion mechanism, while hydrophobic drugs are released by erosion of the outer surface of the system.

Hydrodynamically balanced systems (HBS)

HBS, with gel-forming hydrophilic polymers, are single-unit dosage forms. The most common excipient is hydroxypropyl methylcellulose (HPMC), although sodium carboxymethyl cellulose (NaCMC), carrageenan, hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC), and alginic acid have also been applied. Administration of the drug-mixed polymer is mainly done in a gelatin capsule, which dissolves in the gastric fluid rapidly. A floating mass is produced by hydration and swelling of the polymer's surface (**Figure 2**) (Makwana, Sameja, Parekh, & Pandya, 2012; Rastogi, 2016).

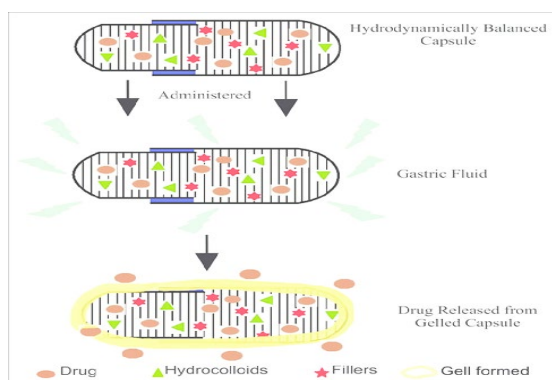


Figure 2. Hydro dynamically Balanced capsule (Rastogi, Kumar, Yadav, Hegde, & Rastogi, 2016). Thanks to Dr. Vaibhav Rastogi for permitting us to use this figure.

Sustained-release HBS tablets (containing hydrophilic hydrocolloids) were first developed by Sheth and Tossounian. Following contact with the gastric fluid, a soft gelatin mass was formed on the tablet surface, creating a water-impermeable barrier. The slowly released drug from the gelatin mass surface remained buoyant on the gastric fluid (Sheth & Tossounian, 1979). In order to prepare ofloxacin HBS capsules, liquid paraffin, lactose, HPMC K4M, and polyvinylpyrrolidone K30 (PVP K30) were used. The capsules were floated without any lag time for more than six hours. Sustained drug release was reported in this period, and its rate was reliant on the amounts of PVP K30, HPMC K4M, and liquid paraffin content (A. K. Nayak, Das, & Maji, 2013).

Bomma et al. prepared floating matrix tablets of norfloxacin, using polymers such as xanthan gum, HPMC K199M, and HPMC K4M in the wet granulation technique. Prolonged drug release was indicated by the tablets while floating over the dissolution medium (Bomma, Naidu, Yamsani, & Veerabrahma, 2009). In addition, Ali J et al. introduced a single-unit floating capsule to deliver metformin using the HBS technology. In this system, different low-density polymers were used. During five hours of examination, the formulation was found to be buoyant on the gastric fluid in rabbits. The plasma level-time AUC increased in the optimized HBS metformin capsules compared to the immediate-release formulation (Ali et al., 2007).

Floating systems based on polypropylene foam powder

Streuble et al. prepared floating microparticles using polypropylene foam powder and investigated their performance *in vitro*. An oil-in-water solvent extraction method was used to prepare the floating microparticles, which consisted of polypropylene foam powder. Verapamil HCl was used as a drug, along with a controlled release polymer (polymethyl methacrylate, Eudragit® RS, or ethyl cellulose). All formulations displayed appropriate buoyancy behavior with a wide range of dissolution profiles (Streubel, Siepmann, & Bodmeier, 2002). The researchers also developed the floating tablets based on polypropylene foam powder in a matrix-forming polymer. The highly porous foam powder presented inherently low-density systems capable of floating for at least 8 hours at 0.1 N HCl at 37 ° C. The release properties were strongly related to drug chemistry and could be modified according to the ratio of the polymer matrix to the foam powder (Streubel, Siepmann, & Bodmeier, 2003).

Flotation chamber filled with air or harmless gas

Microporous compartment systems are designed by the principle of drug encapsulation in a compartment with pores on its walls (top and bottom) attached to an air-containing chamber (Harrigan, 1977). For preventing any interactions between the

undissolved drug and gastric mucus, the peripheral walls were sealed in the device completely. Using a low density of microporous chambers, the delivery system can float on the gastric fluid (Atyabi, Sharma, Mohammad, & Fell, 1996a). When a limited amount of gastric juice enters the pores, the drug dissolves and leaves the dosage form, and is continuously delivered throughout the intestine. (Hafeez, Maurya, Singh, Mittal, & Rana, 2013).

Micro balloons or hollow microspheres

Emulsification-solvent diffusion or simple solvent evaporation method was applied to prepare drug-loaded micro balloons (A. Michaels, 1974). In developing these systems, cellulose acetate, Eudragit

S, polycarbonate, low methoxy pectin, and calcium alginate are used (Kawashima, Niwa, Takeuchi, Hino, & Itoh, 1992). In this regard, Kouchak and Badrian applied the emulsification-solvent diffusion method to prepare a multiple-unit oral floating system for theophylline. After dissolving dibutyl phthalate, theophylline, and ethyl cellulose in the dichloromethane-alcohol mixture, the compounds were added to an aqueous medium. Rapid alcohol diffusion in the aqueous medium and dichloromethane evaporation while stirring accounted for the formation of an interfacial polymer and drug deposition, resulting in the generation of low-density hollow microspheres with pores in the shells (Figure 3).

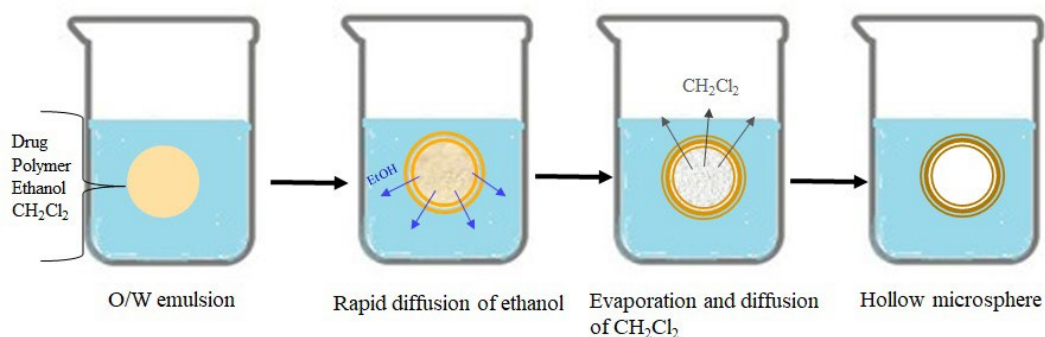


Figure 3. Hollow microsphere formation

The produced microspheres showed a spherical porous shape, which tended to float on the simulated gastric medium for 12 hours or more. In the scanning electronic micrograph (SEM), the hollow porous structure of the micro balloon is indicated. One of the problems in preparation of micro balloons with high drug content is the solubility of the drug in an aqueous phase in the process of microsphere formation. One of the problems in preparing high-drug micro balloons is the solubility of the drug in an aqueous phase in the process of forming microspheres. Kouchak and Badrian succeed in increasing theophylline loading of the micro balloons by adding NaCl 20% to the aqueous phase. The saturated solubility of theophylline decreased considerably at high concentrations of NaCl, which increased the encapsulation efficiency of the-

