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PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/);

STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/);

STROBE statement, a checklist of items that should be included in reports of observational studies (http://www.strobe-statement.org/);

MOOSE guidelines for meta-analysis and systemic reviews of observational studies (Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis of observational Studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008-12).

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Keywords: A list of minimum 3, but no more than 5 key words must follow the abstract. Key words in English should be consistent with "Medical Subject Headings (MESH)" (www.nlm.nih.gov/mesh/MBrowser.html). Turkish key words should be direct translations of the terms in MESH.

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Results: The results of the study should be stated, with tables/figures given in numerical order; the results should be evaluated according to the statistical analysis methods applied. See General Guidelines for details about the preparation of visual material.

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Acknowledgements: Any technical or financial support or editorial contributions (statistical analysis, English/Turkish evaluation) towards the study should appear at the end of the article.

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Review articles can address any aspect of clinical or laboratory pharmaceuticals. Review articles must provide critical analyses of contemporary evidence and provide directions of or future research. Most review articles are commissioned, but other review submissions are also welcome. Before sending a review, discussion with the editor is recommended.

Reviews articles analyze topics in depth, independently and objectively. The first chapter should include the title in Turkish and English, an unstructured summary and key words. Source of all citations should be indicated. The entire text should not exceed 25 pages (A4, formatted as specified above).

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LETTER TO EDITOR

COVID-19 Pandemic and its Impact on Pharmacy Education

COVID-19 Pandemisi ve Eczacılık Eğitimine Etkisi

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Key words: Pharmacy, COVID-19, education

Anahtar kelimeler: Eczane, COVID-19, eğitim

Dear Editor,

The sudden occurrence of Coronavirus Disease-2019 (COVID-19) reached a pandemic state soon after its global emergence in the early spring of the year 2020. It was immediately followed by a worldwide shutdown and the practice of social distancing. In this unprecedented situation, encompassing day-to-day work and employment, all sorts of activities faced interruption in the worst possible way, like never before. Lockdown turned into a significant and unforeseen challenge of the year 2020 for all-particularly the management and administration of workplaces and educational institutes. To ensure means of earning degrees and evade their delay, immediate policies and strategies were implemented. It was mandatory following the government announcement that education must be continued without interruption. Under such circumstances, digital learning emerged and was adopted by all institutes worldwide to ensure continued instruction. This brought forth a sudden shift from traditional on-campus classes to e-classes. Students were kept busy, and any delay was avoided; on the other hand, a panic was initiated among teachers, students, and their families. However, within a short time, teachers across the globe were delivering lectures virtually while students were participating online.[1]

In addition to this, pre-scheduled scientific meetings, workshops, and educational conferences were delayed or canceled, considering universities and private halls' shutdown. The gathering was also devalued, with people allowed to interact only at a safe distance of 6 feet, hindering the exchange of knowledge and information. Keeping the severity of the situation in mind, some essential shifts were the need of the hour and the way forward to combat interruptions. Many organizers successfully switched to virtual events, managed to adhere to the original event schedule, transmitting due information quickly without any added delay, and welcomed a larger community. However, the other side of the picture hides some limitations besides the aforementioned tools and technologies, lending help in the crisis. Besides the support rendered, unobstructed network access, online interruptions, and voice modulations remained some of the technical flaws, other than the absence of emotional connection and real-time simulation still being in question.[2]

It is important to note that our current concerns and observations come from online sessions conducted with the Doctor of Pharmacy (Pharm-D) program, a professional degree instituted on a multidisciplinary curriculum. This course of study entails a series of theoretical sessions, combined with practical demonstrations and exercises. The respective regulatory council binds pharmacy aspirants to reach further afield, such as hospitals, clinics, and industries, and gain real-time experience to strengthen their understanding and bring their skills into practice. In compliance with the same, pharmacy

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students undertake regular laboratory work, internships, and graded field visits, back-to-back during the degree's five-year span.[3]

Besides the scientific and clinical core of the profession, hands-on clinical experience and onsite experimental learning in clinical settings were impeded. Thus, the very first challenge dealt with the suspension of on-campus classes was the adjustment of curricula according to the current needs and choices available. Various platforms were brought into use, such as the Learning Management System, Google Meet, Zoom, etc., as conventional platforms such as Skype and Viber failed to cope with the current crisis. Many senior teachers and non-technical people in pharmacy viewed this as a challenge yet successfully adapted to the changes. The situation was no different for students; while many were quick to learn, those in remote and rural areas were stuck with inadequate computer and internet facilities. Hence, a solution was provided via recorded lectures followed by open-book tests and quizzes to establish sound feedback system.[4]

The pandemic effect on students in health fields such as Pharm-D was manifested in grave consequences comprising the loss of clinical rotations, laboratory demonstrations, industrial internships, and other clerkships. Keeping this in mind, it was essential to provide a suitable alternative and cater to this professional degree's practical needs, as the role of pharmacists in community and hospital settings is critical in any healthcare system. In response to this, e-learning strategies covered the practical needs of the scientific curriculum well, and simulations of laboratory settings were prepared thoroughly and taken up at whatever level possible to confirm the delivery of practical skills. Although the closure of institutes did not impede lecture delivery, and examinations were still administered in earnest. However, upon completion of pharmacy curriculum uniformity in understanding, failed across boards. Many competitive examinations, along with board examinations, were canceled on the national and international levels, and scholarships were withheld or abandoned altogether. Having mentioned the issues and proposed solutions, the professionals graduating this year shall face consequences of the COVID-19 pandemic. Besides the educational interruptions they have faced, they shall be interviewed on strict grounds to assess their knowledge only if they have bypassed the residual effects of the pandemic, such as a major global recession.[5,6]

To conclude, COVID-19, which has acutely disrupted the entire learning process, has also raised concerns regarding pharmacy education and other fields in general. Students from different field have different needs, yet for pharmacy students, practical knowledge is as essential as theory. Despite all efforts made, a significant gap remains, demanding some initiative or policy to provide pharmacy students with hands-on clinical training, industrial exposure, hospital rounds, and retail residencies to stir the required level of confidence before entering professional life.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Effect of Medium pH and Light on Quinidine Production in *Cinchona calisaya* Wedd. Endophytic Fungi

Cinchona calisaya Wedd. Endofit Mantarında Kinidin Üretiminde Ortamın pH'sının ve Işığın Etkisi

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ABSTRACT

Objectives: Quinidine has pharmaceutical importance as an antimalarial, antiarrhythmia, antimicrobial, anticancer, antioxidant, astringent, and bitter flavoring agent. Quinidine is in high demand, yet its production from the bark of the quina tree (*Cinchona calisaya*) is limited. Quinidine production from quina tree fungal endophytes, namely *Aspergillus sydowii*, *Diaporthe* sp., *Diaporthe lithicola, Fusarium oxysporum,* and *F. solani* is lower than the quinidine content of the tree bark. This study attempted to increase quinidine production from these fungi. This research aimed to determine the optimum culture conditions for quinidine production from endophytic fungi.

Materials and Methods: Quinidine was produced by *in vitro* culturing of the fungal endophytes in potato dextrose broth (PDB) medium under different culture conditions, i.e., a combination of an initial medium pH of 6.2 or 6.8, with or without light, in a static condition for 21 days of incubation at room temperature. Production under natural daylight in PDB medium without pH modification was used as the control. At the end of the incubation period, the mycelial mass was separated from the filtrate. The dried biomass and chloroform-extracted filtrate were weighed. Quinidine in the extract was analyzed qualitatively and quantitatively using high-performance liquid chromatograph.

Results: Quinidine production was affected by both light and the initial pH of the medium, depending on the fungal strain used. A significant increment in quinidine production, approximately 1.1-9.3-fold relative to its respective control was obtained from all fungi under their optimum conditions. Quinidine production in most of the fungi was significantly correlated with their biomass production but not with their extract production. Of those five fungi, *F. solani* that was cultured in PDB medium with an initial pH of 6.2 and incubated under continuous light produced the highest concentration of quinidine with low biomass.

Conclusion: The quinidine production of all fungal endophytes studied was affected by the culture conditions. *F. solani* is the most promising fungus for use as a quinidine production agent.

Key words: Fungal endophytes, *in vitro* culture, optimization, light, pH, quinidine

ÖZ

Amaç: Kinidin, antimalaryal, antiaritmik, antimikrobiyal, antikanser, antioksidan, astrinjen ve acı tatlandırıcı olarak kullanıldığı için farmasötik öneme sahiptir. Kinidin yoğun talep görmesine rağmen, kinidin kaynağı olan quina ağacı (*Cinchona calisaya*) kabuğundan üretimi sınırlıdır. Quina ağacı fungal endofitleri olan *Aspergillus sydowii, Diaporthe* sp., *Diaporthe lithicola, Fusarium oxysporum* ve *F. solani*'den kinidin üretimi, ağaç kabuğundaki kinidin içeriğinden daha düşüktür. Bu çalışmada endofitik mantarların kinidin üretiminin belirlenmesi optimum kültür koşullarının belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: Kinidin farklı kültür koşullarında patates dekstroz et suyu (PDB) ortamında fungal endofitlerin *in vitr*o kültürlenmesi yoluyla üretilmiş, başlangıç ortamı olarak pH 6,2 veya 6,8'in kombinasyonları ışıklı/ışıksız olarak kullanılarak statik koşullarda oda sıcaklığında 21 gün süreyle inkübasyona tabi tutularak üretilmiştir. pH'yi değiştirmeden PDB ortamında doğal gün ışığı altında üretim kontrol olarak kullanılmıştır. İnkübasyon süresinin sonunda, misel kütlesi filtrattan ayrılmıştır. Kurutulmuş biyokütle ve kloroform ile ekstre edilmiş filtrat tartılmıştır. Ekstredeki kinidin, kalitatif ve kantitif olarak yüksek basınçlı sıvı kromatografisi kullanılarak analiz edilmiştir.

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Bulgular: Kinidin üretimi, mantar suşuna bağlı olarak ortamın hem ışık hem de ilk pH'sinden etkilenmiştir. Optimum koşullar altında ilgili kontrolüne göre tüm mantarların kinidin üretiminde yaklaşık 1,1-9,3 kat belirgin bir artış elde edilmiştir. Mantarların çoğunda kinidin üretimi, ekstrakt üretimi ile değil, biyokütle üretimi ile anlamlı ölçüde korele bulunmuştur. Bu beş mantardan, ilk pH'si 6,2 olarak ve sürekli ışık inkübasyonu altında PDB ortamında kültürlenen *F. solani*, düşük biyokütle ile en yüksek kinidin konsantrasyonunu üretmiştir.

Sonuç: Çalışılan tüm mantar endofitlerinin kinidin üretimi, kültür koşullarından etkilenmiştir. *F. solani*, kinidin üretim ajanı olarak kullanılacak en umut veren mantardır.

Anahtar kelimeler: Fungal endofitler, *in vitro* kültür, optimizasyon, ışık, pH, kinidin

INTRODUCTION

Quinoline is a major secondary metabolite produced in the bark and root of the quina tree (*Cinchona*, Rubiaceae). There are four members of quinoline, i.e., quinine, quinidine, cinchonine, and cinchonidine. These substances are commonly used as antimalarial drugs.[1] Malaria is a very dangerous disease and a leading cause of death worldwide. Malaria is caused by four species of *Plasmodium.* The most serious form of malaria is caused by *Plasmodium falciparum* and *P. vivax.* The goal of the World Health Organization in 2016-2030 is to either reduce the incidence of malaria by at least 90% or eradicate the disease in all countries of the world.[2]

Quinidine is actually three-fold more effective at treating malaria caused by *P. falciparum* than the other compounds mentioned. Further, it has the widest range of uses out of all members of the quinoline family. In addition to its application as an antimalarial drug, quinidine is also useful as an antimicrobial,[3] anticancer,[4] antioxidant,[5] astringent, bitter flavoring,[6] and antiarrhythmia agent.[7] As an antimalarial drug, quindine binds to free heme to prevent hemozoin formation. The production of hemazoin is a heme detoxification system in the malaria parasite.[8] Free heme is very toxic to the parasite, as it can induce oxidative stress, which leads to the parasite's death. As an antibacterial, quinidine inhibits the internalization or invasion of bacteria.[9] As an anticancer agent, quinidine can arrest MCF-7 human breast cancer cells in the G1 phase of the cell cycle and lead to apoptotic cell death.[10] As an antiarrhythmic drug, quinidine is classified as class Ia sodium channel blocker.[11] Quinidine is very effective at treating atrial fibrillation and short QT syndrome. It can also be used as an anticonvulsant, an anticholinergic drug, and an antagonist of α-adrenergic and muscarinic drugs.[12] Therefore, increased quinidine production is needed to fill the growing pharmaceutical demand.

Currently, quinidine availability is solely dependent on the bark and root of the quina tree. Indonesia has shown a drastic decline in the quina tree population and a consequent decline in quinidine production. Efforts have been made to increase the plant population and quinidine production, i.e., quina tree replanting, tree selection through hybridization and clonal propagation[7,13] and quinidine production through cell culture.[14] However, the quinidine supply is still limited. Therefore, it is necessary to explore other natural resources for quinidine production.

Endophytic fungi can produce secondary metabolites similar to those of their host plant.[15] Endophytic fungi isolated from the *Cinchona* tree are potential sources of quinidine production.[16-21] Some of *Cinchona calisaya* Wedd. endophytic fungi, i.e., *Aspergillus*, *Diaporthe*, and *Fusarium* species were reported to produce quinidine as well as other quinolines.[21] *Diaporthe* sp. CLF-J, a fungal endophyte of *C. ledgeriana* (Howard) Bern. Moens ex Trimen, was reported to produce low levels of quinidine, i.e., <10 µg/L.[18] Therefore, quinidine production should be determined and improved simultaneously by optimizing fungal culture conditions.

The factors affecting secondary metabolite production in fungi are the initial pH of the culture medium[18,22,23] and light intensity.[24-26] The effect of these factors varies from induction to inhibition of secondary metabolite production, depending on the fungal strain used. Presumably, these abiotic factors may induce quinidine production in some *Cinchona* endophytic fungi but not in others. Therefore, the effects of medium pH and light intensity on quinidine production and biomass in five different strains of *C. calisaya* endophytic fungi in submerged fermentation were investigated.

MATERIALS AND METHODS

Fungal culture preparation

The fungal strains used in this study (*Aspergillus sydowii* Institut Pertanian Bogor Culture Collection (IPBCC) 15.1258, *Diaporthe* sp. IPBCC 15.1292, *D. lithicola* IPBCC 15. 1314, *Fusarium oxysporum* IPBCC 15.1250, and *F. solani* IPBCC 15.1247) were isolated in 2012 from healthy leaf, fruit, twig, bark, and petiole of *C. calisaya,* respectively. The trees were grown in the Tea and Quina Research Centre Orchard in Gambung, West Java, Indonesia.[21] The fungal cultures were maintained at -80°C in glycerol trehalose and maintained in the Bogor Agricultural University Culture Collection Indonesia for 3 years. The stock cultures were rejuvenated on potato dextrose agar (PDA) and incubated at 25°C. A 7 day-old culture was used as a working culture (Figure 1).

Optimization of culture conditions for quinidine production

Each fungus was cultivated individually in liquid fermentation medium. Three pieces of mycelial plugs (0.8 cm dia) were taken from the edge of the PDA culture of each fungal colony then transferred into 200 mL potato dextrose broth (PDB), in 250 mL Erlenmeyer flasks. The pH of the PDB was adjusted to either 6.2 or 6.8 prior to sterilization by adding phosphate buffer. The culture conditions for quinidine production by these fungi were the combination of the initial pH of the medium (6.2 and 6.8) either with continuous white light (about 1700 lux) or without light (dark). Production under natural light (daylight) in PDB medium without pH modification (5.1±0.2) was used as a

control. Three replicates were performed for each treatment. The cultures were then incubated for 21 days under static conditions. At the end of the incubation period, quinidine was extracted, weighed, and analyzed. In addition, the final pH value of the medium was measured, and the dry biomass and dry extract were weighed.

Quinidine extraction and analysis

The mycelial mass was separated from the filtrate using filter paper and dried in an oven at 60°C for 24 hours or until a constant dry mass was reached. The biomass dry weight represented biomass production. The filtrate was extracted by adding chloroform ($CHCl_3$, ≥99.8%) at a 1:1 ratio. The mixture was homogenized and allowed to stand for 24 hours. The mixture was transferred to a separatory funnel and allowed to stand for a few seconds to form two layers. Afterward, the bottom layer was removed and evaporated using a rotary evaporator at 45°C and 60 rpm until a pellet was formed.[21] The pellet was weighed and designated as the extract. Finally, the extract was stored at 4°C as the stock material for quinidine analysis.

Quinidine was analyzed using a Shimadzu Prominence 20AD high-performance liquid chromatograph (HPLC). The extract was first dissolved in 1 mL chloroform. About 20 µL of that solution was injected into the HPLC. The analytical conditions was set as follows: Thermo C-18 column (4.6x250 mm), methanol:acetonitrile (80:20) as the mobile phase, 1.0 mL sec^{-1} flow rate, 40°C column temperature, and a detection wavelength of 210 nm.[27] The presence of quinidine was determined on the basis of the peak that appeared at the same retention time (Rt) as the quinidine standard. The concentration of quinidine was determined using a formula representing the correlation between 2, 4, 8, 10, and 12 ppm standard quinidine with their area under the corresponding peak. The formula is $y=mx + b$, where y is the area under the standard quinidine peak, m is the regression coefficient, x is the quinidine concentration, and b is a constant.

Experimental design

A factorial design with a combination of two factors, i.e., light exposure (with or without light) and the initial pH of the medium (pH 6.2 and 6.8) was used in this research. The production condition in PDB medium without pH modification incubated at room temperature was used as a control. The treatments were tested on five *C. calisaya* endophytic fungi, i.e., *A. sydowii*, *Diaporthe* sp., *D. lithicola*, *F. oxysporum*, and *F. solani*. Each treatment was applied in triplicate to produce a total of 75 experimental units.

Statistical analysis

Data were analyzed statistically using analysis of variance followed by Duncan's multiple range test (DMRT), both at the 5% level of significance. Correlation of quinidine production with biomass dry weight and with extract weight were calculated using Pearson's correlation coefficient (r_p). The correlation was categorized into five groups, i.e., weakest (0.00-0.19), weak (0.20-0.39), moderate (0.40-0.69), strong (0.70-0.90), and strongest (0.90-1.00).[28]

RESULTS

Quinidine production

The HPLC chromatogram of all fungal extracts showed two peaks, indicating the presence of quinidine and an unknown substance (Figure 2A-E). Quinidine was detected to have an Rt similar to that of the quinidine standard (Rt 9.9412±1.9 minutes, Figure 2F), and the unknown compound had a peak at ± Rt 5 minutes (Figure 2A-E). The quinidine standard concentration was highly correlated (R^2: 1) with its area under the respective peak; therefore, the regression equation y=123.915x+1442.8 was further used to calculate the quinidine concentration in the extract.

The amount of quinidine varied depending on the fungal strain used and the incubation conditions. *A. sydowii*, *F. oxysporum*, and *F. solani* produced the maximum concentration of quinidine under continuous light incubation with an initial medium pH of 6.2. In contrast, the maximum quinidine production of *Diaporthe* sp. and *D. lithicola* were obtained under dark incubation with an initial medium pH of 6.8. Under optimum conditions, all fungi (*A. sydowii*, *Diaporthe* sp., *D. lithicola*, *F. oxysporum*, and *F. solani*) produced quinidine at their highest concentrations, i.e., 29.026 (Figure 3A), 8.913 (Figure 3B), 11.148 (Figure 3C), 23.967 (Figure 3D), and 65.177 µg (Figure 3E), respectively, in 200 mL PDB medium after 21 days of incubation.

The quinidine production of all fungi increased significantly ($p<0.05$) under optimum conditions. *A. sydowii* (Figure 3A), *Diaporthe* sp. (Figure 3B), *D. lithicola* (Figure 3C), *F. oxysporum* (Figure 3D), and *F. solani* (Figure 3E) increased

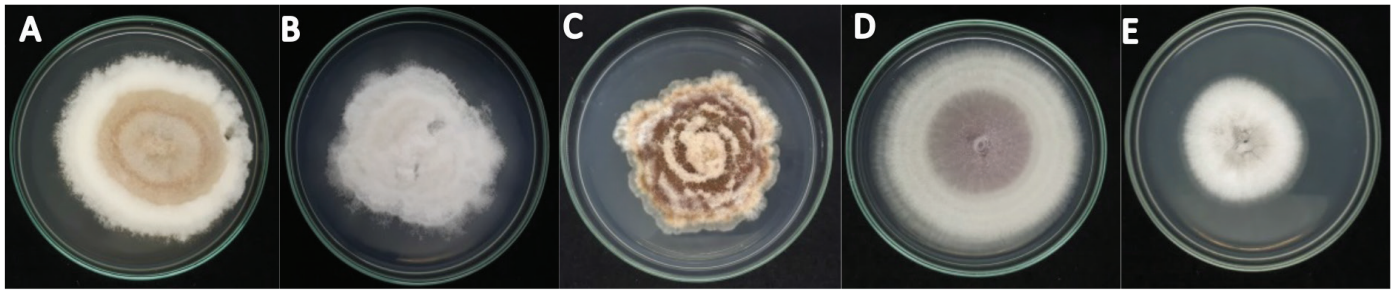

Figure 1. (A) *Aspergillus sydowii* IPBCC 15.1258, (B) *Diaporthe* sp. IPBCC 15.1292, (C) *Diaporthe lithicola* IPBCC 15. 1314, (D) *Fusarium oxysporum* IPBCC 15.1250, (E) *F. solani* IPBCC 15.1247

IPBCC: Institut Pertanian Bogor Culture Collection

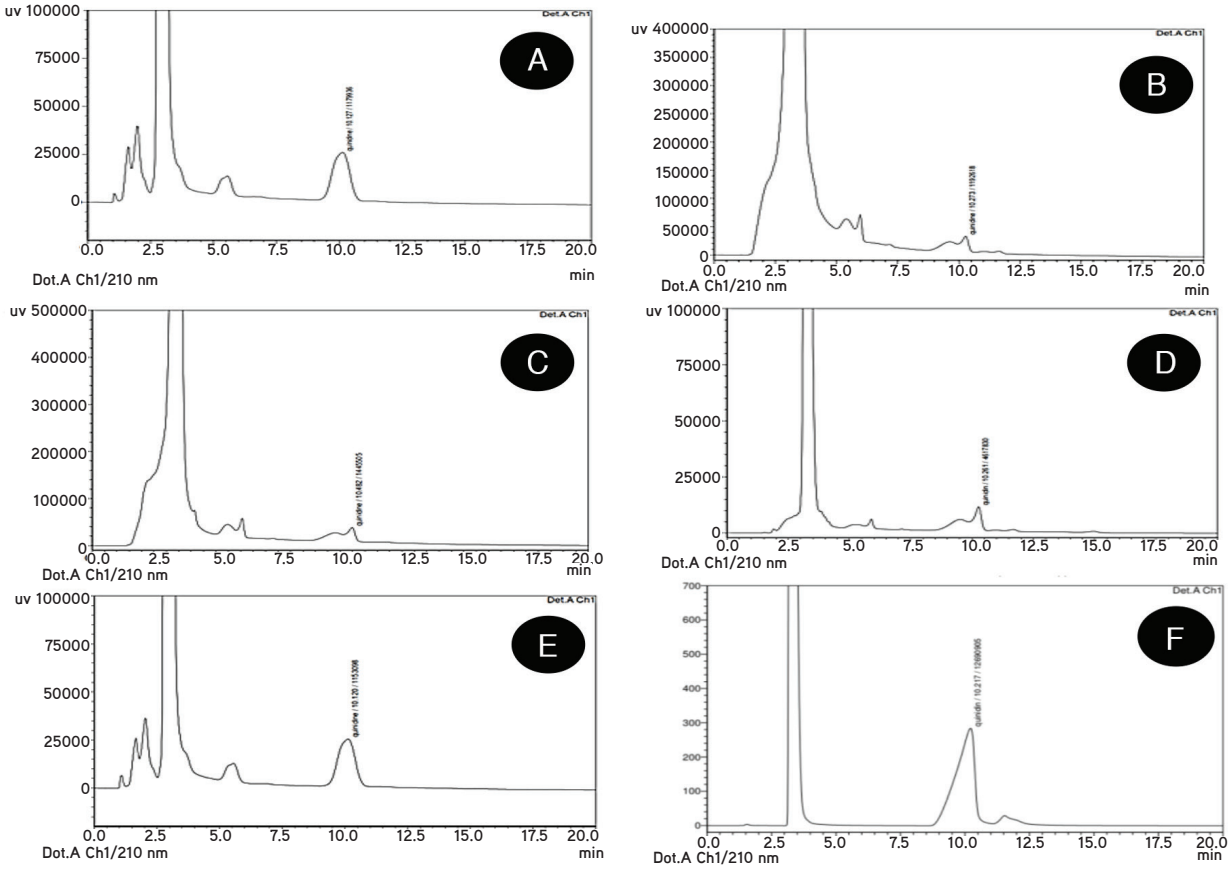

Figure 2. High-performace liquid chromatogram of (A) *Aspergillus sydowii*, (B) *Diaporthe* sp., (C) *Diaporthe lithicola*, (D) *Fusarium oxysporum*, (E) *F. solani,* and (F) quinidine standard

2.7-, 1.6-, 1.1-, 9.3-, and 6.0-fold, respectively, compared with their corresponding controls. The DMRT test ($p<0.05$) indicated that quinidine production in *F. solani* and *Diaporthe* sp. increased significantly under the combined conditions of light and initial medium pH, whereas the other strains were affected only by either light or the initial pH of the medium. Quinidine production in *F. solani* increased significantly with continuous light and an initial medium pH of 6.2, whereas that of *Diaporthe* sp. increased with dark incubation and an initial medium pH of 6.8. Unlike *F. solani* and *Diaporthe* sp., quinidine production in *F. oxysporum* increased under continuous light regardless of the pH, and that of *A. sydowii* and *D. lithicola* was only affected by the initial pH of the medium.

Biomass and extract production and the final pH of the culture medium

The optimum condition for biomass production of each fungus varied and was different from those for quinidine production except for that of *F. oxysporum*. This fungus produced both the maximum quinidine concentration and biomass dry weight (0.247 g) under continuous light and an initial medium pH of 6.2 (Figure 4A). Like *F. oxysporum*, *A. sydowii* grew better (0.387 g) under continuous light but at a different initial medium pH (pH 6.8) (Figure 4B). In contrast, that of *D. lithicola* (0.307 g) occurred under the dark condition with an initial medium pH of 6.2 (Figure 4C). The highest biomass production of all fungi occurred in modified-pH medium, and the highest biomass production of *Diaporthe* sp. (Figure 4D) and *F. solani* (Figure 4E) were obtained under the control conditions, i.e., 0.351 g and 0.470 g, respectively.

The optimum conditions for extract (pellet) production were also different from those for quinidine production, except in *Diaporthe* sp. and *D. lithicola.* Similar to their quinidine production, *Diaporthe* sp. (Figure 5A) and *D. lithicola* (Figure 5B) produced the highest extract dry weight, i.e., 0.765 g and 0.115 g, respectively, under darkness and at an initial medium pH of 6.8. Under these conditions, *F. solani* (Figure 5C) also produced the highest extract dry weight (0.102 g), whereas that of *A. sydowii* (Figure 5D) and *F. oxysporum* (Figure 5E) were obtained under control conditions, i.e., 0.164 g and 0.122 g, respectively. The extract production of each fungus did not reflect the quinidine production. Of the fungi studied, *Diaporthe* sp. produced the highest extract dry weight, but its quinidine production was the lowest (Table 1). Based on extract production, the quinidine content in the extract of *F. solani* was the highest (1551.83 µg/g extract), followed by that of *A. sydowii*, *F. oxysporum*, *D. lithicola*, and *Diaporthe* sp., i.e., 518.32, 230.45, 96.94, and 11.65 µg/g extract, respectively.

Correlation of quinidine with biomass and extract production

Quinidine production can be either negatively or positively correlated with the corresponding biomass production (Table 2). The correlation can also either be significant or insignificant ($p<0.1$). Of the fungi studied, only *F. solani* and *F. oxysporum* showed a significant correlation. In *F. solani,* quinidine production was strongly negatively (r_p: -0.862) correlated with biomass production. In contrast, there was a strong postive correlation between quinidine and biomass production in *F. oxysporum* (r_p: 0.835). In addition, *D. lithicola* showed a negative correlation (r_p: -0.595, moderate), albeit at a lower level of significance ($p<0.2$). In contrast, quinidine production in *A. sydowii* and *Diaporthe* sp. was not correlated with biomass production. The correlation coefficients (r_p) for the latter two fungi were 0.002 (weakest) and 0.199 (weak), respectively.

Quinidine and extract production also showed a similar pattern of correlation. However, most of the fungi (*A. sydowii*,

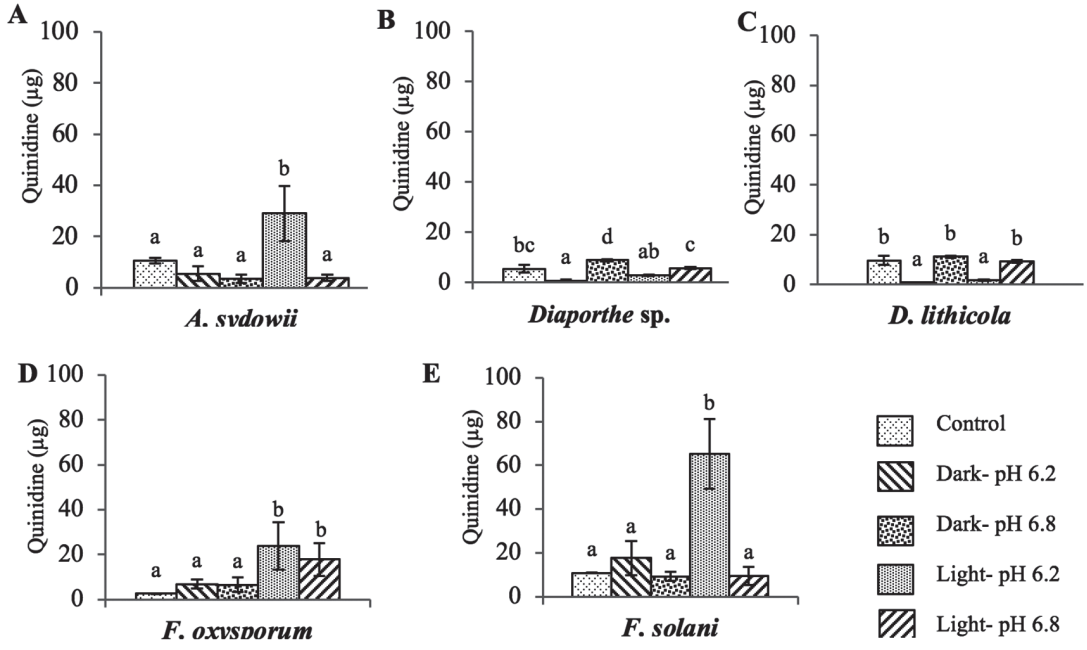

Figure 3. Quinidine production by *Cinchona calisaya* endophytic fungi in 200 mL potato dextrose broth. Bar data followed by the same letter not significantly different in Duncan's multiple range test at the 5% level of significance

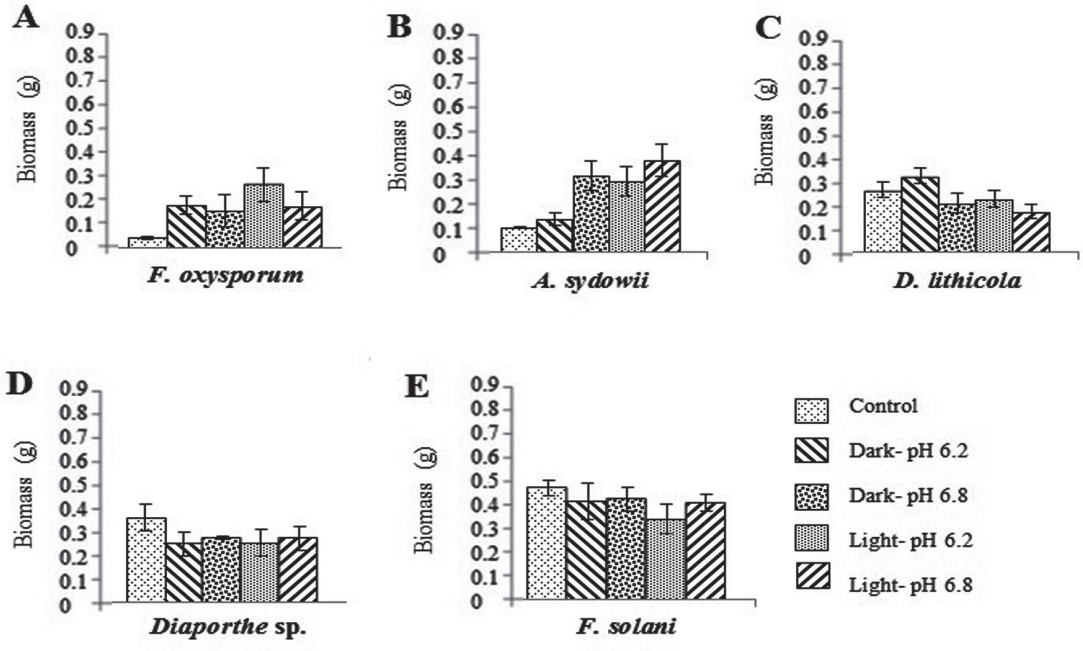

Figure 4. Biomass production by *Cinchona calisaya* endophytic fungi in four treatments in 200 mL potato dextrose broth. A vertical bar above the data block indicates the standard error

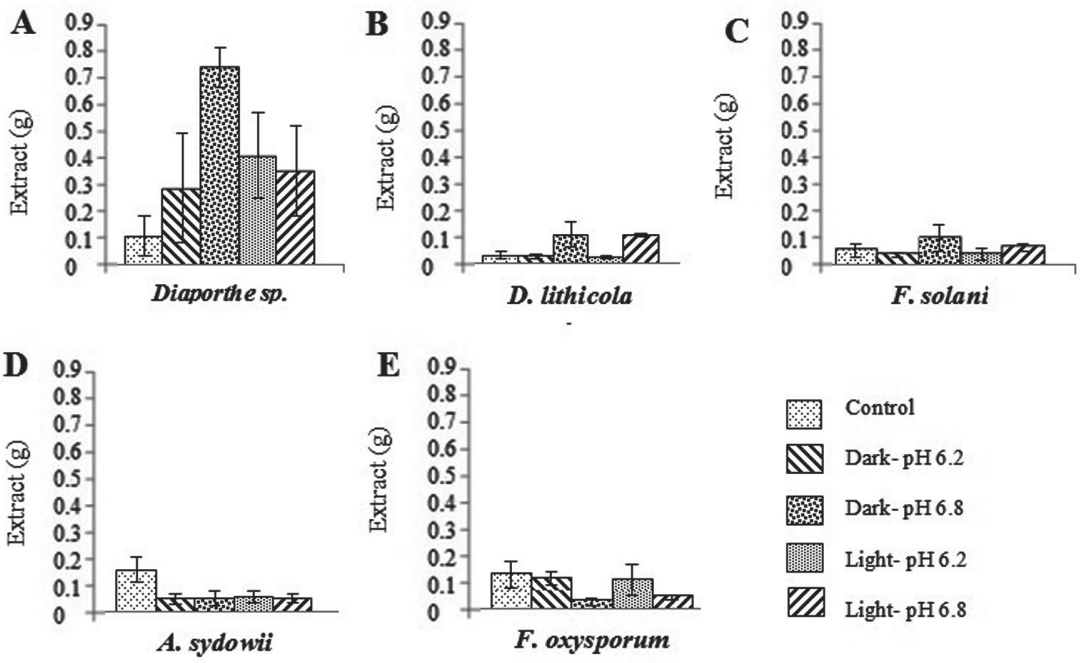

Figure 5. Extract production by *Cinchona calisaya* endophytic fungi in four treatments in 200 mL potato dextrose broth. Vertical line above the data block indicates the standard error

Table 1. Quinidine content in alkaloid extract

Cinchona calisaya endophytic fungi	Optimum condition	Quinidine (µg)*	Extract (g)*	Quinidine extract (µg/g)
Fusarium solani	Light-pH 6.2	65.177	0.042	1551.83
Fusarium oxysporum	Light-pH 6.2	23.967	0.104	230.45
Aspergillus sydowii	Light-pH 6.2	29.026	0.056	518.32
Diaporthe sp.	Dark-pH 6.8	8.913	0.764	11.65
Diaporthe lithicola	Dark-pH 6.8	11.148	0.114	96.94

*: 200 mL production

Table 2. Correlation of quinidine production with biomass production and with extract production based on the Pearson's correlation test

Cinchona calisaya endophytic fungi	Coefficient correlation of quinidine production with	
	Biomass (r_p)	Extract (r_p)
Fusarium solani	-0.862*	-0.493
Fusarium oxysporum	0.835*	-0.120
Aspergillus sydowii	0.002	0.030
Diaporthe sp.	0.199	0.559
Diaporthe lithicola	-0.595**	0.708**

r_p: Pearson's correlation, *: Significant at α 0.1, **: Significant at α 0.2

Diaporthe sp., *F. oxysporum*, and *F. solani*) showed insignificant correlations, except in the case of *D. lithicola*, where the correlation was strongly positive and significant (r_p: 708, p<0.2).