ophylline (Kouchak & Badrian, 2007). Kouchak et al. used the mentioned emulsification-solvent diffusion method to prepare micro-balloon systems containing diclofenac to increase its gastric emptying time. In this study, the solubility of diclofenac decreased by adding HCl 0.1 M into the aqueous phase, resulting in enhanced loading efficiency (Kouchak & Moghimi-pour, 2007; Nayak, Malakar, & Sen, 2010). Rishikesh Gupta et al. introduced an oral multiple-unit famotidine microsphere to target stomach ulcers, using the modified solvent evaporation method. They used the Eudragit S-100 as polymer and dichloromethane and ethanol as solvents. The SEM images indicated the floating cavity and porous surface of the microsphere loaded with famotidine (Figure 4) (Gupta, Prajapati, Pattnaik, & Bhardwaj, 2014).

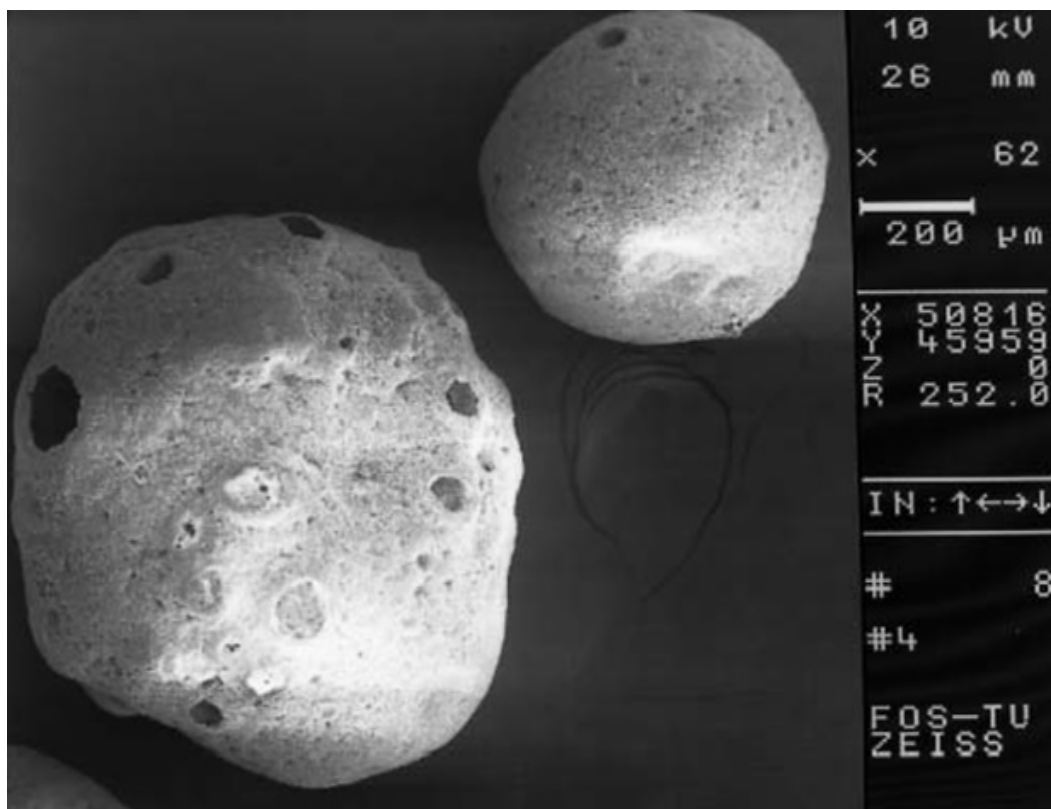


Figure 4. SEM image of hollow microspheres (Kouchak & Badrian, 2007).

Joseph et al. using hollow polycarbonate microspheres, designed a floating dosage form of piroxicam. In *in vivo* studies on healthy rabbits, the pharmacokinetic data revealed that piroxicam-loaded polycarbonate microsphere systems could increase bioavailability and sustained drug delivery over an extended period. In addition (Joseph, Lakshmi, & Jayakrishnan, 2002), Thanoo et al. used polycarbonate in the solvent evaporation method to develop sustained-release floating microspheres. In their study, griseofulvin, aspirin, and p-nitroaniline were applied as the model drugs (Thanoo, Sunny, & Jayakrishnan, 1993).

For the preparation of hollow microspheres, Sato et al. used mixtures of Eudragit S with other hydrophobic or hydrophilic polymers (e.g., HPMC or EC) to improve riboflavin release. An increase in the content of HPMC increased the release of riboflavin, while the floating features of the microspheres reduced. γ -scintigraphy was applied to evaluate riboflavin-containing

micro-balloon. Also, urinary riboflavin excretion was assessed. The floating system showed a significantly higher bioavailability, compared to the non-floating formulation (Sato, Kawashima, Takeuchi, & Yamamoto, 2004a, 2004b; Sato, Kawashima, Takeuchi, Yamamoto, & Fujibayashi, 2004).

Alginate beads

For designing a multiple-unit floating system, Talukdar and Fassihi used cross-linked beads, which consisted of Ca^{2+} ions, an anionic polysaccharide, and sodium alginate. The buoyancy was maintained for more than 12 hours by using the beads (Talukder & Fassihi, 2004). Sodium alginate solution was added to an aqueous calcium chloride solution in this method, resulting in calcium alginate precipitation. After separation and drying the beads via air convection and freeze-drying, a porous system was created (Figure 4) (Kaushik, Chaurasia, Chaurasia, Mishra, & Bhardwaj, 2011; Mahajan, Gupta, & Sharma, 2010). Malleswari

prepared alginate beads of stavudine using the ionotropic gelation method with HPMC and sodium alginate. The beads showed extended drug release (almost 12 hours) and remained buoyant for nearly 12 hours (Malleswari K, 2016). Moreover, Mishra et al. formulated controlled-release gastro retentive floating gel beads of loratadine using pectin and sodium alginate, accompanied by oil (mineral or castor oil), by the emulsion gelation method. They used calcium chloride solution as the cross-linking agent (Mishra & Pathak, 2008).

Additionally, Fell et al. prepared floating alginate beads containing amoxicillin through dropwise addition of alginate to the CaCl_2 solution and freeze-drying the prepared gel beads. The buoyancy of beads with high drug loadings persisted for 20 hours (Whitehead, Collett, & Fell, 2000). Kumar Dey et al. developed amoxicillin-loaded floating mucoadhesive beads containing sunflower oil using sodium alginate and HPMC as matrix polymers and Chitosan as a coating polymer. They used ionotropic gelation method to prepare the beads. Firstly, an aqueous solution of sodium alginate, HPMC, and amoxicillin trihydrate in demineralized water was prepared, and sunflower oil was added dropwise. The emulsion was extruded through 5% (w/v) calcium chloride solution, and the formed beads transferred to chitosan solution to be coated. All beads were able to float for >24 h with a maximum lag time of 46.3 ± 3.2 s. X-ray study in rabbit stomach confirmed the gastric retention of optimized formulation (Dey et al., 2016).