DISCUSSION

Only two of the fungal strains used in this study had ever been investigated for quinoline production.[21] In that previous study, *Diaporthe* sp. and *D. lithicola* were grown under similar conditions to this study, and those fungi were reported to produce quinine.[21] In contrast, quinine was not detected in the current study. Further, only quinidine was detected. All these quinolines (quinine, quinidine, cinchonine, and cinchonidine) are synthesized from the same structure; therefore, the possibility exists of structural changes among these compounds.[7,29,30] Quinidine ws the only quinoline compound produced in this study, as quinidine is probably structurally more stable than quinine.

The quinidine biosynthetic pathway in *C. calisaya* endophytic fungi has not yet been clarified. In *Cinchona,* the quinoline biosynthetic pathway is hypothetically synthesized from tryptophan and geraniol to form two intermediates, strictosidine and cinchonaminal, prior to the formation of cinchonidinone.[7] Cinchonidinone becomes a basal substance for the synthesis of the four quina alkaloids. C-8 epimerization of cinchonidinone via enol will form cinchoninone.[7] Both cinchonidinone and cinchoninone are catalyzed by nicotinamide adenine dinucleotide phosphate oxidoreductase to form cinchonidine and cinchonine, respectively. Moreover, cinchonidinone may form quininone by the hydroxylation and methylation, as do cinchoninone to form quinidinone. Quininone is reduced to quinine, while quinidinone is reduced to quinidine. Quinidine can also be synthesized

from quinine by oxidation, epimerization, and reduction steps. Cinchonidine and quinine can be differentiated by their R groups, a hydrogen in cinchonidine, and a methoxy in quinine. Quinine is diastereoisomer of quinidine, as are cinchonine and cinchonidine.

In this study, the medium became more acidic at the end of the incubation period. This means that the pH of the medium was not buffered. It is expected that a medium with an uncontrolled pH will create conditions conducive to greater quinidine production. With a dynamic pH level that decreases from pH 6.5 to pH 3.8 during incubation, swainsonina alkaloid production was induced to a higher level than at a constant pH (pH 6.5).[31] A preliminary study showed that no quinidine was detected in hyphal cells. The acidity of the medium might influence the permeability of the cell wall, thereby affecting nutrient uptake from the environment into cells.[32] and simultaneously allows the quinidine produced in the cell to be excreted into the medium. This simplifies the harvesting of quinidine. In addition, the pH may also affect the expression of genes associated with the synthesis of secondary metabolites and enzyme activity.[33]

Not only pH but also light exposure affects quinidine production. This is in accordance with studies on other secondary metabolites.[22,34-37] Secondary metabolite production of *Penicillium isariiforme* was inhibited by light,[34] in contrast to that of *A. flavus* and *A. ochraceus*, which were induced by light.[35] *F. verticilloides*, *Isaria farinosa*, and *Emericella nidulans* produced maximum secondary metabolites with dark incubation and an initial synthetic medium pH of 5.6.[36] *F. graminearum* produced a higher quantity of trichothecenes when it was grown under dark conditions rather than under light exposure.[38] *Fusarium* spp. from *Taxus wallichiana* bark produced maximum secondary metabolites under optimum conditions, which were medium pH 6 and daylight incubation,[22] whereas *A. terreus* from seaweed *Codium decorticatum* produced maximum antibacterial compounds at an initial medium pH of 5.5 and with daylight incubation.[23] In addition, blue light inhibited mycotoxin production in *A. flavus*, *A. parasiticus*, and *Alternaria alternata.*[39] Green light stimulated citrinin production by *Monascus ruber*.[37]

Light is a crucial signal for every living cell. It seems that not only secondary metabolite production but also regulation of gene transcription, enzyme activation, nutrient uptake, reproduction, morphogenesis, cell wall components, and metabolism of lipids, nucleic acids, and amino acids involves metabolic pathways.[25] Blue light stimulates sexual reproduction in *Phycomyces blakesleeanus*.[40] Mycelia of *Fujikuroi gibberella* are orange and produce maximum carotenoids when incubated under light conditions, yet the mycelia become white and produce low amounts of carotenoids when incubated under dark conditions.[41] Glucose absorption by *A. ornatus* decreased significantly when it was incubated under light conditions. In this case, light may stimulate biosynthesis of an inhibitor that blocks glucose absorption into fungal cells.[42]

In vitro growth of a microorganism is often correlated with its secondary metabolite production.[43] The correlation can be either negative or positive, as shown in this study. When biomass production was negatively correlated with quinidine production, primary metabolism was more supported than secondary metabolism. Otherwise, primary metabolism is inhibited and shifted to secondary metabolism. When the correlation is positive, the carbon source might be used for both primary and secondary metabolism.

During *in vitro* production, endophytic fungi produced are not only beneficial secondary metabolites but may also produce toxins. *F. oxysporum f.* sp. *lycopersici* has been reported to produce fusaric acid after 3 days of incubation; maximum production was reached after 10 days of incubation in potato sucrose broth.[44] *F. oxysporum* could also produce fumonisin in glucose yeast asparagine malic acid medium at 25°C for a 2-week incubation period.[45] Fumonisin has high solubility in water, methanol, and acetonitrile,[46,47] while quinidine is very soluble in chloroform solvents, moderately soluble in alcohol and benzene, and slightly soluble in water solvents.[48] Another toxin, such as cyclosporine had been reported to be produced by *F. solani f.* sp. *radicicola* in modified liquid culture medium at 25°C for 7 days.[49] *F. solani* M11 produces T-2 toxin, trichothecene, and solaniol in Czapek dox peptone medium at 25°C for 12 days.[50] *A. sydowii* produced sydonic acid in liquid medium with an initial pH 7.2 at 28°C for 3 days,[51] while *Diaporthe* produced phomopsin A in Czapek Dox broth at 24°C for 28 days.[46] Regardless of toxin solubility, as these fungi may produce an unwanted substance, consideration should be taken when *in vitro* production of quinidine using these fungi is brought into practice.

Quinidine is a high-value substance. In this study, quinidine production by *F. solani, F. oxysporum,* and *A. sydowii* was higher than that of *Diaporthe* sp. and *D. lithicola*. Further, the amount of quinidine produced by *Diaporthe* sp. (8.913 µg) and *D. lithicola* (11.148 µg) in 200 mL PDB medium after 21 days of incubation was much higher than by *D. phaseolorum* (3-5 µg/L) and *Schizophyllum* sp. (0.01 µg/L) on synthetic medium pH 6.2 after 4 days of incubation.[18,19] This indicates that all the fungi studied are prospective agents for quinidine production.

The quinidine production from five *C. calisaya* endophytic fungi was still lower than that from *C. calisaya* bark extraction (2.240 µg/g).[52] However, compared with quinidine production in *C. ledgeriana* cell culture (349.38 µg/g), *in vitro* quinidine production from *A. sydowii* and *F. solani* was higher. In contrast, quinidine production from these fungi was lower than in the cell culture (8.078 µg/g) modified by adding 5 ppm of paclobutrazol and incubating it under 10 µmol/m^2/sec light intensity.[14] Nonetheless, quinidine production from endophytic fungi is more beneficial than that from plants, considering that its production requires a shorter time. An attempt to scale up quinidine production should, therefore, be pursued using *F. solani*, which is capable of producing the highest amount of quinidine with low biomass production using PDB medium with an initial pH of 6.2 and incubation under light.

CONCLUSION

In quinidine production by *A. sydowii*, *Diaporthe* sp., *D. lithicola*, *F. oxysporum*, and *F. solani*, fungal endophytes from *C. calisaya*, was affected by light and the initial pH of the medium. Quinidine production by *A. sydowii*, *F. oxysporum*, and *F. solani* were higher with incubation under continuous light and an initial medium pH of 6.2, while *Diaporthe* sp. and *D. lithicola* were higher with incubation under continuous darkness and an initial medium pH of 6.8. *In vitro* culture of *F. solani* in PDB medium with an initial pH 6.2 and with incubation under continuous light produced the highest concentration of quinidine with low biomass and thus could potentially be used for quinidine production. Five endophytic fungi from *C. calisaya*, mainly *F. solani*, are natural resources that could be exploited commercially for quinidine production. Production under optimum light and initial medium pH were considered as economically and environmentally friendly conditions. However, the stability of this quinidine production under optimum conditions should be further investigated to find models for production on an industrial scale.

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ORIGINAL ARTICLE

Comparative Proteomic Analysis of *Escherichia coli* Under Ofloxacin Stress

Ofloksasin Stresi Altında *Escherichia coli*'nin Karşılaştırmalı Proteomik Analizi

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ABSTRACT

Objectives: In the present study, proteomics was utilized to evaluate changes in *Escherichia coli* proteins in response to ofloxacin to understand the mechanism of action of ofloxacin and the mechanisms of ofloxacin resistance in *E. coli*.

Materials and Methods: Proteomics analysis of *E. coli* was performed by using liquid chromatography quadrupole time-of-flight mass spectrometry followed by a data processing step using MaxQuant. Functional classification and pathway analysis showed a systematic effect of ofloxacin over *E. coli* proteome structure.

Results: In total, 649 common proteins were identified in the untreated and ofloxacin-treated groups, while 98 proteins were significantly different in the ofloxacin-treated group. Functional classification and pathway analysis showed that ofloxacin has a systematic effect over ribosomal processes, energy pathways (tricarboxylic acid cycle and glycolysis), membrane proteins, microbial targets, and biofilm formation.

Conclusion: The results showed that ofloxacin affected many cellular processes and pathways. In addition, proteomic analysis revealed that *E. coli* develops resistance mechanism with different biological processes.

Key words: Proteomics, LC/MS, antibiotic resistance, ofloxacin, *E. coli*

ÖZ

Amaç: Bu çalışmada ofloksasinin etki mekanizmasına karşı yanıt olarak *Escherichia coli* proteinlerinde görülen değikliklerin belirlenmesi ve *E. coli*'deki oflotoksin direnç mekanizmalarının değerlendirilmesi için proteomiks kullanılmıştır.

Gereç ve Yöntemler: *E. coli*'nin proteomik analizi sıvı kromatografisi/kuadrupol uçuş zamanlı kütle spektroskopisi kullanarak gerçekleştirilmiş ve sonrasında veri prosesleme aşaması MaxQuant kullanarak yapılmıştır. İşlevsel sınıflandırma ve yolak analizi oflotoksinin *E. coli*'nin proteome yapısına sistematik bir etkisinin olduğunu göstermiştir.

Bulgular: Çalışmada 649 tane sıklıkla rastlanan proteinin yapısı oflotoksin ile muamele edilen ve edilmeyen gruplarda belirlenirken, 98 proteinin oflotoksin ile muamele gören grupta belirgin derecede farklı olduğunu göstermiştir. İşlevsel sınıflandırma ve yolak analizi oflotoksinin ribosomal prosesler, enerji yolakları (trikarboksilik asit siklusu ve glikoliz), membran proteinleri, mikrobiyal hedefler ve biofilm oluşumu üzerine sistematik bir etkisinin olduğunu göstermiştir.

Sonuç: Bu sonuçlar ofloksasinin birçok hücresel prosesi ve yolağı etkilediğini göstermiştir. Ayrıca, proteomik analizler *E. coli*'nin farklı biyolojik prosesler ile direnç mekanizması geliştirdiğini göstermiştir.

Anahtar kelimeler: Proteomik, LC/MS, antibiyotik direnci, ofloksasin, *E. coli*

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INTRODUCTION

Antibiotic resistance is one of the most pressing health issues worldwide. Improper use of antibiotics has led to the emergence of resistant microorganisms.[1,2] A more detailed understanding of antibiotic-bacteria interactions is key to increasing the efficiency of antibiotic use as well as developing novel combination treatment strategies. In recent years, omics-based technologies have emerged in bacterial antibiotic resistance.[3,4] Mass spectrometry (MS)-based proteomics is a powerful method for the comprehensive characterization of proteome structure and to understand the cellular process at the protein level within an organism. In microbiology, proteomics is a promising approach to understanding the effect of antibiotics on bacteria and resistance mechanisms against antibiotics.[5-7]

Ofloxacin is one of the second-generation fluorinated quinolones with broad-spectrum antibacterial activity against most Gram-negative and, Gram-positive bacteria, including anaerobes.[8,9] Ofloxacin inhibits the activity of DNA gyrase and topoisomerase IV in DNA replication. Alterations in the target enzymes (DNA gyrase and topoisomerase IV) by chromosomal mutations is the main mechanism of resistance.[9] However, the contribution of proteins to resistance still requires explanation for a deeper understanding of bacteria-fluoroquinolone interactions.

The present study aimed to evaluate the effects of ofloxacin on *Escherichia coli* at the protein level. Apart from its broad-spectrum activity on clinically important infections, ofloxacin is also renowned for its wide application in livestock production and its high frequency in environmental sources such as surface water.[10,11] Livestock and environmental sources are emerging due to its high capacity for horizontal gene transfer of antimicrobial resistance genes. Thus, the present study is also important for elucidating the potential resistance mechanisms of bacteria against ofloxacin.

MATERIALS AND METHODS

Bacterial culture and minimum inhibitory concentration (MIC) experiments

E. coli ATCC 25922 was cultured on tryptic soy agar (TSA). In order to confirm the MIC value of ofloxacin against *E. coli*, standard broth microdilution was performed according to the method reported by the Clinical Laboratory Standards Institute.[12] The MIC value of ofloxacin was determined and compared with the latest breakpoint tables of European Committee on Antimicrobial Susceptibility Testing (EUCAST).[13] For proteomics experiments, the 0.5x MIC value for ofloxacin was used (0.01 mg/L). *E. coli* ATCC 25922 was cultured on Mueller Hinton Broth (MHB) and incubated at 37°C until the log phase was achieved. The bacterial suspension was prepared using MHB containing 0.01 mg/L ofloxacin to obtain a bacterial concentration of $5x10^5$ cfu/mL bacteria. For the control group, the same concentration of the bacterial culture was also prepared without adding any antibiotics. Flasks were incubated at 37°C for 20 h for proteomics experiments. A bacterial growth curve was constructed for 48 hours by plotting time against the log 10 cfu/mL values. Colony counting for each hour was performed by serial dilution and inoculation of the bacterial suspension into TSA.

Sample preparation for proteomics

After the incubation period, the cultures were centrifuged, and washed with phosphate buffered saline in triplicate. The bacterial cells were disrupted using lysis buffer containing lysozyme. Protein concentrations of ofloxacin in the treated and control groups were calculated by using the Bio-Rad DC assay. The chloroform (Sigma)/methanol (Sigma)/water co-solvent system was used for protein extraction.

Extracted proteins were suspended in 100 mM ammonium bicarbonate solution (Sigma). As a reducing agent, 200 mM dithiothreitol (DTT) (Sigma) was used. Proteins were incubated with DTT at 56°C for 20 min. After reduction, proteins were incubated with 100 mM iodoacetamide (Sigma) at room temperature. Proteins were digested with trypsin (1:100 (w/w) at 37°C for 16 hours. After desalting, tryptic peptides were dissolved in acetonitrile that contained 0.1% formic acid.

Liquid chromatography/quadrupole time-of-flight/MS (LC/QTOF) analysis

Peptides were analyzed in a LC/QTOF/MS platform. They were separated in C18 columns [Zorbax C18 column 150x0.05 mm, 5 column 1 Å (Agilent)] at 55°C performed with an Agilent 1290 high performance LC. A total volume of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used as the mobile phase, and the flow rate was adjusted to 0.070 mL/min. Peptides were eluted with a gradient of 1%-55% mobile phase B over 80 minutes, followed by 55%-85% mobile phase B over 5 minutes. The total gradient run time was 90 minutes. Post run was 25 minutes.

Peptides were analyzed in an Agilent 6530 QTOF/MS system in positive-ion mode of the electrospray ionization source. The capillary voltage was adjusted at 4000 V with a drying temperature of 350°C. Automated MS/MS data of peptides were recorded between 300 m/z and 1400 m/z above the 1500-count threshold.

The six most intense ions were selected for MS/MS analysis. The ion charge states were +2, +3, and +4. The fragmentation energy was adjusted to 45 V. Twenty micrograms of protein was loaded onto the LC/MS system for each run. Four technical replicates were analyzed for each of ofloxacin-treated and control groups.

Protein identification and quantification

The recorded MS/MS data were processed using Maxquant (Max Planck Institute, Germany, https://www.maxquant.org). The *E. coli* database was downloaded from UniprotKB. Peptides and proteins were identified by matching the recorded MS/MS data with *in silico* MS/MS data. In the matching process, 20 ppm mass tolerance was used for the first and main search. Carboamidomethylation on cysteine and oxidation on methionine were chosen as fixed and variable modifications, respectively. Two missed cleavages were allowed. In the identification process, the false discovery rate (FDR) value was selected as 0.01 for reliable identification.

Statistical analysis for comparative proteomics

The label-free quantification algorithm in Maxquant multilevel feedback queue (MLFQ) was used for semi-quantification between ofloxacin-treated and control groups.

Perseus software (https://www.biochem.mpg.de/5111810/perseus) was used for the statistical analysis. Principal component analysis (PCA) showed the overall effects of ofloxacin on *E. coli*. A two-sample t-test ($p<0.05$) was performed with 250 randomizations and a FDR of 1%, to identify the differentially expressed proteins between the control and ofloxacin-treated groups.

Gene list, protein-protein interaction, and pathway analysis

Panther, the gene list software (http://pantherdb.org), was used to classify differentially expressed proteins according to cellular component, molecular function, and biological process. We also investigated the relationships of downregulated and upregulated proteins in a protein map by using the String functional tool (https://string-db.org). In addition, differentially expressed proteins were evaluated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/mapper.html) to observe the ofloxacin-induced metabolic pathways.

RESULTS

LC/QTOF/MS proteomics assay

The determined MIC value for *E. coli* ATCC 25922 was 0.02 mg/L, which was in accordance with the EUCAST breakpoints. Thus, the 0.5x MIC value of 0.01 mg/L was used for proteomics experiments. The growth curve of *E. coli* ATCC 25922 after treatment with 0.01 mg/L of ofloxacin was constructed over 48 hours. The colony counts for treated bacterial cells were significantly different from those of the control at 6, 20, and 48 hours (Figure 1).

Proteins were extracted and digested from the control and ofloxacin-treated groups and analyzed in four technical replicates by using the LC/QTOF/MS system. Chromatograms are shown in Figure 2. Proteins were identified with a conservative threshold (FDR ≤0.01 and matched peptide numbers ≥2). In total, 649 common proteins were identified in the control and ofloxacin-treated groups. In the present study, we used micro-LC-based proteomics analysis. Micro LC-based proteomics is less sensitive but more robust and reproducible than nano LC-based proteomics. Thus, we identified fewer proteins than we would have using nano LC based proteomics.

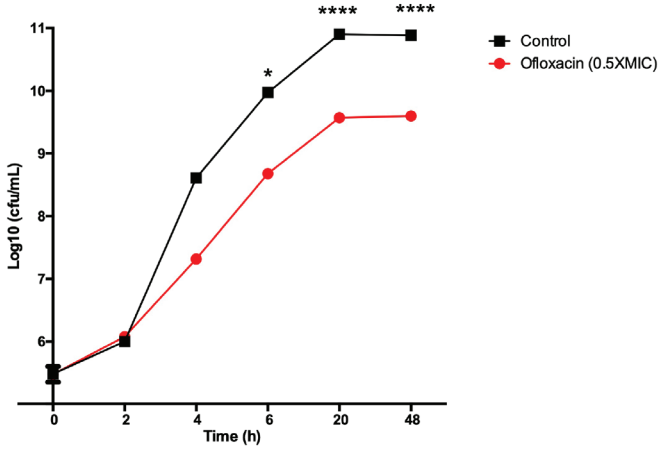

Figure 1. Growth curve of *Escherichia coli* ATCC 25922 with or without 0.02 mg/L ofloxacin treatment (0.5x MIC). Sampling for proteomic experiments was conducted at the 20th hour. Two-Way ANOVA was performed for multiple comparisons

*: $p<0.05$, ****: $p<0.0001$, MIC: Minimum inhibitory concentration

The label-free protein quantification algorithm of Maxquant MLFQ was used for semi-quantitative proteomics. The MLFQ intensities of proteins were evaluated statistically. First, treated and untreated groups were analyzed individually through a multi-scatter plot analysis to observe the reproducibility of technical replicates. The Pearson's correlation coefficients were between 0.885 and 0.968 for the untreated group. For the treated group, the correlation coefficients ranged from 0.871 to 0.946. These results indicated highly reproducible findings between technical replicates. PCA was used to observe the global effect of ofloxacin on the *E. coli* proteome. In addition, PCA analysis was used to observe the similarity between the technical replicates of ofloxacin-treated and control groups (Figure 3). PCA results showed that ofloxacin significantly impacts proteome structure in *E. coli* (PC1 50.8%, PC2 19.2%).

The MLFQ intensities of proteins were evaluated using the statistical software Perseus. The two-sample t-test showed alterations in the expression levels of 98 proteins. A total of 30 proteins showed increased and 68 proteins showed decreased expression (altered proteins and fold changes are given in the supplementary material).

Gene ontology (GO) analysis of differentially expressed proteins

Differentially expressed proteins were classified into GO categories. Proteins were first classified according to their location inside cells. Differentially expressed proteins were mostly located in the cytosol (34.88%), plasma membrane (23.26%), and ribosome (23.26%). Moreover, some of them were categorized as integral membrane (9.30%), mitochondrial (4.65%), adenosine triphosphate (ATP) synthase complex (2.33%), and nucleus-related proteins (2.33%) (Figure 4A).

The proteins were also evaluated to understand the effect of ofloxacin stress on biological processes. Proteins were mostly

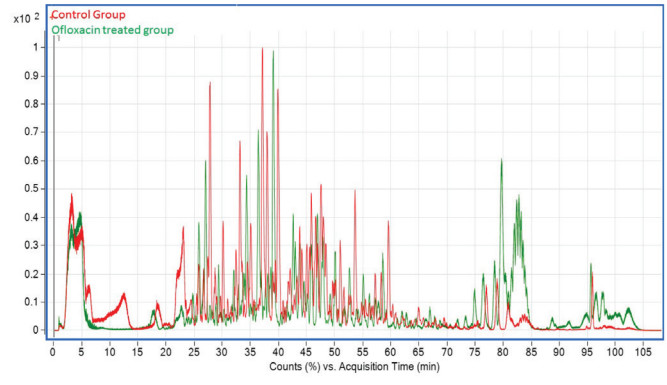

Figure 2. Chromatograms of control and ofloxacin-treated groups

involved in metabolic processes (47.4%), cellular processes (36.6%), biogenesis (7.3%), localization (6.1%), and biological regulation (2.1%) (Figure 4B). As expected, metabolic processes were mainly altered in *E. coli* under ofloxacin stress. Since primary metabolic processes are essential for cell viability, those processes were analyzed in detail (Figure 4C).

According to the molecular functions of proteins (Figure 4D), it was determined that differentially expressed proteins have catalytic (44.6%), binding (21.5%), structural molecular (18.5%), translation regulatory (3.1%), transporter (9.2%), antioxidant (1.5%), and receptor activities (1.5%).

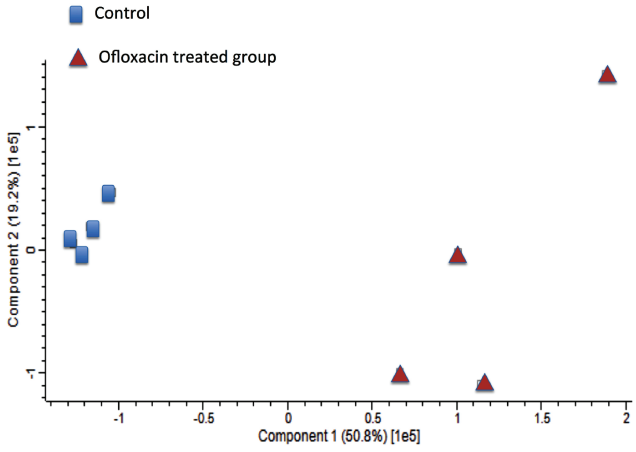

Figure 3. PCA analysis of treated and untreated groups. The blue zone shows the technical replicates of untreated groups, and the red zone shows the technical replicates of the treated group

PCA: Principal component analysis

Protein interaction analysis

The string protein interaction tool was used to observe interactions between differentially expressed proteins in a protein map. Up- and downregulated proteins were analyzed separately (Figure 5A, 5B).

KEGG pathway analysis

Next, we evaluated increasing and decreasing proteins together in the KEGG pathway (FDR <0.05). The ofloxacin-induced metabolic pathways are presented in Figure 6. KEGG pathway analysis showed that the ofloxacin treatment mainly affects cells' metabolic functions (especially energy metabolism) and ribosomes. Other affected ofloxacin pathways include the following: Biosynthesis of secondary metabolites, microbial metabolism in diverse environments, carbon metabolism, biosynthesis of amino acids, pyruvate metabolism, methane metabolism, and purine metabolism.

DISCUSSION

In the present study, changes in the proteome structure of *E. coli* were evaluated in response to ofloxacin exposure. PCA analysis showed that ofloxacin has a widespread effect on *E. coli*. Ninety-eight proteins were found to be altered in the ofloxacin-treated group.

The differentially expressed proteins were classified functionally in GO analysis to understand the systematic effect of ofloxacin on *E. coli* at the protein level. First, we classified

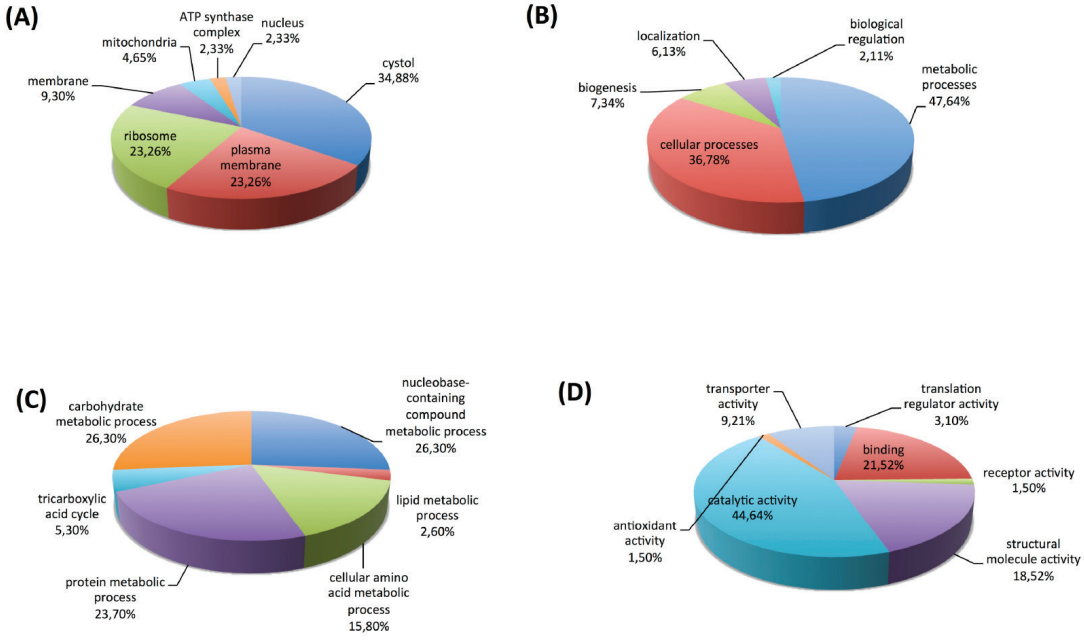

Figure 4. (A) Classification of differentially expressed proteins according to cellular component; (B) functional classification of differentially expressed proteins in cellular component; (C) ofloxacin-induced primary metabolic processes (nucleobase-containing compound metabolic process); (D) functional classification of differentially expressed proteins according to their molecular functions

ATP: Adenosine triphosphate

proteins according to their location (cellular component) in cells. We observed that many differentially expressed proteins in the ribosome were dysregulated, which indicated that ofloxacin has a veritable effect on protein and enzyme synthesis, in addition to its main mechanism of action. In cellular component annotation, another important aspect is the induction of membrane proteins by ofloxacin. Membrane proteins are important regulators involved in the various metabolic processes within cells, additionally facilitating antibiotic resistance. According to our results, five outer membrane proteins, MipA, LamB, OmpW, TorA, and RseB, were found to be downregulated in the ofloxacin-treated group. In recent years, the role of these proteins in antibiotic resistance has been studied extensively, and it has been found that they could act as both positive and negative regulators of antibiotic resistance. Lin et al.[14] showed that increased levels of ompW were related to antibiotic resistance. They also found the outer membrane protein LamB to be downregulated under antibiotic stress.[14] MipA is another extensively studied membrane protein. Li et al.[15] showed that MipA is a novel antibiotic resistance related protein in *E. coli*, conferring resistance to kanamycin. The downregulation of MipA promotes resistance mechanisms. Thus, the downregulation of LamB and MipA could be one of the key factors in the potential resistance mechanism against ofloxacin. A downregulation of outer membrane proteins could cause a decrease in antibiotic intake by bacterial cells. We also classified proteins according to their biological processes. It was observed that ofloxacin has a dramatic effect on metabolic processes. When we analyzed the metabolic processes in detail, we found that ofloxacin affects primary metabolic processes, which are key systems related to cell viability. In carbohydrate metabolic processes, most of the proteins were found to be downregulated.

We analyzed the differentially expressed proteins according to their molecular functions. It was observed that majority of the proteins in the ofloxacin-treated group were involved in catalytic and binding activities.

Nucleic-acid binding proteins modulate transcription processes in bacterial cells. These proteins directly affect cell viability and growth, and some of them are essential for drug-induced DNA repair. In our work, we observed that ofloxacin induces several nucleic-acid binding proteins. Elongation factor G (FusA) is one of the most important proteins involved in translational elongation. It mediates the translocation of mRNA and tRNA through the ribosome and is essential for protein synthesis. Several studies showed that FusA is the main target of some antimicrobial agents.[16] Another nucleic-acid binding protein, RpoB, is the key protein in drug-resistance development. Several studies have showed that mutation in RpoB is one of the major determinants of rifampin resistance.[17] Our results showed that the expression level of RpoB was decreased in the ofloxacin-treated groups. DNA starvation/stationary phase protection protein (Dps), a nucleic-acid binding protein, provides protection to cells under stress conditions. Several studies on the function of Dps in antibiotic resistance indicated that it has multiple properties that function in stress protection: DNA binding, iron sequestration, and ferroxidase activity.[18-20] Therefore, Dps has recently emerged as a potent antimicrobial target. According to our results, the expression level of Dps decreased in the ofloxacin-treated group.

We observed that the NAD-dependent DNA ligase A (LigA), which is an important element in the DNA repair process, was upregulated in the treated group. The DNA repair process is one of the most important steps in the development of antibiotic resistance. The overexpression of LigA may promote DNA repair against ofloxacin-induced stress. In recent years,

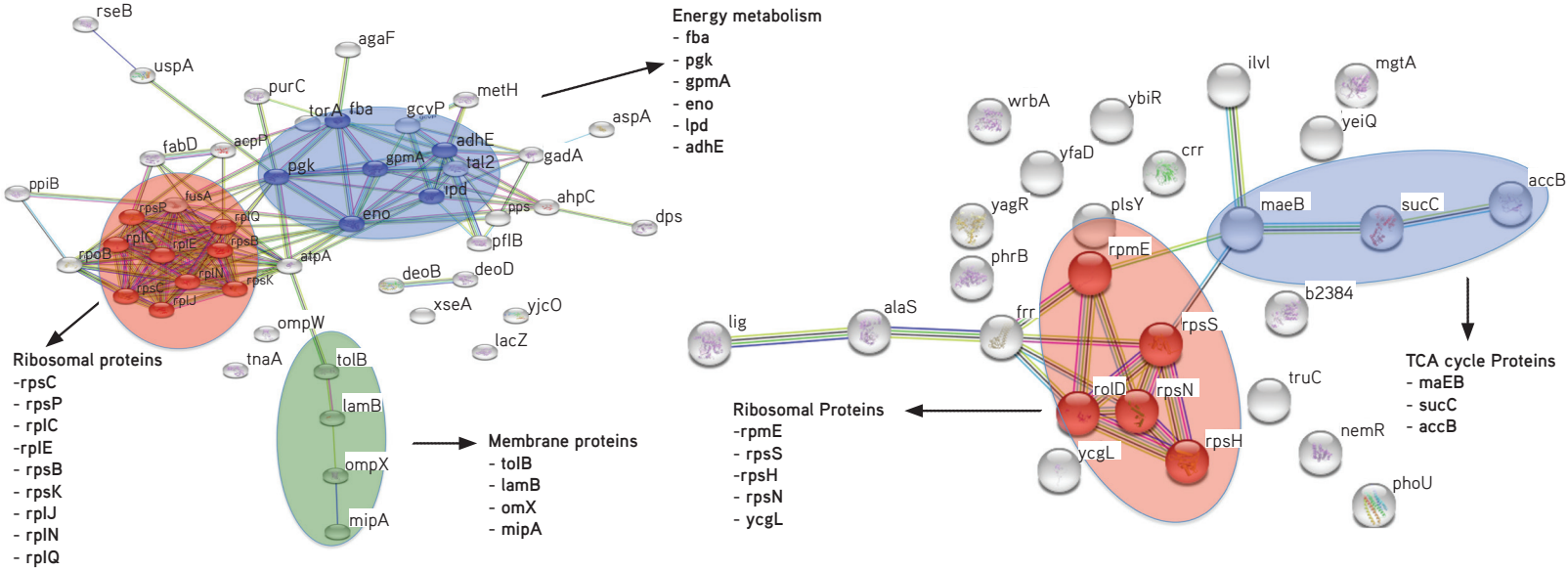

Figure 5. (A) Map of downregulated proteins. Red represents the proteins involved in ribosomal proteins, whereas the blue mark represents glycolytic metabolism. Moreover, membrane proteins are marked in green. (B) Map of upregulated proteins. Ribosomal proteins are marked red, and TCA cycle proteins are marked in the blue zone

TCA: Tricarboxylic acid

LigA has been evaluated as a potential antimicrobial target, and LigA inhibitors have shown promising results.[21,22] We believe that combination therapies including ofloxacin and LigA inhibitors can be more effective for resistant strains of *E. coli*.

We also observed that ofloxacin affects the expression levels of various heterocyclic-compound-binding proteins. Tryptophanase, (TnaA), which is responsible for the production of indole, is an important factor in biofilm formation and antibiotic resistance.[23] Previous studies have shown that the decrease in TnaA levels interrupts inter-species biofilm signaling and subsequently decreases biofilm formation.[24] In our study, TnaA was decreased in the presence of subinhibitory concentrations of ofloxacin. This result suggests that ofloxacin treatment may be considered in the treatment of biofilm-forming infections.

ATP synthase subunit alpha is another heterocyclic-compound-binding protein. Previous studies showed that inhibition of ATP synthase proteins caused dysregulation of energy metabolism and suppressed bacterial growth. ATP synthase is a promising target of many antibiotics.[25] In our study, the presence of the ATP synthase subunit alpha decreased in the ofloxacin-treated groups.

In protein interaction analysis, We investigated interactions of altered proteins in biological pathways. When downregulated proteins were evaluated with String analysis, it was observed that the disruption of ribosomal metabolism was likely to be closely related to energy metabolism, which was also largely affected by the reduction in outer membrane proteins such as LamB and MipA. As discussed above, MipA and LamB are important regulators in membrane-based resistance processes. The exact the interactions between ribosomal proteins, energy metabolism, and membrane proteins have not yet been elucidated. It can be hypothesized that the reduction in MipA and LamB may also lead to reduced intake of the essential factors related to ribosomal and energy metabolism, in addition to reduced antibiotic intake. KEGG pathway analysis also confirmed that ofloxacin treatment mainly affects cells' metabolic functions (especially energy metabolism) and ribosomes.

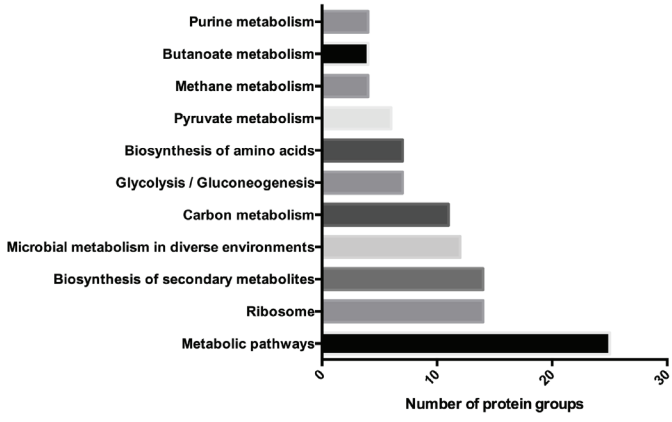

Figure 6. Ofloxacin-induced pathways (FDR <0.05)
FDR: False discovery rate

According to the String analysis of upregulated proteins, the ribosomal proteins RpmE and RpsS were related to pyruvate metabolism and (tricarboxylic acid) TCA cycle proteins (MaeB, SucC, AccB). Rosato et al.[26] showed that the TCA cycle is upregulated under antibiotic stress, and dysregulated production of TCA cycle-mediated reactive oxygen species had an effect on DNA integrity and subsequently triggered activation of the SOS response. Due to the SOS response, RecA is upregulated for DNA repair,[27] which may result in enhanced mutagenesis and the development of resistance to antibiotics as a defense mechanism. We demonstrated upregulation of TCA cycle proteins; however, we did not observe RecA in our study, which might have been due to the concentration and exposure time of ofloxacin.