Effervescent systems

Effervescent floating systems can produce CO_2 and decrease device density. These systems remain buoyant for a long time in the stomach. Some of the FDDS that use an effervescent mechanism in their design are given in the following sections.

Solid core coated by the effervescent layer

Gas-producing agents (such as citric acid and tartaric acid) are utilized as internal effervescent layers in these systems for gas generation (Figure 5). The stoichiometric citric acid ratio to sodium bicarbonate is reported to be optimally 0.76:1 (Michaels, Bashwa, & Zaffaroni, 1975).

In this regard, Elsamaligy and Bodmeier designed a multiple unit effervescent extended-release drug delivery system. In addition to adequately controlling the release of drugs with different solubility, this system showed fast and long buoyancy. Fluidized bed-coating methods were applied for preparing the pellet systems, and were evaluated from the points of drug release, floatation, medium uptake, and swelling in HCl 0.1 M. In addition, two pellet systems were studied. The first system included drug-layered sugar cores, NaHCO_3 layer, and polymeric top coating. The second pellet system was coated by three layers, including Eudragit® RL 30D top coating, NaHCO_3 layer, and drug-containing Eudragit® RS 30D coating. The Eudragit® RL coating led to adequate medium penetration into the pellet and high CO_2 entrapment efficiency (Elsamaligy & Bodmeier, 2015).

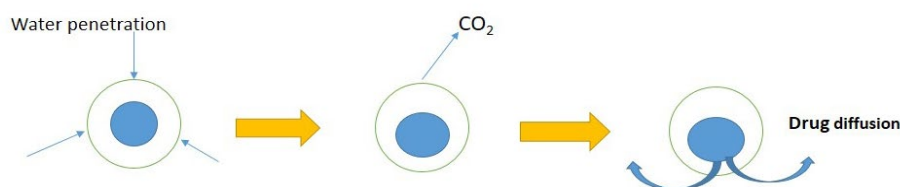


Figure 5. Gas generation of a solid core coated by effervescent layer after placing in water.

Gastro retentive controlled-release (GCR) effervescent tablets

Tadros developed ten gastro retentive formulations, using HPMC K15M and, or sodium alginate as release-retarding polymers, along with NaHCO_3 or CaCO_3 as gas formers. The tablets showed acceptable swelling, floating, and adhesive properties (Tadros, 2010). In addition, Goole et al. developed 3-mm floating mini tablets containing glyceryl palmitostearate as a meltable binder and tartaric acid, NaHCO_3 , and CaCO_3 as effervescent agents. After preparing the tablets via melt granulation and compression, the coating was done with Eudragit® RL 30D and a flexible membrane was produced. The buoyancy of mini-tablets was independent of the medium pH; it occurred within 10 minutes and continued for more than 13 hours (Goole, Amighi, & Vanderbist, 2008; Goole, Deleuze, Vanderbist, & Amighi, 2008).

Floating capsules containing effervescent agents

Li et al. prepared floating capsules consisting of an effervescent mixture, Carbopol 934, and HPMC with different viscosity grades. They found that the presence of Carbopol, HPMC viscosity, and polymer-polymer interactions significantly influenced the buoyancy and release features of dosage forms (Li, Lin, Daggy, Mirchandani, & Chien, 2002, 2003).

Bicarbonate-loaded Ion exchange resin (IER) system

Bicarbonate-loaded IER beads can be used to formulate gastro retentive systems. For this purpose, an ionic drug and bicarbonate ions are attached to anionic resin beads, and the beads were coated with a semipermeable membrane. After reaching the acidic stomach environment, chloride and bicarbonate ions are exchanged. As carbon dioxide is released and trapped in the membrane, the density of the beads decreases and they move to the top of gastric fluid (Anand, Kandarapu, & Garg, 2001; Klausner, Eyal, Lavy, Friedman, & Hoffman, 2003).

Kouchak and Atyabi prepared a multiple-unit oral floating dosage system, using Amberlite-IRA900 as the IER system. It included loading IER beads with

diclofenac and bicarbonate ions using a batch method and coating them with Eudragit RS or ethyl cellulose. In the batch method, the ion exchange resin beads and the solution containing the ion exchange candidate ions are mixed in a vessel and allowed to equilibrate. In contact with the simulated gastric fluid, the beads could generate CO_2 , and the entrapped gas caused the coated beads to float (Kouchak & Atyabi, 2004). In this regard, Atyabi et al. introduced a similar system using Dowex as an ion exchange resin and loaded it with bicarbonate ions and theophylline (as the model drug). The beads were coated with a semipermeable membrane to overcome rapid CO_2 loss (Atyabi et al., 1996a; Atyabi, Sharma, Mohammad, & Fell, 1996b).

Raft forming system

The raft forming system consists of an effervescent liquid with in-situ gel properties and buoyancy capability (Ibrahim, 2009). In this system, the CO_2 is generated along with a viscous alginate gel in contact with gastric fluids. This continuous layer of gel, called raft, can remain intact and buoyant over the stomach contents for a long time to facilitate sustained release of drugs (Fayaz et al., 2018; Vinod et al., 2010). So, an antacid raft forming system acts as a barrier between the stomach and the esophagus and prevents the reflux of gastric content into the esophagus. In addition, the alginate layer can adhere to the gastric mucosa due to its bioadhesive nature (Fayaz et al., 2018).

Kerdsakundee et al. developed new raft forming systems containing solid dispersions of curcumin-Eudragit EPO to provide a long-acting gastric ulcer treatment. These formulations were composed of curcumin-Eudragit EPO solid dispersions, sodium alginate as a gelling polymer, and calcium carbonate for creating divalent Ca^{2+} ions and carbon dioxide gas. The solvent evaporation method was used to prepare the solid dispersions. These new raft forming formulations at 40 mg/kg once daily displayed a higher therapeutic effect on the gastric ulcer than the standard antisecretory agents: lansoprazole (1 mg/kg, twice daily) and a curcumin suspension (40 mg/kg, twice daily) (Kerdsakundee, Mahattanadul, & Wiwattanapatapee, 2015).