CONCLUSION

The present study, which was designed to evaluate the effect of ofloxacin on *E. coli* proteomics and to elucidate additional resistance mechanisms, showed that ofloxacin has systematic effects on ribosomal processes, energy pathways, and various antimicrobial targets. Moreover, we found that various mechanisms may play a role in ofloxacin resistance, which requires confirmation by further dose- and time-dependent studies.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

In Vitro Physiological Effects of Betahistine on Cell Lines of Various Origins

Betahistinin Farklı Orijinlere Sahip Hücreler Üzerindeki *İn Vitro* Fizyolojik Etkileri

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ABSTRACT

Objectives: Betahistine is a histamine analog commonly prescribed for symptomatic treatment of vertiginous symptoms. *In vitro* studies have shown that betahistine was not toxic at the prescribed doses in a nasal epithelial cell line. However, the effect of betahistine on other cell types has not been studied. In this study, we aimed to investigate some of the physiological effects of betahistine on L929 fibroblast, A549 lung cancer, human umbilical vein endothelial (HUVEC), and Ishikawa endometrial cell lines.

Materials and Methods: Cellular proliferation was assed assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, apoptosis was evaluated by acridine orange-ethidium bromide staining, and cellular migration was assed assessed by scratch assay.

Results: Betahistine treatment (0.1-0.5 mg/mL, 24 hours) can inhibit cell proliferation and induce apoptosis in HUVEC, A549, Ishikawa, and L929 cell lines. Betahistine (≥0.1 mg/mL) significantly increased the number of apoptotic cells (HUVEC: 26.3%, A549: 17.3%, L929: 8.6%, and Ishikawa: 2.3%). Betahistine at doses over 0.1 mg/mL significantly suppressed the cell migration rate in all of the cell lines. In contrast, exposure to a low dose of betahistine (0.025 mg/mL) induced migration rates of HUVEC and Ishikawa cells by 81% and 48%, respectively.

Conclusion: Betahistine may alter the processes of cellular proliferation, apoptosis, and cellular migration in a cell line- and dose-dependent manner. In this sense, proliferative and metastatic properties of certain cancer cells can potentially be altered in response to betahistine treatment.

Key words: Betahistine, cellular-migration, apoptotsis, proliferation, cancer

ÖZ

Amaç: Betahistin vertigo semptomlarının semptomatik tedavisi amacıyla sıklıkla reçete edilen bir histamin analoğudur. *İn vitro* çalışmalar, betahistinin reçete edilen dozlarda nazal epiteliyal hücreler üzerinde toksik etkisi olmadığını göstermiştir. Ancak, betahistinin diğer hücre hatlarındaki üzerindeki etkileri hakkında çalışma bulunmamaktadır. Bu çalışmada, betahistinin L929 fibroblast, A549 akciğer kanseri, insan umbilikal ven endoteliyal (HUVEC) ve Ishikawa endometriyal hücre hatları üzerindeki bazı fizyolojik etkilerini araştırmayı amaçladık.

Gereç ve Yöntemler: Hücre proliferasyonu 3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum-bromür yöntemiyle, apoptoz akridin turuncusu-etidyum ikili boyama yöntemiyle ve hücre göçü hızı çizik deneyi ile araştırılmıştır.

Bulgular: Betahistin uygulamasının (0,1-0,5 mg/mL, 24 saat), HUVEC, A549, Ishikawa ve L929 hücre hatlarında hücre proliferasyonunu baskılayabileceği ve apoptozu indükleyebileceği belirlenmiştir. Betahistin'nin (≥0,1 mg/mL) apoptotik hücrelerin sayısında önemli bir artışa neden olmuştur (HUVEC: %26,3, A549: %17,3, L929: %8,6 ve Ishikawa: %2,3). Betahistin 0,1 mg/mL dozun üzerinde tüm hücre hatlarında hücre göçü hızını önemli derecede baskılamıştır. Buna karşılık, düşük dozlarda betahistin uygulaması (0,025 mg/mL) HUVEC ve Ishikawa hücre hatlarında hücre göçü hızını sırasıyla %81 ve %48 oranlarında artırmıştır.

Sonuç: Betahistinin hücre proliferasyonu, apoptoz ve hücre göçü hızını hücre tipi ve doza bağımlı şekilde etkileyebilir. Bu kapsamda, betahistin uygulamasına yanıt olarak bazı kanser hücrelerinin proliferatif ve metastatik özellikleri de potansiyel olarak değişebilir.

Anahtar kelimeler: Betahistin, hücre göçü, apoptoz, proliferasyon, kanser

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INTRODUCTION

Betahistine is a histamine analog used for symptomatic treatment of vertiginous symptoms related to Ménière's disease.[1] It is considered to exert its effects partially by acting as an antagonist of H3 receptors. Betahistine can enhance the release of histamine in the central nervous system.[2] Histamine is a neuromodulatory transmitter that regulates important cerebral activities, including vestibular functions.[3] Additionally, betahistine is reported to improve the microcirculation of the inner ear.

In vitro cell culture models are valuable tools for biocompatibility and drug toxicity studies prior to the use of animal models.[4] For example, the commercially available nasal epithelial cell line RPMI 2650 has been widely studied in drug toxicology tests.[5] There is a limited number of reports on the possible physiological effects of betahistine in cell culture models. Pilicheva et al.[6] showed that betahistine was not toxic at prescribed doses in a nasal epithelial cell line. Toxicity was observed only at very high concentrations (>50 mg/mL), which are not achievable under *in vivo* conditions. However, systemic administration of betahistine can have effects on many other cell types. Although *in vitro* cell culture tests suggest that use of betahistine is safe for nasal epithelial cells, the possible effect of this drug on other cell types, such as cancer, endothelial, or fibroblast cell lines, has not been extensively studied. Therefore, we aimed to investigate the effect of betahistine on L929 fibroblast, A549 lung cancer, human umbilical vein endothelial (HUVEC) endothelial, and Ishikawa endometrial cell lines. For this purpose, viability, the changes in the ratio of apoptotic cells, and cell migration rates were compared.

MATERIALS AND METHODS

Cell culture and chemicals

HUVEC, human Asian endometrial adenocarcinoma (Ishikawa), pulmonary adenocarcinoma human alveolar epithelial (A549), and murine fibroblast (L929) cell lines were cultured in high-glucose Dulbecco's Modified Eagle's medium (DMEM) (Sigma, 5546) supplemented with P/S (50 U/mL penicillin and 50 µg/mL streptomycin; Biological Industries, 03-031-1B), 1% 2 mM L-glutamine (Biological Industries, BI03-020-1B), and 10% FBS (Biowest, S1810-500). Cells, $1.5x10^6$ from each cell line, were seeded in 10 cm plates and split after 72 hours. Commercially available betahistine tablets (Betaserc®, Abbott Healthcare SAS, Châtillon-sur-Chalaronne, France) were ground (~100 mg) in a muller and then dissolved in a 100 mL volumetric flask containing 50 mL 0.1N HCl (pH: 1.2). The flasks were immersed in a water bath, maintained at 75°C for 2-3 min, and shaken until all the pellets were completely melted. The flasks were cooled for 1 hour (room temperature), and 0.1N HCl was added to bring the volume to 100 mL. The resulting suspensions were filtered through a 0.45 µm syringe filter.[7] Betahistine stock solutions (24 mg/mL) were kept at -20°C. A non-treated (NT) control group (vehicle) was prepared by diluting 8.28 mL of HCl (Sigma 320331) in 1000 mL DMEM.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A 12 mM (5 mg/mL) stock solution of MTT (Neofrox 3580 MTT) was prepared as described by Mosmann.[8] Approximately 10^4 cells were seeded in each well of a 96-well plate in a volume of 100 µL. The MTT assay was carried out as follows: 10 µL from the 12 mM MTT stock solution was added to each well and incubated at 37°C for 4 hours (final concentration in incubation medium was 0.5 mg/mL). Medium alone (100 µL) was included as a negative control. After 4 hours of incubation with MTT, 75 µL of medium was removed from the wells, and then formazan crystals were dissolved with 50 µL of dimethyl sulfoxide by mixing thoroughly with a pipette. Following an additional incubation at 37°C for 10 minutes, the samples were mixed again briefly, and the absorbance at 540 nm was recorded.

Acridine orange/ethidium bromide (AO/EtBr) double staining

The AO/EtBr dual staining technique was performed as described by Liu et al.[9] Briefly, cells were seeded in a 96-well plate at a density of approximately 10^4 cells/well. Following incubation with betahistine for 48 hours, cells were trypsinized, and 10-25 µL cell suspensions were transferred onto glass slides. One microliter of AO/EtBr staining solution (a mixture of dyes containing 100 µg/mL AO and 100 µg/mL EtBr) was added to cell suspensions, and then the samples were covered with a coverslip. The cell morphology was examined under a fluorescent microscope (Carl-Zeiss/Axio observer 3., Zen 2.3 Blue Edition software) within 20 minutes after addition of the Ao/EtBr stain. For statistical analysis, at least 200 cells were counted, and the results were expressed as mean values obtained from at least three independent experiments. In the asay, both live and dead cells are stained with AO, while EtBr stains only dead cells that have lost membrane integrity. Live cells appear uniformly green, whereas early apoptotic cells show green dots in their nuclei. Late apoptotic cells stain orange and show condensed and/or often fragmented nuclei. Necrotic cells stain orange, with a nuclear morphology resembling that of viable cells, but without condensed chromatin.[9]

In vitro scratch assay

For the evaluation of cell migration rates, an *in vitro* scratch assay was carried out according to the protocol described by Liang et al.[10] Cell lines were seeded at $1x10^5$ cells/well into 6-well plates in growth medium overnight. Briefly, a scratch on the surface of the well was made with a 10 µL sterile pipette tip in 6-well plates. Following gentle washing (to remove the detached cells) with culture medium, photos of the scratch were taken at different time points (0-24 hours) under a microscope at 10x magnification (Carl-Zeiss/Axio observer 3). The gap size was analyzed using ImageJ software, and the rate of cell migration was calculated by comparing the cell-free areas of the scratches at 24-hour post-wounding and the area of the scratches at 0 hour. The percent changes in the migration rates were compared against the vehicle (NT control group). The results were expressed as means of triplicate experiments.

Statistical analysis

Statistical analysis was performed using GraphPad (Prism 5) software. Multiple comparisons were made using Tukey's procedure. $P<0.05$ was considered to indicate statistical significance. Analysis of variance was used for significant differences in the apoptotic index among groups.

RESULTS

Firstly, we investigated the effects of betahistine on cell viability by comparing the changes in proliferation rates of A549 (human pulmonary adenocarcinoma basal epithelial), HUVEC, Ishikawa (human endometrial adenocarcinoma), and L929 (murine fibroblast) cell lines by MTT assay. For this purpose, several doses (25, 50, 100, 250, and 500 µg/mL for 24 hours) were tested. Analysis of data obtained from proliferation assay studies showed that low levels of betahistine treatment (25 µg/mL, 24 hours) slightly induced proliferation rates in all of the cell lines tested (HUVEC by 109.8%, A549 by 107.7%, L929 by 116.2%, and Ishikawa by 153.5%) (Figure 1a-d). Betahistine (100 µg/mL) inhibited the proliferation rate by 35% in the endothelial HUVEC cell line at (Figure 1c), while A549, L929, and Ishikawa cell lines seemed to be more resistant to 100 µg/mL betahistine treatment (Figure 1a, b, d).

Betahistine treatment (250 µg/mL) resulted in a significant inhibition in A549 (56.86%), L929 (56.88%), and Ishikawa (43.21%), cell lines (Figure 1c). As seen in Figure 1c, HUVEC cell line seemed to be more resistant to 250 µg/mL betahistine when compared with the other cell lines (33.5% inhibition). On the other hand, 500 µg/mL betahistine treatment resulted in an almost 50% reduction in the proliferation rates of all of the cell lines tested.

Next, we compared the changes in the ratio of apoptotic cells using the "AO/EtBr" double staining protocol. For this purpose, the changes in the ratio of apoptotic and necrotic cells upon exposure to 25, 50, 100, 250, and 500 µg/mL (24 hours) of betahistine were evaluated. Our findings clearly suggested that lower doses of betahistine (25 µg/mL, 24 hours) did not significantly induce apoptosis in any of the cell lines tested (Figure 2a-d). However, as seen in Figure 2a-d, 100 µg/mL or higher concentrations of betahistine significantly increased the number of apoptotic and necrotic cells (HUVEC: Apoptotic cell 26.33%, A549: Apoptotic cells 17.33%, L929: Apoptotic cells 8.6%, and Ishikawa: Apoptotic cells 2.3%). L929 fibroblast and endothelial HUVEC cells were among the most sensitive cell lines (HUVEC: Apoptotic cells 26.33%, A549: 17.33% apoptotic cells), while endometrial Ishikawa cells seemed to be more resistant to betahistine (Ishikawa: 2.3% apoptotic cells) (Figure 2a-d). This result suggests that stromal and endothelial tissues may be at higher risk when betahistine is used at high concentrations. Representative microscopy images from AO/EtBr-stained samples are presented in Figure 3a-h.

Then, we investigated whether or not betahistine treatment could induce changes in cell migration rates. For this purpose, we tested the effect of 25, 50, 100, 250, and 500 µg/mL of betahistine (24 hours) treatment on cell migration rates in L929, A549, HUVEC, and Ishikawa cell lines using the *in vitro* scratch assay technique (Figure 4, 5). Our findings indicated that low-

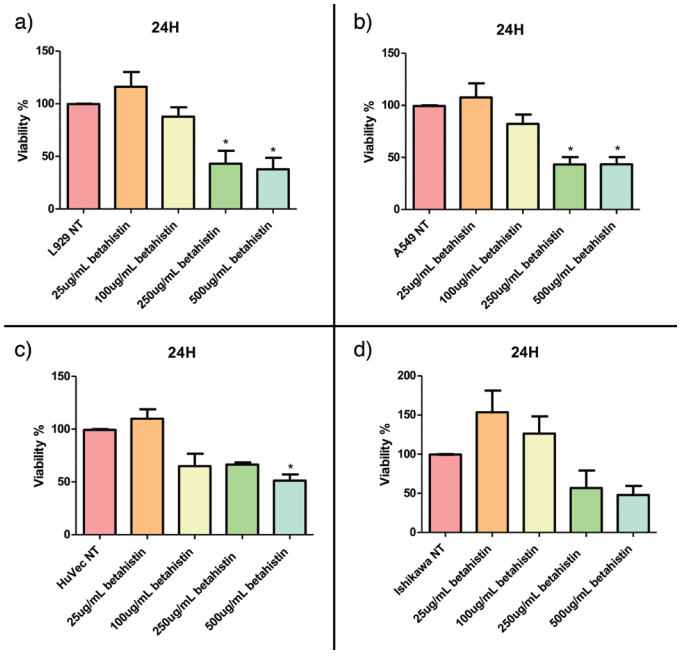

Figure 1. The effect of betahistine on cell proliferation. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. MTT assays were performed 24 hours after treatment with the indicated doses of betahistine. Relative % changes in proliferation rates were compared against the vehicle (NT) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NT: Non-treated

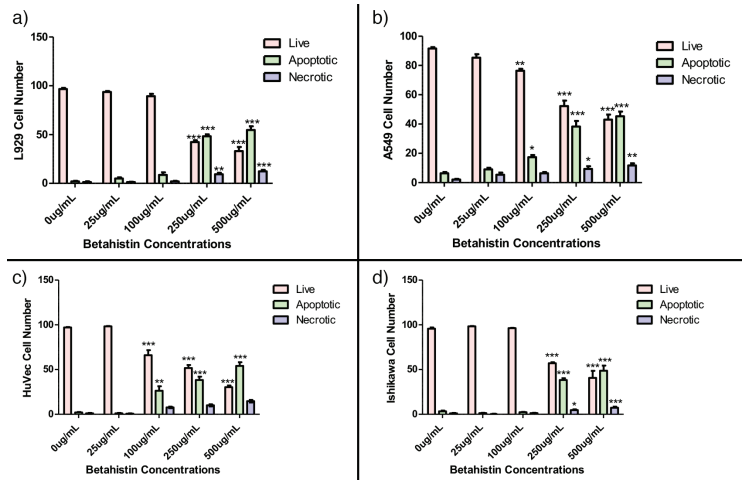

Figure 2. Betahistine induces apoptosis at high concentrations. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. AO/EtBr double staining was performed 24 hours after treatment with the indicated doses of betahistine. Percentage changes in the ratio of apoptotic cells were compared against the vehicle (NT control) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*, **, ***: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, AO/EtBr: Acridine orange/ethidium bromide, NT: Non-treated

dose betahistine treatment (25 µg/mL, 24 hours) induced cell migration rates in both HUVEC (by 81%) and Ishikawa cell lines (by 48%) (Figure 4c, d). In contrast, the cell migration rate was reduced by 67% in the L929 fibroblast cell line, while A549 cells did not seem to be affected by treatment with 25 µg/mL of betahistine for 24 hours (Figure 4a, b). However, as seen in Figure 4a-d, 100 µg/mL or higher doses (24 hours) of betahistine exposure significantly suppressed the cell migration rate in all of the cell lines tested. Representative microscopy images from scratch assay experiments are presented in Figure 5a-l.

DISCUSSION

Betahistine is a commonly prescribed drug for the treatment of vertiginous symptoms related to Ménière's disease.[11] Betahistine is a structural analog of histamine that acts as a weak partial postsynaptic histamine H1 receptor agonist and presynaptic H3 receptor antagonist, with no effect on postsynaptic H2 receptors.[12] The proposed mode of action of betahistine in Menière's disease involves increased blood flow to the inner ear, which in turn shifts the balance of production and re-absorption of endolymph toward absorption.[13] Indeed, Ihler et al.[14] demonstrated that betahistine exerted a dose-dependent effect on the increase in blood flow in cochlear capillaries in Guinea pigs.

In some clinical cases, increasing doses of betahistine are administered for relatively long periods of time up to a year.[15] Although long-term betahistine treatment at high doses is reported to be clinically safe,[16] very little is known about its *in vitro* cytotoxic effects. Betahistine, which has been in clinical use for over 40 years, has shown an excellent safety profile within the dose range of 8-48 mg daily.[17] Only a total of three cases of neoplasm have been reported in relation with the use of betahistine.[18] For example, although histamine is shown to be involved in the regulation of cancer-associated biological processes during cancer development,[19] no data are available for the histamine analog betahistine.

Only a single *in vitro* study suggested that betahistine was not toxic at prescribed doses, and toxicity was observed only at extremely high concentrations (>50 mg/mL) in a nasal epithelial cell line.[6] However, systemic administration of betahistine may also affect other cell types. For example, the possible effect of this drug on other cell types, such as cancer, endothelial, or

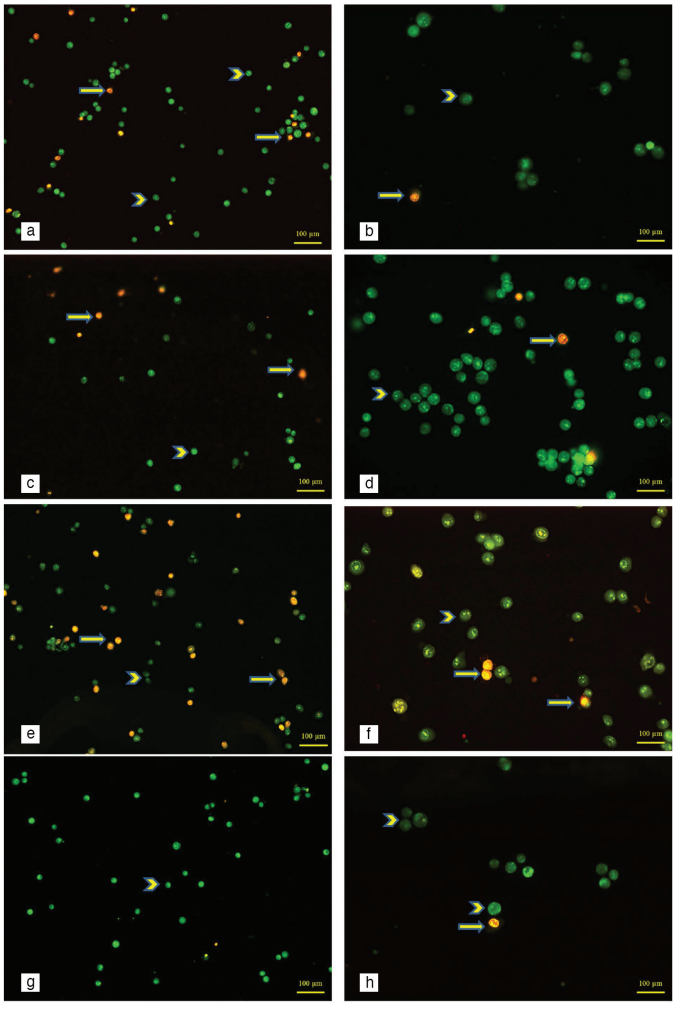

Figure 3. Representative microscope images from AO/EtBr double staining. A) Magnification: 10x L929 fibroblast cells, b) magnification: 40x L929 fibroblast cells, c) magnification: 10x A549 cells, d) magnification: 40x A549 cells, e) magnification: 10x HUVEC cells, f) magnification: 40x HUVEC cells, g) magnification: 10x Ishikawa cells, and h) magnification: 40x Ishikawa cells (100 µg/mL betahistine). Arrows point to apoptotic cells, and arrow heads point to live cells

AO/EtBr: Acridine orange/ethidium bromide, HUVEC: Human umbilical vein endothelial

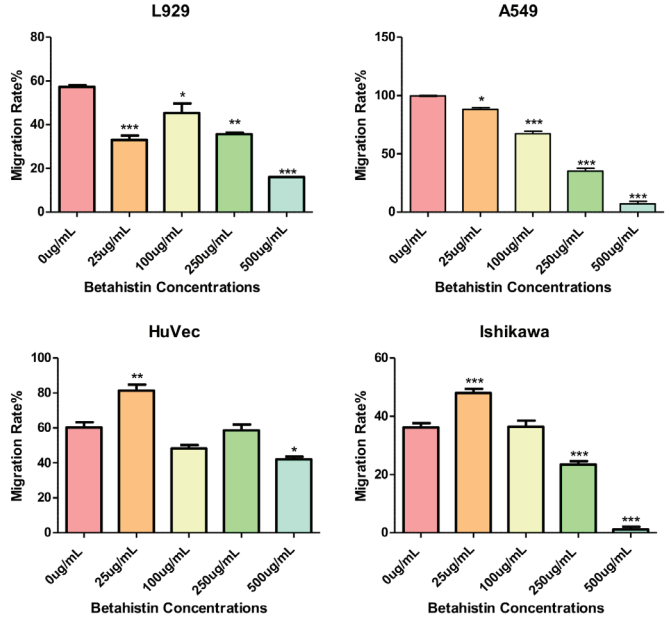

Figure 4. Betahistine reduces the cell migration rate at high concentrations. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. A scratch assay was performed 24 hours after treatment with the indicated doses of betahistine. The rate of migration (how soon the gap was closed) in 24 hours was calculated by measuring the gap at 0 and 24 hours after scratcing the plates. % changes in the migration rates were compared against the vehicle (NT control) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*, **, ***: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, NT: Non-treated

fibroblast cell lines, has not been extensively studied. In this study, we investigated some physiological effects of betahistine on L929 fibroblasts, A549 lung cancer, HUVEC endothelial, and Ishikawa endometrial cell lines.

Previously, Pilicheva et al.[6] reported betahistine toxicity at very high concentrations (>50 mg/mL) in the RPMI 2650 nasal epithelial cell line. In contrast with this previous report, we found that 500 µg/mL (0.5 mg/mL) betahistine treatment resulted in an almost 50% reduction in proliferation rates in all of the cell lines tested (Figure 1). Our findings clearly show that betahistine treatment at 0.1-0.5 mg/mL can inhibit cell proliferation in a cell-type-dependent fashion. These observations imply that betahistine administration may impact the process of wound healing. Interestingly, however, 25 µg/mL betahistine seemed to increase the proliferation rate of Ishikawa cells significantly (Figure 1).

Data from the AO/EtBr double staining protocol suggested that treatment with betahistine (25 µg/mL, for 24 hours) did not induce apoptosis in A549, L929, HUVEC, or Ishikawa cell lines (Figure 2). However, at concentrations of 100 µg/mL or higher, betahistine significantly increased the number of apoptotic and necrotic cells in all of the cell lines tested, and L929 fibroblast and endothelial HUVEC cells were found to be the most sensitive cell lines (Figure 2a-d), suggesting that primarily stromal and endothelial tissues may be affected when betahistine is used at high concentrations. This subject has yet to be investigated,

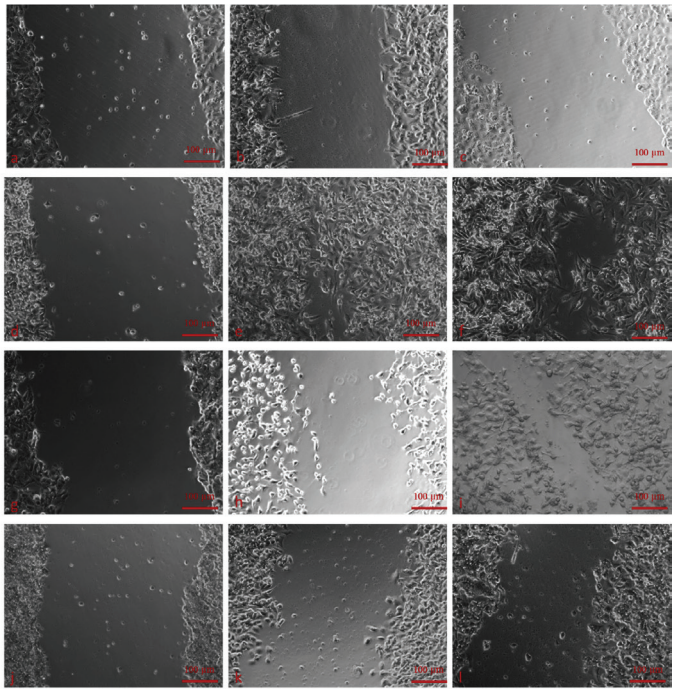

Figure 5. Representative microscope images from the *in vitro* scratch assay. a) 0 hour L929 fibroblast cells, b) 24 hour 0 µg/mL betahistine L929 fibroblast cells, c) 24 hour 25 µg/mL betahistine L929 fibroblast cells, d) 0 hour A549 cells, e) 24 hour 0 µg/mL betahistine A549 cells, f) 24 hour 25 µg/mL betahistine A549 cells, g) 0 hour HUVEC cells, h) 24 hour 0 µg/mL betahistine HUVEC cells, i) 24 hour 25 µg/mL betahistine HUVEC cells, j) 0 hour Ishikawa cells, k) 24 hour 0 µg/mL betahistine Ishikawa cells, and l) 24 hour 25 µg/mL betahistine Ishikawa cells (magnification: 10x)

HUVEC: Human umbilical vein endothelial

and clarification of molecular mechanisms underlying this observation can have important clinical and pharmacological implications.

We also demonstrated that betahistine at ≥100 µg/mL can exert inhibitory effects on the migration rate of all of the cell lines tested in this study (Figure 4). Especially, fibroblasts were among the most sensitive to betahistine treatment. This finding suggests that betahistine administration may have implications in cellular migratory processes such as wound healing or cellular extravasation. Although the underlying molecular mechanisms remains elusive, Tang et al.[20] showed that betahistine suppressed Th17 expansion in lymph nodes of collagen-induced arthritis mice. A betahistine-induced decrease in cell migration rates might be due to the reduced proliferative capacity upon betahistine treatment. Thus, further tests should be performed to clarify whether or not the effect of betahistine on cellular migration is independent of the proliferation rate.

Intriguingly, we found that treatment with 25 µg/mL betahistine-induced cell migration in endothelial (HUVEC) and endometrial (Ishikawa) cell lines (Figure 4). Similarly, the proliferation rates of these cell lines were also increased upon treatment with 25 µg/mL betahistine. These observations point to the possibility that betahistine treatment at low doses can induce proliferation and cell migration rates in certain types of cancer cells. Thus, betahistine treatment can pose a risk for cancer patients (especially for tumors with endometrial origin). However, we could not find any reports investigating the possible link between cancer and betahistine treatment. Thus, further experimental and clinical studies are required to investigate this hypothesis.

Study limitations

In this study, we found that betahistine administration may alter the processes of cellular proliferation, apoptosis, and migration in a cell line- and dose-dependent manner, suggesting that betahistine can potentially affect cellular processes such as wound healing or proliferative properties in certain cell types. However, we were not able to provide any molecular information on the underlying mechanisms that might have affected apoptosis, cellular migration, or proliferation in the cell lines tested. Moreover, animal experiments should also be performed to further test the validity of our *in vitro* observations in living systems.

CONCLUSION

Our findings demonstrate that high doses of betahistine seem to inhibit proliferation and cellular migration and induce apoptosis in HUVEC, human Asian endometrial adenocarcinoma (Ishikawa), pulmonary adenocarcinoma human alveolar epithelial (A549), and murine fibroblast (L929) cell lines. Our preliminary *in vitro* findings suggest that betahistine administration may alter cellular migration and therefore can potentially impact the metastatic properties of some cancer cells in a cell type- and dose-dependent manner. Animal models can be useful for understanding the molecular mechanisms underlying betahistine-induced physiological changes.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Cytotoxic and Antileishmanial Effects of Various Extracts of *Capparis spinosa* L.

Capparis spinosa L.'nin Farklı Ekstrelerinin Sitotoksik ve Antileishmanial Etkileri

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ABSTRACT

Objectives: Cutaneous leishmaniasis (CL) is considered as one of the most critical infections worldwide, in which the protozoa of the genus *Leishmania* infects a person. Today, the common and selective drugs for the treatment of CL are antimonial compounds present some limitations to their usage. The objective of this study is to investigate the cytotoxic and antileishmanial effects of various extracts of *Capparis spinosa* L. on the *in vitro* model.

Materials and Methods: The primary phytochemical analysis of the *C. spinosa* extracts was performed to assess the presence of tannins, alkaloids, saponins, flavonoids, terpenoids, and glycosides. Furthermore, the *in vitro* cytotoxic and antileishmanial effects of *C. spinosa* extracts on *Leishmania tropica* promastigote were evaluated. Additionally, these effects on the J774-A1 macrophage cells by colorimetric cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay were also assessed.

Results: In this study, the findings of primary phytochemical screening of the *C. spinosa* extracts demonstrated the existence of flavonoids, tannins, terpenoids, glycosides, and alkaloids in this plant. Importantly, the findings indicated that the aqueous and methanolic extracts of *C. spinosa* exhibit a high potency to inhibit the growth of *L. tropica* promastigotes with inhibitory concentration 50 values of aqueous and methanolic extracts being 28.5 and 44.6 µg/mL, respectively. Based on the obtained results, *C. spinosa* extracts did not display a considerable cytotoxicity on the J774-A1 macrophage cells.

Conclusion: The obtained findings exhibited remarkable antileishmanial effects of *C. spinosa* extracts on *L. tropica*, thereby indicating the ability of *C. spinosa* as a herbal product to be developed as a new antileishmanial drug. Nevertheless, supplementary investigations will be obligatory to achieve these findings, especially in human subjects.

Key words: Herbal medicines, *in vitro, Leishmania tropic*a, macrophage, promastigote

ÖZ

Amaç: *Leishmania* protozoa türünün insanı enfekte etmesiyle görülen kutanöz leishmania (CL) dünya genelindeki en kritik enfeksiyonlardan biri kabul edilmektedir. Bugün, CL'nin tedavisi için sıklıkla kullanılan ve seçilmiş ilaçlar olan antimonial bileşiklerin kullanımlarında bazı kısıtlamalar vardır. Bu çalışmanın amacı of *Capparis spinosa* L.'nin farklı ekstrelerinin *in vitro* modelde sitotoksik ve antileishmanial etkilerinin incelenmesidir.

Gereç ve Yöntemler: *C. spinosa* ekstrelerinin primer fitokimyasal analizi tannen, alkaloid, saponin, flavonoid, terpenoid ve glikosidlerinin değerlendirmek için yapılmıştır. Ayrıca, *C. spinosa* ekstrelerinin *Leishmania tropica* promastigotu üzerindeki *in vitro* sitotoksik ve antileishmanial etkileri değerlendirilmiştir. Ek olarak, bu etkiler kolorimetrik hücre canlılığı 3-(4,5-dimetil-tiyazolil-2,5-)difeniltetrazolyum bromid yöntemiyle J774-A1 makrofaj hücrelerinde de belirlenmiştir.

Bulgular: Bu çalışmada, *C. spinosa* ekstrelerinin primer fiotokimyasal izlemlenmesi bu bitkide tannen, alkaloid, saponin, flavonoid, terpenoid ve glikosidlerinin varlığını göstermiştir. Önemli olarak, bulgular *C. spinosa*'nın sulu ve metanolik ekstrelerinin sırasıyla 28,5 ve 44,6 µg/mL inhibitor konsantrasyon 50 değerleriyle *L. tropica* promastigotunun büyümesini yüksek bir potens ile inhibe ettiğini göstermiştir. Elde edilen verilere göre, *C. spinosa* ekstreleri J774-A1 makrofaj hücrelerinde belirgin bir toksisite göstermemiştir.

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Sonuç: Elde edilen bulgular *C. spinosa* ekstrelerinin *L. tropica* üzerinde belirgin antileishmanial etkiler gösterdiğini ve *C. spinosa*'nın bir herbal ürün olarak yeni antileishmanial ilaç için geliştirilmesi için kullanılabileceğini göstermiştir. Bununla birlikte, özellikle insanlarda bu verilerin ek araştırmalarla doğrulanması gerekmektedir.

Anahtar kelimeler: Herbal ilaç, *in vitro, Leishmania tropica*, macrofaj, promastigot

INTRODUCTION

In present times, cutaneous leishmaniasis (CL) is one of the main parasitic infections worldwide, in which human beings are infected by the *Leishmania* protozoan parasites. The most important characteristics of this disease are chronic and prolonged ulcers that leave scars even after recovery.[1] Every year, about 1.5 million people become infected with this disease; hence, it can be considered as a main health and economic challenge.[2] Previous studies have demonstrated that in Iran, the common types of CL are anthroponotic CL (*Leishmania tropica*) and zoonotic CL (*L. major*).[3]

Today, the common and selective chemotherapies for CL treatment are antimonial compounds such as meglumine antimoniate and sodium stibogluconate; however, recent studies have suggested some restrictions about the use of these drugs such as excessive side effects and parasitic resistance to these agents.[4,5] Therefore, it is highly believed that the discovery of a new drug with same efficacy to the current agents and even higher than them along with lower toxicity can be a priority for researchers.

From centuries ago, the use of natural compounds has been considered for the treatment of several diseases such as infectious ones.[6,7] *Capparis spinosa* L. from the family of *Capparidaceae*, which is called "Kabar" in Persian, widely grows in the various parts of the world, especially in Iran. Previous studies have shown that various parts of this plant represent some biological and medicinal effects such as antimicrobial, antioxidant, and anticancer activities.[8] Therefore, the objective of this study is to investigate the *in vitro* cytotoxic and the leishmanicidal activities of extracts of *C. spinosa*.

MATERIALS AND METHODS

Parasite strain

Here, we obtained the *L. tropica* (MHOM/IR/2002/Mash2) strain from the Leishmaniasis Research Center (Kerman, Iran). The promastigotes were cultured in the NNN medium, and then subcultured in RPMI-1640, complemented with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 15% heat-inactivated fetal calf serum.

Collection of plant materials

We collected the aerial parts of *C. spinosa* from the mountains of Lorestan Province, Iran. The materials were recognized by a botanist, and a voucher specimen was deposited at the herbarium of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.

Preparation of extracts

After chopping the fruits into smaller portions and drying them in shade, the fruits were powdered. Afterward, the powdered materials were extracted using the technique of percolation with methanol and water for 3 days at 21°C. The obtained extracts were allowed to pass through a filter paper to remove the excess particles. Finally, by means of a rotary evaporator (Heidolph, Germany), the extracts were vacuum concentrated at 50°C and kept at -20°C until testing.[9-11]

Phytochemical analysis

The primary phytochemical analysis of the both *C. spinosa* extracts was conducted to assess the presence of tannins, alkaloids, flavonoids, saponins, terpenoids, and glycosides via following reagents and chemicals:[12] Alkaloids with Mayer and Dragendorff's reagents, flavonoids by using Mg and HCl, tannin with 1% gelatin and 10% NaCl solutions, terpenoids with chloroform, and concentrated sulfuric acid, glycosides with $FeCl_2$ and H_2SO_4, and saponin with the ability of producing suds.