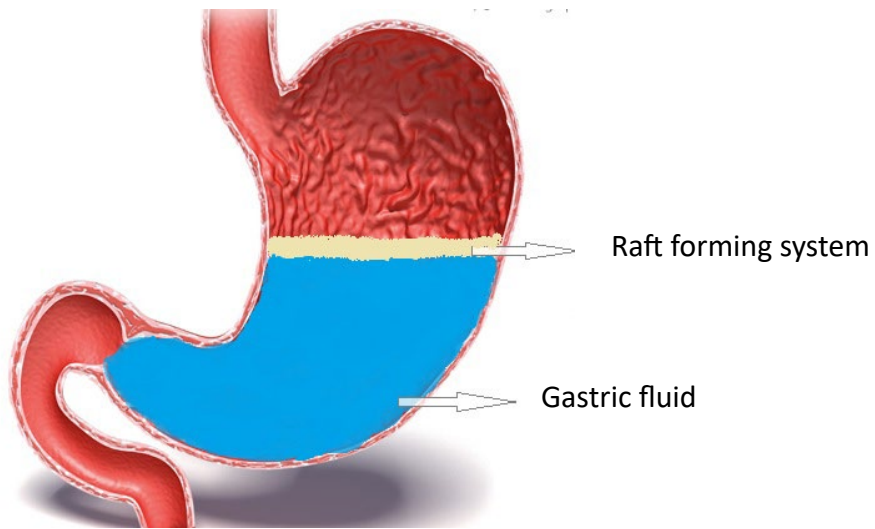


Figure 6. Schematic image of Raft forming system

Novel techniques for preparation of FFDS

Recently, researchers have used novel methods to design floating systems. Regardless of whether their mechanism is effervescent or non- effervescent, some of them are described in the following section (**Figure 7**).

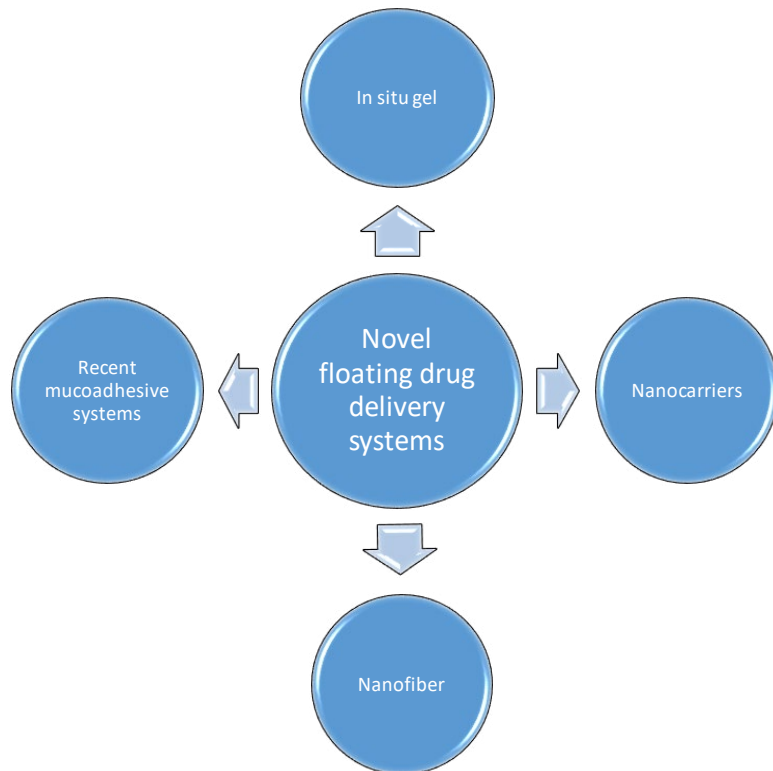


Figure 7. Some of the novel floating drug delivery systems

In situ gelling

Rajinikanth, et al. designed a gastric floating system based on in situ gelling mechanism for delivery of amoxicillin to eradicate *Helicobacter pylori*. Calcium carbonate and gellan were used as the gas-releasing agent and the gelling polymer, respectively. Dissolution of calcium carbonate in the acidic environment of the stomach produces calcium ions and leads to gelation of gellan gum. *In vivo* study showed ten times effectiveness of the floating system against *Helicobacter pylori* compared to the amoxicillin suspension (Rajinikanth, Balasubramaniam, & Mishra, 2007). This author, in another similar study, made a floating in situ gelling system of clarithromycin using gellan and calcium carbonate. The increased clarithromycin stability and prolonged gastrointestinal residence time led to eradication of *H. pylori* (Rajinikanth & Mishra, 2008).

Nanocarriers

Nana Chen, et al. prepared novel nanomicelles-loaded gastro retentive beads. At first, emodin-loaded nanomicelles were prepared by pluronic F127 and Tween 80 using the thin-film hydration method. The nanomicelles were coated with chitosan and tested against human gastric carcinoma. Secondly, nanomicelles-loaded floating mucoadhesive beads (NFM-Beads) were developed by ionotropic gelation method using sodium carboxymethylcellulose and aluminum chloride. NaHCO₃ as a floating agent was trapped in this network. The release, swelling, degradation, mucoadhesion, and floating ability of the samples were investigated *in vitro*. Also, gastric retention was evaluated *in vivo*. They concluded that the NFM-Beads system could improve the therapeutic potency of emodin against gastric cancer (Chen et al., 2019).

The insertion of liquid crystalline (LC) monomers can give molecularly imprinted polymers (MIPs) reversible deformation properties in response to different environmental factors. Recently, it has been shown that liquid crystalline -molecularly imprinted polymers (LC-MIPs) have buoyancy behavior on the aqueous medium due to their solvent-responsive deformation. LC-MIPs have a much higher ca-

capacity compared to usual MIPs because of their low cross-linking structure. LC-MIPs were first used as an FDDS for S-amlodipine delivery (Zhang et al., 2017).

Li-Ping Zhang, et al. fabricated a novel floating interaction-controlled DDS using LC-MIP coated multiwalled carbon nanotubes (MWCNTs) and used levofloxacin as a model template. The levofloxacin-loaded MWCNT@LC-MIP showed a combination of buoyancy and controlled release properties, which provided high relative bioavailability (Zhang, Tan, Huang, & Liu, 2018).

Nanofiber-based effervescent pouches

Serdar Tort, et al. reported a nanofiber-based effervescent approach for producing FDDS. They embedded polyethylene oxide (PEO)/sodium bicarbonate (NaHCO₃) cast films into Pramipexole-loaded electrospun nanofibers fabricated from Eudragit RL and RS polymers. The PEO/ NaHCO₃ film released CO₂ gas after contact with gastric acid and produced self-inflating nanofiber pouches filled gas bubbles. Nanofiber formulations showed floating lag times lower than 1 second and total floating times more than 72 h. The system provided the sustained release of pramipexole more than overnight (Tort, Han, & Steckl, 2020).

Recent mucoadhesive systems in floating drug-delivery

Zn-pectinate-sterculia gum interpenetrating polymer network (IPN) beads were prepared by concurrent ionotropic gelation with zinc acetate and covalent crosslinking with glutaraldehyde. The pectinate-sterculia gum (SG) blend beads could enhance the duration of gastric retention, which was achieved by a combination of floatation mechanism and mucoadhesion. Bera, et al. utilized this system for intragastric ziprasidone HCl delivery. The density of ziprasidone HCl -loaded IPN beads was significantly lower than the density of gastric fluids (0.608 -0.911 ± 0.19 g/cm³). The use of zinc acetate as an ionic crosslinker may be the cause of this low density. The optimized beads displayed a floating lag time of less than 2 min and buoyancy ability of more than 63% at eight h. They showed good mucoadhesive with the goat gas-

tric mucosa. (See **Table 1.**) (Bera, Boddupalli, & Nayak, 2015).