Antileishmanial effects of C. spinosa extracts

To determine the antileishmanial effects of *C. spinosa* extracts, we used the colorimetric cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method as explained by some researchers.[13-15] After adjusting the promastigotes from the logarithmic growth phase to 10^6 cells per each mL, 0.1 mL of suspension of promastigotes was put in a 96-well plate. In the next step, promastigotes were treated with the various concentrations of each plant extract (0-200 µg/mL) at 25°C±1°C for 3 days. After finishing the exposure time, 0.01 ml of MTT solution (5 mg/mL) was poured into wells and again incubated at 25°C for 4 hours. Next, to solve the formazan crystals and subsequently generate the purple color, 0.1 mL of isopropanol was added into wells. At the end, an ELISA reader (BioTek-ELX800) was used and the absorbance level of wells was determined at 490 nm. The complete medium containing promastigote and no extract was considered as a positive control, whereas a complete medium with no parasite and extract considered as a negative control (blank).

Cytotoxic effects

To assess the cytotoxic effects, the J774-A1 cells cultured at Dulbecco's modified eagle's medium were adjusted at $5x10^5$ cell per mL. Then, they were treated in 96-well plates with different concentrations of each extract (0-5.000 µg/mL) at 37°C in 5% CO_2 for 48 hours. Finally, the cytotoxic effects of extracts were measured by the colorimetric MTT assay as mentioned above.[15-17]

Statistical analysis

We performed experiments in triplicates. The collected data were analyzed by SPSS software version 22.0. Moreover,

[cytotoxic concentration for 50% (CC_{50})of macrophages] and inhibitory concentration 50 (IC_{50}) (50% ICs for promastigotes) were measured by the linear regression method. Furthermore, the selectivity index (SI) was measured as the equation of CC_{50} for J774-A1/IC_{50} for promastigotes to assess the toxicity and activity of *C. spinosa* extracts. Additionally, One-Way ANOVA test was applied to assess the variations among the test and control groups. Furthermore, $p<0.05$ was considered to be statistically significant for this study.

RESULTS

Phytochemical analysis

In this study, the findings referred to the primary phytochemical screening of the *C. spinosa* methanolic and aqueous extracts demonstrated the presence of tannins, flavonoids, terpenoids, glycosides, and alkaloids in this plant.

Antileishmanial effects of C. spinosa extracts

Figure 1 shows the antileishmanial effects of different extracts of *C. spinosa* on *L. tropica* promastigote. The obtained findings showed that different extracts of *C. spinosa*, mostly methanolic extract, displayed effective antileishmanial effects on *L. tropica* promastigote in a dose-dependent manner ($p<0.05$). The obtained IC_{50} values of aqueous and methanolic extracts on *L. tropica* promastigote were 28.5 and 44.6 µg/mL, respectively. Meglumine antimoniate also as control drug revealed effective antileishmanial effects with the IC_{50} value of 35.7 µg/mL on *L. tropica* promastigotes.

Cytotoxic activity

Based on the obtained results, *C. spinosa* extracts did not display considerable cytotoxicity on the J774-A1 macrophage cells. As shown in Table 1, the CC_{50} values of aqueous and methanolic extracts of *C. spinosa* on J774-A1 macrophage cells were 261.3 and 373.6 µg/mL, respectively. Table 1 presents the SI values of different extracts of *C. spinosa*.

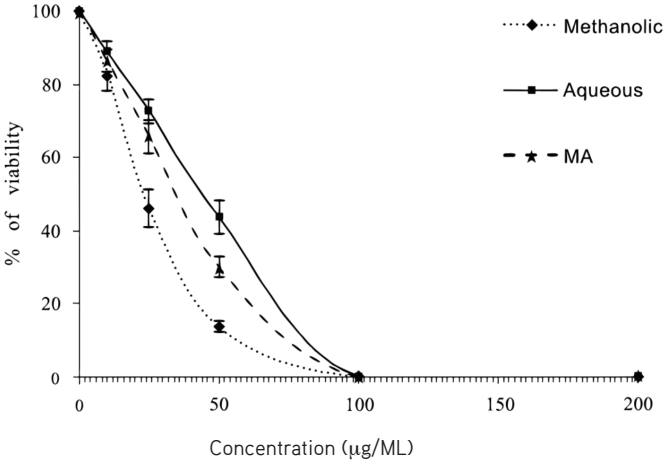

Figure 1. Antileishmanial effects of various extracts of *Capparis spinosa* on the viability rate of *Leishmania tropica* promastigote. Data are expressed as mean ± SD (n=3)

SD: Standard deviation

DISCUSSION

Since long ago, herbal medicines have been recognized as one of the main therapeutic agents worldwide. In recent years, the therapeutic and preventive use of medicinal plants has attracted increased attention because of low post-consumption complications and the various biological properties.[18-20]

So far, researchers have demonstrated the antileishmanial effects of a broad spectrum of medicinal herbs, such as black cumin, garlic, savory, pistacia, berberis, myrtle, periwinkle, black beans, and others, on CL.[21] Although previous investigations have reported a number of pharmacological benefits of *C. spinosa* such as antioxidant, anticancer, and antibacterial activities, there is no documented study regarding the antiparasitic effects of this plant. Thus, we decided to investigate the *in vitro* antileishmanicidal and cytotoxic activities of *C. spinosa* extracts. The results revealed that different extracts of *C. spinosa*, mostly methanolic extract, displayed effective antileishmanial effects on *L. tropica* promastigote in a dose-dependent manner ($p<0.05$). The obtained IC_{50} values for methanolic and aqueous extracts on *L. tropica* promastigote were 28.5 and 44.6 µg/mL, respectively.

In this study, the results of the primary phytochemical analysis of the *C. spinosa* extracts indicated the existence of tannins, flavonoids, terpenoids, glycosides, and alkaloids in this plant. Previous studies on phytochemical analysis *C. spinosa* have proven that this plant contains high amounts of bioactive components, such as alkaloids, flavonoids, steroids, terpenoids, and tocopherols.[8] Moreover, a study conducted by Tlili et al.[22] on the phytochemical analysis of *C. spinosa* showed that aerial parts of this plants are rich in quaternary ammonium compounds, alkaloids, phenolic compounds, and glycosides, such as glucosinolates, further indicating various pharmacological properties useful in modern medicine.

Regarding the antileishmanial effects of polyphenolic compounds, Antwi et al.[23] demonstrated that rosmarinic acid (as a phenolic compound) exerted antileishmanial effect through iron chelation that results in the morphological changes and cell cycle arrest against the promastigote and intracellular amastigote forms of *L. donovani.* Monzote et al.[24] demonstrated the potent antileishmanial activity of ten phenolic compounds including cinnamic acid, coumaric acid isomers, gallic acid, sinapic acid, gentisic acid, morin, rutin extrasynthese, and ellagic acid, vanillic acid against intracellular amastigotes

Table 1. CC_{50} values of various extracts of *Capparis spinosa* on the J774-A1 macrophage cells as well as their IC_{50} and selectivity index values on *Leishmania tropica* promastigotes

Drug	IC_{50} (µg/mL)	CC_{50} (µg/mL)	SI
Methanolic extract	28.5	261.3	9.1
Aqueous extract	44.6	373.6	8.4
Meglumine antimoniate	35.7	261.3	7.3

SI: Selectivity index, IC_{50}: Inhibitory concentration 50, CC_{50}: Cytotoxic concentration 50%

as well as experimental CL in BALB/c mice infected with *L. amazonensis*.

Regarding antileishmanial activity of alkaloids, Delorenzi et al.[25] showed that indole alkaloid coronaridine have shown considerable antileishmanial effects, which led to the growth of promastigote and amastigote forms. Through change in their mitochondrial functions. Tasdemir et al.[26] also demonstrated that some flavonoid compounds exert potent antileishmanial and antitrypanosomal effects against *Trypanosoma brucei rhodesiense, Trypanosoma cruzi*, and *L. donovani in vitro* and *in vivo.*

Arruda et al.[27] demonstrated that nerolidol as a sesquiterpene (terpenoids) prevented the growth of *L. amazonensis, L. braziliensis*, *L. chagasi* promastigotes, and *L. amazonensis* amastigotes with IC_{50} values of 85, 74, 75, and 67 μM, respectively; whereas a reduction of lesion sizes was observed in *L. amazonensis*-infected BALB/c mice treated with nerolidol. Considering the mechanisms of the antimicrobial action of polyphenolic compounds, some studies have shown that antimicrobial mechanisms of polyphenolic compounds are associated with their lipophilia as well as their effects on protein synthesis.[28-31] Previously, Puupponen-Pimiä et al.[32] have shown that polyphenolic compounds, through their disruptive action on the external membrane, can inhibit the growth of bacteria.[32-34] Therefore, although the accurate antileishmanial mechanisms of *C. spinosa* is unclear, we can suggest that antiparasitic effects of this plant is referred to the existence of polyphenolic compounds in it. Here, we found that *C. spinosa* extracts did not display considerable cytotoxicity on the J774-A1 macrophage cells; moreover, the SI values above ten of methanolic and aqueous extracts of *C. spinosa* revealed their immunity against the macrophages and specificity to the parasite, according to Weninger et al.[35,36]

CONCLUSION

The obtained findings exhibited remarkable antileishmanial effects of *C. spinosa* extracts on *L. tropica,* thereby indicating the ability of *C. spinosa* as a natural ingredient to create a new antileishmanial drug. Nevertheless, supplementary investigations will be obligatory to achieve these findings, especially in human subjects.

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ORIGINAL ARTICLE

QSAR Studies on Neuraminidase Inhibitors as Anti-influenza Agents

Anti-influenza Ajanları Olarak Nöraminidaz İnhibitörlerinin QSAR Çalışmaları

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ABSTRACT

Objectives: The present study aimed to establish significant and validated quantitative structure-activity relationship (QSAR) models for neuraminidase inhibitors and correlate their physicochemical, steric, and electrostatic properties with their anti-influenza activity.

Materials and Methods: We have developed and validated 2D and 3D QSAR models by using multiple linear regression, partial least square regression, and k-nearest neighbor-molecular field analysis methods.

Results: 2D QSAR models had q^2: 0.950 and pred_r^2: 0.877 and 3D QSAR models had q^2: 0.899 and pred_r^2: 0.957. These results showed that the models werere predictive.

Conclusion: Parameters such as hydrogen count and hydrophilicity were involved in 2D QSAR models. The 3D QSAR study revealed that steric and hydrophobic descriptors were negatively contributed to neuraminidase inhibitory activity. The results of this study could be used as platform for design of better anti-influenza drugs.

Key words: QSAR, neuraminidase inhibitors, thiazolidine-4-carboxylic acid derivatives, anti-influenza activity

ÖZ

Amaç: Bu çalışma nöraminidaz inhibitörlerinin belirgin ve valide nicel yapı-aktivite ilişkisi (QSAR) modellerini kurmayı ve bu bileşiklerin fizikokimyasal, sterik ve elektrostatik özelliklerini anti-influenza aktiviteleriyle korele etmeyi amaçlamıştır.

Gereç ve Yöntemler: Çoklu regresyon, parsiyel en düşük kare regresyon ve k-en yakın komşu moleküler alan analizi yöntemlerini kullanarak 2D ve 3D QSAR modellerini geliştirdik ve valide ettik.

Bulgular: Geliştirilen 2D QSAR modeli için q^2: 0,950 ve pred_r^2: 0,877 bulunurken, 3D QSAR modeli için q^2: 0,899 ve pred_r^2: 0,957 bulundu. Bu sonuçlar modellerinin tahmin gücünün olduğunu gösterdi.

Sonuç: Hidrojen sayısı ve hisrofilisite gibi parametreler 2D QSAR modellerine dahil edildi. 3D QSAR modelleri sterik ve hisrofobik tanımlayıcıların nöraminidaz inhibitör aktivitesine negatif etki ettiği belirlendi. Bu çalışmanın sonuçları influenzaya karşı ilaç tasarlamak için bir platform olarak kullanılabilir.

Anahtar kelimeler: QSAR, nöraminidaz inhibitörleri, tiyazolidin-4-karboksilik asit deriveleri, anti-influenza aktivitesi

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INTRODUCTION

Quantitative structure-activity relationship (QSAR) is a technique of indirect drug designing. It is a method of quantification of the relationship of structure with biological activities of a set of molecules having common parent structure and useful in lead optimization. Magnitudes of particular physical properties are considered in classical QSAR. Steric, electrostatic, and hydrophobic properties are covered in 3D QSAR. The objective of the current study was to utilize the reported biological data of a series of anti-influenza compounds to develop predictive QSAR models and to explore the relationship between the ligand properties and biological activity.

Influenza virus is the causative agent for the contagious respiratory infectious disease influenza. Influenza A, B, and C are the three types of flu virus. The worst influenza pandemic occurred in 1918, and it caused 40-100 million deaths worldwide.[1] Recently new subtypes such as H7N7 and H7N2, H9N2, and H7N9 have also been identified to cause human infection.[2-4] The four major classes of anti-influenza drugs available now are inhibitors of hemagglutinin, M2 ion channel blockers, inhibitors of viral RNA polymerase, and inhibitors of neuraminidase.[5]

The number of QSAR studies[6-12] has been reported for various classes of influenza inhibitors. In the current study, we had selected thiazolidine-4-carboxylic acid derivatives to provide structural insight responsible for selectivity of these derivatives toward influenza by QSAR analysis. Due to their high structural diversity and broad-type biological activity, these compounds were selected for the present study. The developed 2D and 3D QSAR models could be used to design new anti-influenza compounds.

MATERIALS AND METHODS

CS Chem Office 2004 (Cambridge Soft Corp., Cambridge, USA) and Vlife MDS 4.3 (VLife Sci. Tech. Lim, Pune, India) are the modeling software used in the present study. The neuraminidase inhibition activity (pIC_{50}: -log10 IC_{50}) of 28 thiazolidine-4-carboxylic acid derivatives (Table 1) was taken from the research reported by Liu et al.[13]

Energy minimization

Using CS Chem Office, the structure of thiazolidines was sketched by, and the 3D structure optimization was done in Vlife MDS by following the method reported by Veerasamy et al.[14] Merck molecular force field energy minimized stable structure of individual compound was stored as Sybyl.mol2 files and used to compute various 2D independent descriptors.

2D QSAR analyses

An auto-scaling method was used to reduce the number of descriptors to 200.[14] The data set was split into training set and prediction set by adopting the sphere exclusion (dissimilarity values 2 and 2.5) and random selection methods (10 trials, 70%, 75%, 80%, and 85%).[15] 2D QSAR equations using multiple linear regression (MLR) and partial least squares regression (PLS) methods were built as per the method reported by Veerasamy et al.[14]

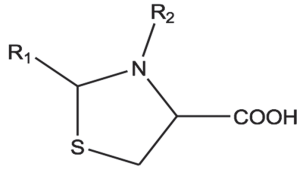
Model quality and validation

The methods compiled by Veerasamy et al.[14] were used to check the model quality and validation of the QSAR models.[16]

3D QSAR analyses

Compound 23 was used as a scaffold to align the molecules using template alignment method. The method reported by

Table 1. The structures of neuraminidase inhibitor derivatives with their activities



Compound no	R_1	R_2	pIC_{50}
1	C_6H_5-	H	4.672
2	(2-OH) C_4H_4-	H	4.695
3	(2-COOH) C_4H_4	H	4.742
4	(4-CN) C_4H_4	H	4.631
5	(2-NO_2) C_4H_4-	H	4.648
6	(2-OH, 3-CH_3O) C_4H_3-	H	4.91
7	C4H3O-	H	4.366
8	C_6H_5-	H	5.123
9	(2-OH) C_4H_4-	$ClCH_2CO$-	5.234
10	(2-COOH) C_4H_4	$ClCH_2CO$-	4.971
11	(4-CN) C_4H_4	$ClCH_2CO$-	5.063
12	(2-NO_2) C_4H_4-	$ClCH_2CO$-	5.116
13	(2-OH, 3-CH_3O) C_4H_3-	$ClCH_2CO$-	5.101
14	C_4H_3O-	$ClCH_2CO$-	4.889
15	C_6H_5-	$PhCH_2CO$-	5.917
16	(2-OH) C_4H_4-	$PhCH_2CO$-	6.187
17	(2-COOH) C_4H_4	$PhCH_2CO$-	5.717
18	(4-CN) C_4H_4	$PhCH_2CO$-	5.607
19	(2-NO_2) C_4H_4-	$PhCH_2CO$-	5.728
20	(2-OH, 3-CH_3O) C_4H_3-	$PhCH_2CO$-	5.790
21	C_4H_3O-	$PhCH_2CO$-	5.539
22	C_6H_5-	NH_2CH_2CO-	6.276
23	(2-OH) C_4H_4-	NH_2CH_2CO-	6.678
24	(2-COOH) C_4H_4	NH_2CH_2CO-	6.553
25	(4-CN) C_4H_4	NH_2CH_2CO-	6.092
26	(2-NO_2) C_4H_4-	NH_2CH_2CO-	5.991
27	(2-OH, 3-CH_3O) C_4H_3-	NH_2CH_2CO-	6.854
28	C_4H_3O-	NH_2CH_2CO-	6.009

Veerasamy et al.[14] was used to generate rectangular grid around the aligned molecules. The selected field descriptors were electrostatic, steric, and hydrophobic. For the electrostatic and steric field, 10.0 and 30.0 kcal/mole were used as the cutoff values.[14]

"A methyl probe of charge +1 at the lattice points of the grid was used to compute steric, electrostatic and hydrophobic interaction energies".[14] The k-nearest neighbor (kNN)-molecular field analysis and PLS methods with each one of the following variable selection methods (stepwise forward-backward) or (genetic algorithm) or simulated annealing) were used to generate 3D QSAR models. The variable selection methods were discussed somewhere else.[14] The methods compiled by Veerasamy et al.[14] were used to validate the 3D QSAR models.[16]

RESULTS AND DISCUSSION

2D QSAR

A data set of 28 thiazolidines and their influenza neuraminidase inhibitory (pIC_{50}) activity in Table 1 was utilized in the present *in silico* study. Two of the best and significant models obtained by using various feature selection and development methods were equations (1) and (2). The used criteria were 80% random selection, stepwise forward-backward variable selection, and MLR.

pIC_{50}: 2.704+0.157 (±0.004) hydrogen count - 6.833 (±0.477) SK average

Hydrophilicity + 0.314 (±0.047) SsssNE-index - 0.279 (±0.050) SsCH3E-index equation (1)

Test set compounds: 1, 6, 16, 21, 26, 27

n=22, r^2: 0.968, r^2 se: 0.132, q^2: 0.950, q^2 se: 0.165, $F_{4,17}$: 128.955, pred r^2: 0.877, pred_r^2 se: 0.308, Z score r^2: 6.941, Z score Q^2: 4.150, best rand R^2: 0.518, best rand Q^2: 0.158

pIC_{50}: 3.139+1.0825 T_N_N_3 - 0.1862 SsCH3E-index + 0.1566 hydrogens count equation (2)

Test set compounds: 3, 11, 16, 19, 22, 27

n=22, r^2 = 0.945, r^2 se: 0.161 q^2: 0.923, q^2 se: 0.190, $F_{3,18}$: 164.005, pred r^2: 0.908, pred_r^2 se: 0.281, Z score r^2: 8.915, Z score Q^2: 7.203, best rand R^2: 0.526, best rand Q^2: 0.340

Equation (1) could explain 96.8% and predict 87.7%, and equation (2) could explain 94.5% and predict 90.8% of the variance of the influenza virus neuraminidase inhibitory data. Thus, the selected good model was equation (1). The absence of intercorrelation between the descriptors was also observed. The parameters (hydrogen count, SK average hydrophilicity, SsssNE-index, SsCH3E-index) were involved, and the calculated influenza virus neuraminidase inhibitory activity by equation (1) is given in Table 2. The correlation between descriptors in 2D QSAR model equation (1) is given in Table 3. The correlation of experimental and predicted activities is graphically represented in Figure 1.