Evaluation Tests

Various parameters are usually evaluated for gastro retentive formulations, including floating duration, specific gravity, dissolution profile, content uniformity, and friability. Particle size, flow properties, mechanical properties, and surface morphology are also assessed in multiparticulate drug delivery systems (Arunachalam et al., 2011).

In vitro evaluation

Size and shape measurements

The particle shape and size majorly influence the

dissolution and bioavailability of drugs. Different methods, including air elutriation analysis, photo analysis, sieve analysis, optical microscopy, laser diffraction methods, colter counter, ultrasound attenuation spectroscopy, and sedimentation techniques, are used to measure the particle size (Narang, 2011).

Buoyancy test

This test is commonly carried out in simulated gastric fluid (SGF) at 37°C. The floating or buoyancy lag time refers to the time needed for the dosage form to float on the medium surface. Also, the total buoyancy time is the total quantity of time when the dosage form is buoyed on the dissolution medium (Prajapati, Jani, Khutliwala, & Zala, 2013).

Table 1.: polymers used in new floating dosage forms

Floating Dosage Form	polymer(s)	Drug	Ref.
In situ gelling solution	Gellan	Amoxicillin	(Kerdsakundee, 2015)
In situ gelling solution	Gellan	Clarithromycin	(Rajinikanth, 2007)
NFM-Beads ¹	Pluronic F127 Chitosan Sodium carboxymethylcellulose	Emodin	(Rajinikanth & Mishra, 2008)
(LC-MIP) particles ²	Methacrylic acid Ethylene glycol dimethacrylate 4- Methyl phenyl dicyclohexyl ethylene	S-amlodipine	(Chen, 2019)
MWCNT@LC-MIP particles ³	Methacrylic acid Ethylene glycol dimethacrylate 4-Methyl phenyl dicyclohexyl ethylene	Levofloxacin	(Zhang, 2017)
Electrospun nanofibers	Polyethylene oxide Eudragit RL Eudragit RS	Pramipexole	(Zhang, 2018)
IPN beads ⁴	Sterculia gum LM-pectin	Ziprasidone HCl	(Tort, 2020)

Resultant weight

- 1 nanomicelles-loaded floating mucoadhesive beads
- 2 Liquid crystalline-molecularly imprinted polymer
- 3 Multiwalled carbon nanotubes (MWCNTs) coated LCMIP
- 4 Interpenetrating polymer network beads

To describe buoyancy, floating time and bulk density are considered the most critical parameters. However, mere measurement of density is insufficient for identifying buoyancy, as thickness varies in media with changes in the resultant weight through time (Narang, 2011; Rathee P, 2011).

Drug release

For *in vitro* evaluation of drug release, dissolution tests are carried out using a USP apparatus: apparatus I (Paddle), apparatus II (basket), apparatus III (modified disintegration testing apparatus), or apparatus IV (flow-through cell). These tests are performed in 0.1M HCl (900 mL) at a stirring rate of 50 or 100 rpm at 37±0.5°C (Jawale, Bairagi, Jaybhai, & Deshmukh, 2010).

Surface topography

Atomic force microscopy (AFM), SEM, and contact profilometer were used to determine the surface topography and structure (Arunachalam et al., 2011; Ichikawa, Watanabe, & Miyake, 1991; Sharma, Agarwal, Gupta, & Khinchi, 2011).

Moisture content measurement

The moisture content is seldom necessary. It indicates whether a product has standard features for production and trade. There are various techniques for determining the moisture content of formulations, such as freeze-drying, vacuum drying, Karl Fischer titration, thermogravimetric methods, and physical methods (Arunachalam et al., 2011; Klausner et al., 2003; Sato, Kawashima, et al., 2004a).

Swelling index

The swelling index is measured by evaluating water uptake (WU) or weight gain after submerging in an aqueous medium, especially 0.1 M HCl for a specific time. After removing the dosage form at regular intervals, weight changes are determined relative to time (Narang, 2011; Sharma et al., 2011). WU is determined based on the weight gain percentage:

$$WU = (W_t - W_o) * 100 / W_o$$

Where W_o and W_t are the initial weight of dosage

form and weight at time t , respectively. Also, dimensional changes can be determined, considering the increase in the thickness and, or diameter of the tablet over time (Prajapati et al., 2013; Sharma et al., 2011).

Drug content assessment

The drug content represents the amount of drugs in each unit. It is measured using HPLC, HPTLC, spectroscopy, near-infrared spectroscopy, microtitrimetric methods, or inductively coupled plasma atomic emission spectrometer (Arunachalam et al., 2011; Tanwar, Naruka, & Ojha, 2007).

Encapsulation efficiency

A significant physicochemical feature of the dosage form is its encapsulation efficiency. Various methods, including ultrafiltration, gel filtration, dialysis bag diffusion, ultracentrifugation, and microdialysis, have been suggested to evaluate encapsulation efficiency (Arunachalam et al., 2011; Bajpai, Bajpai, & Sharma, 2007).

Fourier-transform infrared spectroscopy (FTIR)

FTIR is commonly applied to detect polymeric, organic, or inorganic materials and functional groups. The FTIR spectra of pure drugs, polymers (or other ingredients), and drug-loaded formulations are used to evaluate drug interactions with the polymer (Arunachalam et al., 2011; Sonar, Jain, & More, 2007).

***In vivo* Evaluation**

Radiology and scintigraphy

X-ray radiography and gamma scintigraphy can help determine the dosage form position in the gastrointestinal tract; therefore, it is possible to identify the dosage form passage in this tract and predict the gastric emptying time. By integrating a radio-opaque material in a solid dosage form, X-ray visualization will be enabled to evaluate gastric retention at different intervals. Barium sulfate is recognized as commonly radio-opaque marker. Also, indirect observation is facilitated by a scintiscanner using radionuclide γ -emission in a formulation. The emitting ma-

terial is mostly ^{99}Tc (Horton, Ross, & Darling, 1965; Shalaby, Blevins, & Park, 1992). However, Razavi et al. used samarium (III) oxide ($^{153}\text{Sm}_2\text{O}_3$) to radiolabel the metformin HCl-loaded floating tablet to trace the dosage form via gamma scintigraphy in the gastrointestinal tract. This study was performed on New Zealand white rabbits (Razavi et al., 2015).

Gastroscopy

Gastroscopy is an oral endoscopy, which uses video systems or fiber optics to visualize the effect of dosage form on residence time in the stomach. Moreover, it provides an accurate analysis of the gastro retentive system of drug delivery (Prajapati et al., 2013; Soni et al., 2011).

MRI imaging

MRI, as a relatively safe *in vivo* approach, is used to evaluate the gastro-retention of a system. It can be used to determine the site of ingested dosage form by radio waves and magnetic fields. In this method, compounds, which have optimal paramagnetic features (such as ferrous oxide), are integrated into the dosage form for imaging (Bagul, Patil, Shirsath, Nikam, & Gujar, 2012; Steingoetter et al., 2003).

^{13}C octanoic acid breath test

This test is used to measure the gastric emptying time of GRDDS systems. Octanoic acid is a medium-chain fatty acid, which is rapidly absorbed in the duodenum, with subsequent hepatic oxidation to $^{13}\text{CO}_2$. In this molecule, ^{13}C isotope replaces the carbon atom, which enters CO_2 and comes out in the breath. After oral administration of a floating system containing ^{13}C octanoic, the appearance of $^{13}\text{CO}_2$ in the breath is mainly associated with the gastric emptying of the dosage form in the duodenum (Perri, Pastore, & Annese, 2005). Its analysis requires the collection of several respiratory samples before the use of ^{13}C octanoic acid and then at regular intervals of 15 minutes and 4 hours later, the ratio of $^{13}\text{CO}_2/^{12}\text{CO}_2$ can also be determined by mass spectrometry (IRMS) or infrared spectrometry (IR) (Bruno et al., 2013).

The breath test is a non-invasive, non-operator-dependent, reproducible method without any biological hazards. Moreover, this method is more cost-effective than other methods (Bagul et al., 2012; Jackson et al., 2004).

CONCLUSION

The inability to localize and restrain an oral dosage form in the upper gastrointestinal tract, i.e., stomach, duodenum, and jejunum and the highly variable nature of gastric emptying time result in unpredictable bioavailability. FDDS has been suggested as a potential approach for prolonging gastric retention, controlled delivery, and enhancing the bioavailability of a drug. In this study, we reviewed different floating systems, which have been developed so far. Two major classes, including effervescent and non-effervescent FDDS and also application of *in situ* gels, nanocarriers, nanofibers, and recent mucoadhesive systems in the fabrication of floating dosage forms, were described in detail. In addition, *in vitro* and *in vivo* evaluating methods for assessment of efficiency of floating systems were discussed.

CONFLICT OF INTEREST

All the authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis and experimenting (Kouchak M.), preparing the study text and literature Research (Feizoleslam F., Veisy M.), reviewing the text (Shariat Razavi F.), Analysis, interpretation of the data, and revising the final manuscript (Kouchak M.).

ABBREVIATIONS

Floating drug delivery systems (FDDS)
Gastro retentive drug delivery systems (GRDDS)
Hydro dynamically balanced systems (HBS)
Hydroxypropyl methylcellulose (HPMC)
Sodium carboxymethyl cellulose (NaCMC)
Carrageenan, hydroxyethyl cellulose (HEC)
Hydroxypropyl cellulose (HPC)

Polyvinylpyrrolidone K30 (PVP K30)
Gastro retentive controlled-release (GCR)
Bicarbonate-loaded Ion exchange resin (IER)
Nanomicelles-loaded floating mucoadhesive beads (NFM-Beads)
Liquid crystalline -molecularly imprinted polymers (LC-MIPs)
Multiwalled carbon nanotubes (MWCNTs) coated LCMIP (MWCNT@LC-MIP)
Polyethylene oxide (PEO)
Pectinate- sterculia gum (SG)
Interpenetrating polymer network beads (IPN beads)
Simulated gastric fluid (SGF)
Atomic force microscopy (AFM)
Water uptake (WU)
Fourier-transform infrared spectroscopy (FTIR)

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REFERENCES

- Ali, J., Arora, S., Ahuja, A., Babbar, A. K., Sharma, R. K., Khar, R. K., Baboota, S. (2007). Formulation and development of hydrodynamically balanced system for metformin: in vitro and in vivo evaluation. *European Journal of Pharmaceutics and Biopharmaceutics*, 67(1), 196-201.
- Anand, V., Kandarapu, R., & Garg, S. (2001). Ion-exchange resins: carrying drug delivery forward. *Drug Discovery Today*, 6(17), 905-914.
- Arunachalam, A., Karthikeyan, M., Konam, K., Prasad, P. H., Sethuraman, S., Ashutoshkumar, S., & Manidipa, S. (2011). Floating drug delivery systems: A review. *International Journal of Research in Pharmaceutical Sciences*, 2(1), 76-83.
- Atyabi, F., Sharma, H., Mohammad, H., & Fell, J. (1996a). Controlled drug release from coated floating ion exchange resin beads. *Journal of Controlled Release*, 42(1), 25-28.
- Atyabi, F., Sharma, H., Mohammad, H., & Fell, J. (1996b). In vivo evaluation of a novel gastric retentive formulation based on ion exchange resins. *Journal of Controlled Release*, 42(2), 105-113.
- Badoni, A., Ojha, A., Gnanarajan, G., & Kothiyal, P. (2012). Review on gastro retentive drug delivery system. *The Pharma Innovation*, 1(8, Part A), 32.
- Bagul, U. S., Patil, R. V., Shirsath, Y. A., Nikam, A. J., & Gujar, K. N. (2012). Stomach specific drug delivery systems: a review. *International Journal of Pharmaceutical Research and Development*, 4(4), 147-150.
- Bajpai, S. K., Bajpai, M., Sharma, L. (2007). Prolonged gastric delivery of vitamin b2 from a floating drug delivery system: An in vitro study. *Iranian Polymer Journal*, 16(8), 521-527
- Bera, H., Boddupalli, S., Nayak, A. K. (2015). Mucoadhesive-floating zinc-pectinate-sterculia gum interpenetrating polymer network beads encapsulating ziprasidone HCl. *Carbohydrate Polymers*, 131, 108-118.
- Bomma, R., Naidu, R. A. S., Yamsani, M. R., Veerabrahma, K. (2009). Development and evaluation of gastroretentive norfloxacin floating tablets. *Acta Pharmaceutica*, 59(2), 211-221.
- Bruno, G., Lopetuso, L., Ianiro, G., Laterza, L., Gerardi, V., Petito, V., . . . Scaldaferrri, F. (2013). 13C-octanoic acid breath test to study gastric emptying time. *European Review for Medical and Pharmacological Sciences*, 17(Suppl 2), 59-64.

- Chen, N., Li, Q., Li, J., Ren, Y., Wu, G., Liu, Y., Shi, Y. (2019). Development and evaluation of a new gastroretentive drug delivery system: Nanomicrospheres-loaded floating mucoadhesive beads. *Journal of Drug Delivery Science and Technology*, 51, 485-492.
- Dey, S. K., De, P. K., De, A., Ojha, S., De, R., Mukhopadhyay, A. K., Samanta, A. (2016). Floating mucoadhesive alginate beads of amoxicillin trihydrate: A facile approach for H. pylori eradication. *International Journal of Biological Macromolecules*, 89, 622-631.
- Elsamaligy, S., & Bodmeier, R. (2015). Development of extended release multiple unit effervescent floating drug delivery systems for drugs with different solubilities. *Journal of Drug Delivery Science and Technology*, 30, 467-477.
- Fayaz, M. W., Chasta, P., Sheikh, T. H., Rather, M. A., Kumar, A. H., Mustafa, A. (2018). Gastroretentive Drug Delivery System. *Journal of Drug Discovery and Development (ISSN: 2581-6861)*, 2(1), 11-17.
- Garg, R., & Gupta, G. (2008). Progress in controlled gastroretentive delivery systems. *Tropical Journal of Pharmaceutical Research*, 7(3), 1055-1066. doi: 10.4314/tjpr.v7i3.14691
- Goole, J., Amighi, K., Vanderbist, F. (2008). Evaluation and floating enhancement of levodopa sustained release floating minitabets coated with insoluble acrylic polymer. *Drug Development and Industrial Pharmacy*, 34(8), 827-833.
- Goole, J., Deleuze, P., Vanderbist, F., Amighi, K. (2008). New levodopa sustained-release floating minitabets coated with insoluble acrylic polymer. *European Journal of Pharmaceutics and Biopharmaceutics*, 68(2), 310-318.
- Gupta, R., Prajapati, S. K., Pattnaik, S., Bhardwaj, P. (2014). Formulation and evaluation of novel stomach specific floating microspheres bearing famotidine for treatment of gastric ulcer and their radiographic study. *Asian Pacific Journal of Tropical Biomedicine*, 4(9), 729-735.
- Hafeez, A., Maurya, A., Singh, J., Mittal, A., Rana, L. (2013). An overview on floating microsphere: Gastro Retention Floating drug delivery system (FDDS). *The Journal of Phytopharmacology*, 2(3), 1-12.
- Harrigan, R. M. (1977). Drug delivery device for preventing contact of undissolved drug with the stomach lining: Google Patents.
- Horton, R., Ross, F., Darling, G. (1965). Determination of the emptying-time of the stomach by use of enteric-coated barium granules. *British Medical Journal*, 1(5449), 1537.
- Ibrahim, H. K. (2009). A novel liquid effervescent floating delivery system for sustained drug delivery. *Drug Discoveries & Therapeutics*, 3(4).
- Ichikawa, M., Watanabe, S., Miyake, Y. (1991). A new multiple-unit oral floating dosage system. I: Preparation and in vitro evaluation of floating and sustained-release characteristics. *Journal of Pharmaceutical Sciences*, 80(11), 1062-1066.
- Jackson, S., Leahy, F., McGowan, A., Bluck, L., Coward, W., Jebb, S. (2004). Delayed gastric emptying in the obese: an assessment using the non-invasive ¹³C-octanoic acid breath test. *Diabetes, Obesity and Metabolism*, 6(4), 264-270.
- Jawale, S., Bairagi, A., Jaybhai, S., Deshmukh, V. (2010). A Review of Floating Drug Delivery System.
- Joseph, N., Lakshmi, S., Jayakrishnan, A. (2002). A floating-type oral dosage form for piroxicam based on hollow polycarbonate microspheres: in vitro and in vivo evaluation in rabbits. *Journal of Controlled Release*, 79(1-3), 71-79.

- Kaushik, K., Chaurasia, D., Chaurasia, H., Mishra, S. K., Bhardwaj, P. (2011). Development and characterization of floating alginate beads for gastroretentive drug delivery system. *ACTA Pharmaceutica Scientia*, 53(4), 551-562.
- Kawashima, Y., Niwa, T., Takeuchi, H., Hino, T., Itoh, Y. (1992). Hollow microspheres for use as a floating controlled drug delivery system in the stomach. *Journal of Pharmaceutical Sciences*, 81(2), 135-140.
- Kerdsakundee, N., Mahattanadul, S., Wiwattanapatapee, R. (2015). Development and evaluation of gastroretentive raft forming systems incorporating curcumin-Eudragit® EPO solid dispersions for gastric ulcer treatment. *European Journal of Pharmaceutics and Biopharmaceutics*, 94, 513-520.
- Klausner, E. A., Eyal, S., Lavy, E., Friedman, M., Hoffman, A. (2003). Novel levodopa gastroretentive dosage form: in-vivo evaluation in dogs. *Journal of Controlled Release*, 88(1), 117-126.
- Kouchak, M., & Atyabi, F. (2004). Ion-exchange, an approach to prepare an oral floating drug delivery system for diclofenac. *Iranian Journal of Pharmaceutical Research*, 2, 93-97
- Kouchak, M., & Badrian, A. (2007). Preparation and in vitro evaluation of a microballoon delivery system for theophylline. *Iranian Journal of Pharmaceutical Research*, 6 (1), 35-42
- Kouchak, M., & Moghimipour, E. (2007). Preparation and evaluation of controlled release floating microspheres of diclofenac sodium.
- Li, S., Lin, S., Daggy, B. P., Mirchandani, H. L., Chien, Y. W. (2002). Effect of formulation variables on the floating properties of gastric floating drug delivery system. *Drug Development and Industrial Pharmacy*, 28(7), 783-793.
- Li, S., Lin, S., Daggy, B. P., Mirchandani, H. L., Chien, Y. W. (2003). Effect of HPMC and Carbopol on the release and floating properties of Gastric Floating Drug Delivery System using factorial design. *International Journal of Pharmaceutics*, 253(1-2), 13-22.
- Mahajan, R., Gupta, V., Sharma, J. (2010). Comparison and suitability of gel matrix for entrapping higher content of enzymes for commercial applications. *Indian Journal of Pharmaceutical Sciences*, 72(2), 223.
- Makwana, A., Sameja, K., Parekh, H., Pandya, Y. (2012). Advancements in controlled release gastroretentive drug delivery system: A review. *Journal of Drug Delivery and Therapeutics*, 2(3).
- Malleswari K, D. R. R. B., Himabindu K. (2016). Preparation and in vitro evaluation of stavudine floating sodium alginate beads. *World Journal of Pharmacy and Pharmaceutical Sciences*, 5(7), 823-834.
- Michaels, A. (1974). Drug delivery device with self actuated mechanism for retaining device in selected area: Google Patents.
- Michaels, A. S., Bashwa, J. D., Zaffaroni, A. (1975). Integrated device for administering beneficial drug at programmed rate: Google Patents.
- Mishra, S., & Pathak, K. (2008). Formulation and evaluation of oil entrapped gastroretentive floating gel beads of loratadine. *Acta Pharmaceutica*, 58(2), 187-197.
- Narang, N. (2011). An updated review on: floating drug delivery system (FDDS). *International Journal of Applied Pharmaceutics*, 3(1), 1-7.

- Nayak, A., Malakar, J., Kumar Sen, K. (2010). *Gastroretentive drug delivery technologies: Current Approaches and Future Potential* (Vol. 1).
- Nayak, A. K., Das, B., Maji, R. (2013). Gastroretentive hydrodynamically balanced systems of ofloxacin: In vitro evaluation. *Saudi Pharmaceutical Journal*, 21(1), 113-117.
- Nayak, A. K., Malakar, J., Sen, K. K. (2010). Gastroretentive drug delivery technologies: Current approaches and future potential. *Journal of Pharmaceutical Education and Research*, 1(2), 1.
- Perri, F., Pastore, M., Annesse, V. (2005). 13C-octanoic acid breath test for measuring gastric emptying of solids. *Eur Rev Med Pharmacol Sci*, 9(5 Suppl 1), 3-8.
- Prajapati, V. D., Jani, G. K., Khutliwala, T. A., Zala, B. S. (2013). Raft forming system—an upcoming approach of gastroretentive drug delivery system. *Journal of Controlled Release*, 168(2), 151-165.
- Rajinikanth, P., Balasubramaniam, J., Mishra, B. (2007). Development and evaluation of a novel floating in situ gelling system of amoxicillin for eradication of *Helicobacter pylori*. *International Journal of Pharmaceutics*, 335(1-2), 114-122.
- Rajinikanth, P., & Mishra, B. (2008). Floating in situ gelling system for stomach site-specific delivery of clarithromycin to eradicate *H. pylori*. *Journal of Controlled Release*, 125(1), 33-41.
- Rastogi, V. (2016). Mathematical Optimization and Investigation on Polymeric Blend of Chitosan and Hydroxy Propyl Methyl Cellulose K4M for Sustained Release of Metronidazole. *Asian Journal of Pharmaceutics (AJP): Free Full Text Articles From Asian J Pharm*, 10(2).
- Rastogi, V., Kumar, A., Yadav, P., Hegde, R., Rastogi, P. (2016). *Mathematical Optimization and Investigation on Polymeric Blend of Chitosan and Hydroxy Propyl Methyl Cellulose K4M for Sustained Release of Metronidazole* (Vol. 9).
- Rathee P, J. M., Garg A, Nanda A, Hooda A. (2011). Gastrointestinal mucoadhesive drug delivery system: A review. *Journal of Pharmacy Research*, 4(5), 1448-1453.
- Razavi, M., Karimian, H., Yeong, C. H., Chung, L. Y., Nyamathulla, S., Noordin, M. I. (2015). Gamma scintigraphic evaluation of floating gastroretentive tablets of metformin HCl using a combination of three natural polymers in rabbits. *Drug Design, Development and Therapy*, 9, 4373.
- Rishikesh Gupta, S. K. P., Snigdha Pattnaik, Peeyush Bhardwaj. (2014). Formulation and evaluation of novel stomach specific floating microspheres bearing famotidine for treatment of gastric ulcer and their radiographic study. *Asian Pacific Journal of Tropical Biomedicine*. 4 (9), 729-735.
- Sato, Y., Kawashima, Y., Takeuchi, H., Yamamoto, H. (2004a). In vitro and in vivo evaluation of riboflavin-containing microballoons for a floating controlled drug delivery system in healthy humans. *International Journal of Pharmaceutics*, 275(1-2), 97-107.
- Sato, Y., Kawashima, Y., Takeuchi, H., Yamamoto, H. (2004b). In vitro evaluation of floating and drug releasing behaviors of hollow microspheres (microballoons) prepared by the emulsion solvent diffusion method. *European Journal of Pharmaceutics and Biopharmaceutics*, 57(2), 235-243.
- Sato, Y., Kawashima, Y., Takeuchi, H., Yamamoto, H., Fujibayashi, Y. (2004). Pharmacoscintigraphic evaluation of riboflavin-containing microballoons for a floating controlled drug delivery system in healthy humans. *Journal of Controlled Release*, 98(1), 75-85.
- Shalaby, W. S., Blevins, W. E., Park, K. (1992). Use of ultrasound imaging and fluoroscopic imaging to study gastric retention of enzyme-digestible hydrogels. *Biomaterials*, 13(5), 289-296.

- Sharma, N., Agarwal, D., Gupta, M., Khinchi, M. (2011). A comprehensive review on floating drug delivery system. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 2(2), 428-441.
- Sheth, P. R., & Tossounian, J. L. (1979). Novel sustained release tablet formulations: Google Patents.
- Shivakumar, H., Gowda, D. V., Kumar, T. (2004). Floating controlled drug delivery systems for prolonged gastric residence: a review. *Ind. J. Pharm*, 38(45), 172-178.
- Singh, B. N., & Kim, K. H. (2000). Floating drug delivery systems: an approach to oral controlled drug delivery via gastric retention. *Journal of Controlled Release*, 63(3), 235-259.
- Sonar, G. S., Jain, D., More, D. (2007). Preparation and in vitro evaluation of bilayer and floating-bioadhesive tablets of rosiglitazone maleate. *Asian J Pharm Sci*, 2(4), 161-169.
- Soni, R. P., Patel, A. V., Patel, R. B., Patel, M., Patel, K., Patel, N. (2011). Gastroretentive drug delivery systems: a review. *International Journal of Pharma World Research*, 2(1), 1-22.
- Steingoetter, A., Weishaupt, D., Kunz, P., Mäder, K., Lengsfeld, H., Thumshirn, M., ... Schwizer, W. (2003). Magnetic resonance imaging for the in vivo evaluation of gastric-retentive tablets. *Pharmaceutical Research*, 20(12), 2001-2007.
- Streubel, A., Siepmann, J., Bodmeier, R. (2002). Floating microparticles based on low density foam powder. *International Journal of Pharmaceutics*, 241(2), 279-292.
- Streubel, A., Siepmann, J., Bodmeier, R. (2003). Floating matrix tablets based on low density foam powder: effects of formulation and processing parameters on drug release. *European Journal of Pharmaceutical Sciences*, 18(1), 37-45.
- Tadros, M. I. (2010). Controlled-release effervescent floating matrix tablets of ciprofloxacin hydrochloride: Development, optimization and in vitro-in vivo evaluation in healthy human volunteers. *European Journal of Pharmaceutics and Biopharmaceutics*, 74(2), 332-339.
- Talukder, R., & Fassihi, R. (2004). Gastroretentive delivery systems: hollow beads. *Drug Development and Industrial Pharmacy*, 30(4), 405-412.
- Tanwar, Y. S., Naruka, P. S., Ojha, G. R. (2007). Development and evaluation of floating microspheres of verapamil hydrochloride. *Revista Brasileira de Ciências Farmacêuticas*, 43(4), 529-534.
- Thanoo, B., Sunny, M., Jayakrishnan, A. (1993). Oral Sustained-release Drug Delivery Systems using Polycarbonate Microspheres Capable of Floating on the Gastric Fluid. *Journal of Pharmacy and Pharmacology*, 45(1), 21-24.
- Tort, S., Han, D., Steckl, A. J. (2020). Self-inflating floating nanofiber membranes for controlled drug delivery. *International Journal of Pharmaceutics*, 579, 119164.
- Vinod, K., Vasa, S., Anbuazaghan, S., Banji, D., Padmasri, A., Sandhya, S. (2010). Approaches for gastroretentive drug delivery systems.
- Whitehead, L., Collett, J. H., Fell, J. T. (2000). Amoxicillin release from a floating dosage form based on alginates. *International Journal of Pharmaceutics*, 210(1-2), 45-49.
- Zhang, L.-P., Tan, X.-X., Huang, Y.-P., Liu, Z.-S. (2018). Floating liquid crystalline molecularly imprinted polymer coated carbon nanotubes for levofloxacin delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, 127, 150-158.

Zhang, L.-P., Wang, X.-L., Pang, Q.-Q., Huang, Y.-P., Tang, L., Chen, M., Liu, Z.-S. (2017). Solvent-responsive floating liquid crystalline-molecularly imprinted polymers for gastroretentive controlled drug release system. *International Journal of Pharmaceutics*, 532(1), 365-373.