The good internal prediction of selected model was exhibited by q^2: 0.950, and the external prediction power was also confirmed by pred r^2: 0.877 (pred r^2 >0.6). The low randomized r^2 (0.118) and q^2 (0.158) values confirmed the robustness of the model, and the results were not due to a chance correlation.

The positive contribution of descriptor hydrogen count in the selected model clearly suggests that influenza virus neuraminidase inhibitory activity could be increased with an increase in the number of hydrogen atoms in a compound. The SK average hydrophilicity is influencing the activity variation and is indirectly proportional to the activity. The SK average hydrophilicity reveals the importance of average hydrophilic value on the Van der Waals surface. The SsssNE-index has positive effect on the activity. It is an electrotopological state index descriptor. The SsssNE-index highlights the significance of the number of nitrogen atoms connected with three single bonds in a molecule. The SsCH3E-index has a positive effect on the activity, and it shows the importance of the number of -CH_3 groups connected with one single bond in a molecule.

3D QSAR

The criteria used were 80% random training and test selection method, stepwise forward-backward variable selection, and kNN method.

pIC_{50}: S_775 (-0.229-0.163); H_582 (-0.056-0.011) equation (3)

Test set compounds: 1, 3, 10, 11, 26

k-nearest neighbour: 4

n=22, q^2: 0.896, q^2 se: 0.232, pred r^2: 0.931, pred_r^2 se: 0.182

pIC_{50}: H_682 (-0.060-0.016); S_775 (-0.229-0.128) equation (4)

Test set compounds: 1, 3, 10, 11, 26

k-nearest neighbour: 4

n=23, q^2: 0.899, q^2 se: 0.224, pred r^2: 0.957, pred_r^2 se: 0.153

Equation (3) could predict 93.1%, and equation (4) could predict 95.7% of the variance of the influenza virus neuraminidase inhibitory data. Thus, the selected good model was equation (4). The parameters involved in the selected model (steric and

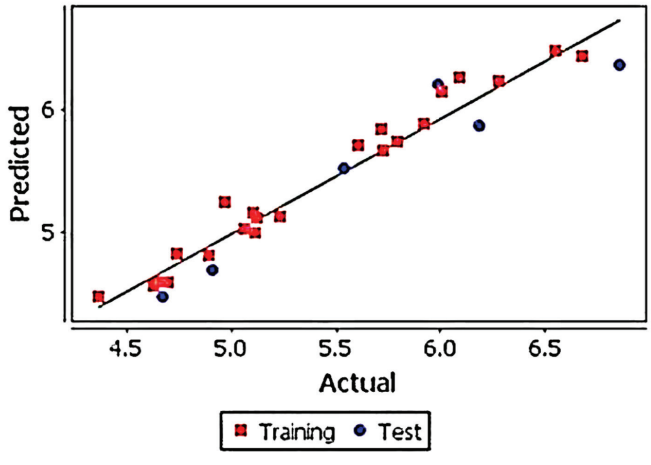


Figure 1. Fitness plot between the experimental and predicted activities for 2D QSAR model equation (1)

QSAR: Quantitative structure-activity relationship

Table 2. Descriptors and predicted activity of 2D QSAR model equation (1)

Compound no	Hydrogen count	SK average hydrophilicity	SsCH3E-index	SsssNE-index	Actual activity (pIC_{50})	Predicted activity (pIC_{50})
1a	11	-0.007	0	0	4.672	4.477
2	11	-0.025	0	0	4.695	4.598
3	11	-0.059	0	0	4.742	4.833
4	10	-0.044	0	0	4.631	4.573
5	10	-0.048	0	0	4.648	4.602
6a	13	-0.057	1.573	0	4.91	4.694
7	9	-0.053	0	0	4.366	4.475
8	12	0	0	1.675	5.123	5.112
9	12	-0.008	0	1.587	5.234	5.137
10	12	-0.027	0	1.51	4.971	5.248
11	11	-0.015	0	1.605	5.063	5.033
12	11	-0.015	0	1.469	5.116	4.993
13	14	-0.029	1.539	1.552	5.101	5.156
14	10	-0.01	0	1.492	4.889	4.806
15	17	0	0	1.655	5.917	5.889
16a	17	0	0	1.567	6.187	5.862
17	17	0	0	1.49	5.717	5.837
18	16	0	0	1.585	5.607	5.711
19	16	0	0	1.449	5.728	5.668
20	19	0	1.536	1.532	5.79	5.735
21a	15	0	0	1.472	5.539	5.518
22	14	-0.125	0	1.509	6.276	6.228
23	14	-0.159	0	1.421	6.678	6.43
24	14	-0.169	0	1.344	6.553	6.477
25	13	-0.156	0	1.44	6.092	6.263
26a	13	-0.153	0	1.303	5.991	6.198
27a	16	-0.167	1.513	1.386	6.854	6.364
28	12	-0.167	0	1.326	6.009	6.140

a: Indicates test set compounds, QSAR: Quantitative structure-activity relationship

Table 3. Correlation matrix for descriptors in 2D QSAR model equation (1)

	pIC_{50}	Hydrogen count	SK average hydrophilicity	SsssNE-index	SsCH3E-index
pIC_{50}	1	-	-	-	-
Hydrogen count	0.685	1	-	-	-
SK average hydrophilicity	-0.584	0.066	1	-	-
SsssNE-index	0.623	0.567	-0.004	1	-
SsCH3E-index	0.111	0.360	-0.061	0.006	1

QSAR: Quantitative structure-activity relationship, SK: S- SlogP, K- Kellog

hydrophobic) and the calculated influenza virus neuraminidase inhibitory activity by equation (4) are given in Table 4 and correlation in Table 5. Figure 2 shows the contribution plot for steric and hydrophobic interactions in lattice. The good internal prediction of the model was confirmed by q^2: 0.899. The external prediction power of the model was confirmed by pred r^2: 0.957 (pred r^2 >0.6).

Hydrophobic descriptors like H_682 with a negative range around the chemical structure of neuraminidase inhibitor indicate that more hydrophobicity is not favorable on those sites for the influenza virus neuraminidase inhibitory activity of the compounds. Steric descriptor like S_775 with a negative range around the chemical structure of neuraminidase inhibitor indicates that the bulky groups are not favorable on those sites for the influenza virus neuraminidase inhibitory activity. Figure 3 shows the plots of predicted vs. observed values of pIC_{50}.

Table 4. Descriptors and predicted activity of 3D QSAR model equation (4)

Compound no	S_775	H_682	Actual activity (pIC_{50})	Predicted activity (pIC_{50})
1a	-0.049	0.075	4.672	4.585
2	-0.045	0.039	4.695	4.638
3	-0.035	0.015	4.742	4.723
4	-0.05	0.033	4.631	4.653
5a	-0.036	0.026	4.648	4.650
6	-0.042	0.015	4.91	4.585
7	-0.052	0.034	4.366	4.720
8	-0.14	0.107	5.123	5.364
9a	-0.131	0.078	5.234	5.057
10	-0.129	0.047	4.971	5.089
11a	-0.134	0.064	5.063	5.144
12a	-0.127	0.058	5.116	5.087
13	-0.136	0.049	5.101	5.092
14	-0.102	0.088	4.889	5.144
15	-0.124	0.176	5.917	5.762
16	-0.076	0.169	6.187	5.758
17	-0.103	0.14	5.717	5.874
18	-0.127	0.14	5.607	5.621
19	-0.171	0.141	5.728	5.589
20	-0.067	0.126	5.79	5.602
21	-0.152	0.211	5.539	5.744
22	-0.164	-0.016	6.276	6.331
23	-0.229	-0.029	6.678	6.429
24	-0.163	-0.06	6.553	6.261
25	-0.122	-0.034	6.092	5.993
26a	-0.171	-0.05	5.991	6.246
27	-0.26	-0.062	6.854	6.386
28	-0.128	-0.046	6.009	6.018

a: Indicates test set compounds, QSAR: Quantitative structure-activity relationship

CONCLUSION

Significant and predictive QSAR models were developed for thiazolidine neuraminidase inhibitor. 2D QSAR model evidenced the influence of structural properties and neuraminidase inhibitory activity of thiazolidines. The engendered 3D QSAR contour maps evidenced the influence of ligand features on the enzyme neuraminidase. It is concluded that modifications in the

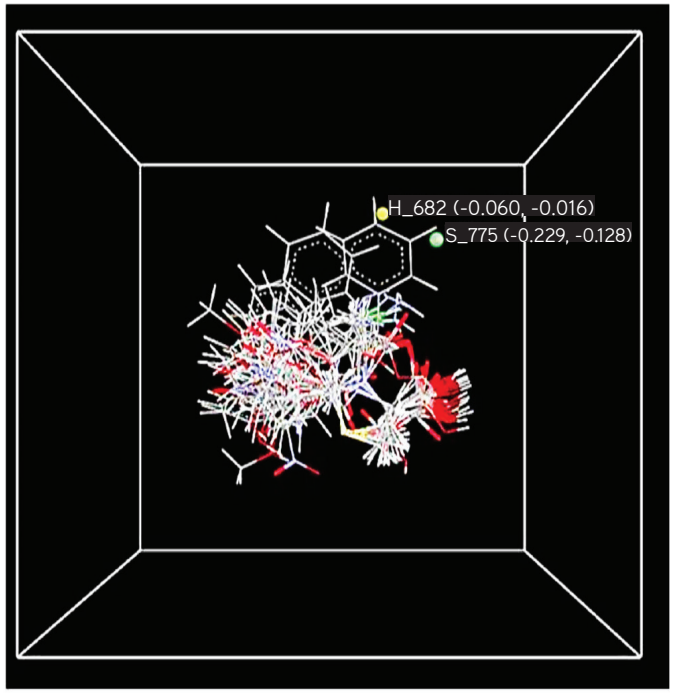


Figure 2. Contribution plot for steric and hydrophobic interactions in lattice for 3D QSAR model equation (4)

QSAR: Quantitative structure-activity relationship

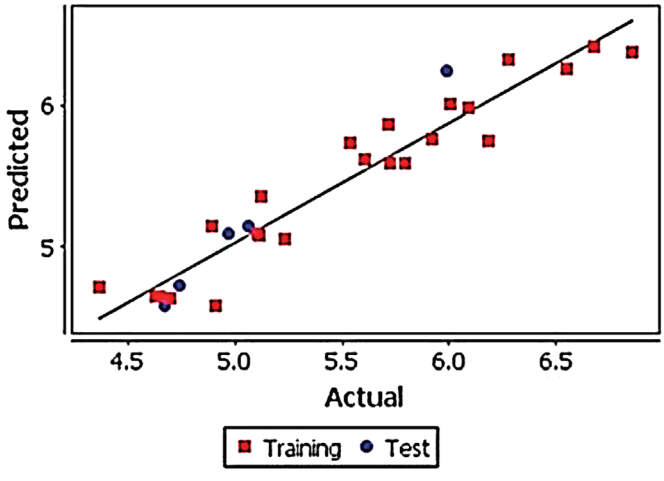


Figure 3. Fitness plot between the experimental and predicted activities for 3D QSAR model equation (4)

QSAR: Quantitative structure-activity relationship

Table 5. Correlation matrix for descriptors in 3D QSAR model equation (4)

	pIC_{50}	H_682	S_775
pIC_{50}	1	-	-
H_682	-0.219	1	-
S_775	-0.756	0.232	1

QSAR: Quantitative structure-activity relationship, pIC_{50}: Negative logarithmic concentration of 50% inhibition, H_682: Hydrophobic descriptor at point 682, S_775: Steric descriptor at point 775

structure of thiazolidines based on the information obtained from the present study could lead to new thiazolidines with potent neuraminidase inhibitory activity. Further in silico tests, such as molecular docking, and kinetic and dynamic studies can be carried out for a better understanding of the mechanism of action. The field is also further open for designing, synthesis, and biological evaluation of potent anti-influenza virus compounds, pharmacokinetic studies, and clinical studies to establish those molecules as drug.

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ORIGINAL ARTICLE

Comparative Morphological and Anatomical Studies on *Morus* Species (Moraceae) in Turkey

Türkiye'deki *Morus* Türleri (Moraceae) Üzerinde Karşılaştırmalı Morfolojik ve Anatomik Çalışmalar

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ABSTRACT

Objectives: *Morus alba* L., *Morus nigra* L., and *Morus rubra* L. are widely cultivated in many countries due to their nutritive, economic, and medicinal value. In this study, comparative morphological and anatomical studies on three common *Morus* L. species found in Turkey were carried out. According to the results, differences regarding the morphological and anatomical features of these species were described, and the data were displayed in detailed photographs.

Materials and Methods: Specimens collected from different provinces of Turkey were studied. In the anatomical studies, investigations were performed on transversal and superficial sections of the leaves. All sections were stained with chloral hydrate and Sartur solution and were then examined using an Olympus BH2 light microscope.

Results: Significant diagnostic characteristics were found, such as trichome types, stomatal measurements, the stomatal index, and the density ratio of the parenchyma and collenchyma layers. Some morphological features of the leaves also showed prominent differences.

Conclusion: Our results may contribute to the taxonomy of *Morus* species for future work and be helpful in species diagnosis.

Key words: Moraceae, *Morus*, anatomy, morphology, Turkey

ÖZ

Amaç: *Morus alba* L., *Morus nigra* L. ve *Morus rubra* L. türleri gıdai, ekonomik ve tıbbi değerleri nedeniyle birçok ülkede yaygın olarak yetiştirilmektedir. Bu çalışmada, Türkiye'de bulunan *Morus* L. türleri üzerinde karşılaştırmalı morfolojik ve anatomik incelemeler yapılmıştır. Sonuçlara göre, türlerin morfolojik ve anatomik özellikleri ile ilgili farklılıklar tanımlanmış ve elde edilen veriler detaylı fotoğraflarla gösterilmiştir.

Gereç ve Yöntemler: Türkiye'nin farklı illerinden toplanan örnekler çalışılmıştır. Anatomik çalışmalarda, yaprakların enine ve yüzeysel kesitleri üzerinde incelemeler gerçekleştirilmiştir. Tüm kesitler kloralhidrat ve Sartur çözeltisi ile boyanmış ve ardından Olympus BH2 ışık mikroskobu kullanılarak incelenmiştir.

Bulgular: Trikom tipleri, stomatal ölçümleri, stomal indeksi, parenkima ve kollenkima tabakalarının yoğunluk oranları gibi belirgin diagnostik karakterler bulunmuştur. Yaprakların bazı morfolojik özellikleri de belirgin farklılıklar göstermiştir.

Sonuç: Sonuçlarımız, gelecek çalışmalar için *Morus* türlerinin taksonomisine katkıda bulunabilir ve türlerin ayırt edilmesinde yardımcı olabilir.

Anahtar kelimeler: Moraceae, *Morus*, anatomi, morfoloji, Türkiye

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INTRODUCTION

The genus *Morus* L. belongs to the Moraceae (mulberry) family, which contains 37 genera and nearly 1,100 species distributed throughout tropical and temperate regions worldwide.[1] *Morus* species are generally known as mulberries, and their distribution is extent to East, West, and South East Asia, South Europe, the South of North America, the Northwest of South America, and some parts of Africa. It can be said that they have a high adaptation capacity for various environmental conditions.[1,2] Mulberries are under cultivation in many different world regions, such as tropical, subtropical, and temperate zones of Asia, Europe, North and South America, and Africa. These species have economic value in most countries because of their use in sericulture. Moreover, they have been widely used as traditional folk medicine, particularly in China and India.[3,4]

Mulberries are grown for the production of edible fruits in other countries like Turkey and Greece.[5] They have a long history of cultivation, having been cultivated as food plants for more than 400 years in Turkey, one of the most important centers of diversity.[6] In Turkey, the best known species are black mulberry (*Morus nigra* L.), white mulberry (*Morus alba* L.), and purple mulberry (*Morus rubra* L.).[3,7,8] Besides the traditional medicinal use of various part of these species, their fruits are also used in making syrup, jam, pulp, ice-cream, vinegar, and natural dyes.[2,6] Flavonoids, anthocyanin and alkaloids contained in the most of parts of mulberries ensure several pharmacological activities such as antidiabetic, antioxidant, antiinflammatory, antimutagenic, anticarcinogenic, and hepatoprotective properties.[4,9]

According to the APG IV classification system, the family Moraceae belongs to the order Rosales within the Rosids clade.[10] Diagnostic indicators of Moraceae include the presence of milky latex, a distinct stipule, anatropous ovules, apical placentation, compound fruits (achenes or syconous), and a cystolith.[11] The genus *Morus* has also attracted the attention of many researchers due to its interesting breeding system, interspecific hybridization, wide distribution range, naturalization in different areas, invasiveness of some taxa, and taxonomic uncertainty within the genus.[12] Taxonomists have reported various species numbers; thus, the taxonomy of *Morus* has been unstable. In the first instance, Linnaeus defined seven species belonging to the genus, but then Burea[13] and Koidzumi[14] identified 5 and 24 species, respectively. We currently know that *Morus* comprises about 14 species throughout the world.[15] Although many taxonomic studies and revisions have been conducted on *Morus*, taxonomic difficulties related to the genus still remains.[7,16,17]

Morphological and anatomical properties are basic tools that have been used in taxonomic studies for centuries.[18,19] Despite certain morphological differences, sometimes fruits of *M. nigra* and *M. rubra* may not be identified by local people, and sellers replace *M. nigra* with an another less expensive black fruit.[20] Moreover, some taxa show minor differences in leaf morphology.[7] Anatomical studies of leaves provide many important diagnostic characteristics, such as the size, shape, and orientation of stomata, guard cells, and subsidiary cells; type and shape of trichomes; and structure of epidermal cells.[21] For these reasons, determining the morphological and anatomical differences between species could be helpful in resolving diagnostic challenges.

Several studies have been performed on the morphology and anatomy of *Morus*.[1,22-26] However, the leaf anatomy and morphology of *Morus* species from Turkey have not been investigated. The present study aims to investigate the morphological and anatomical features of *M. alba*, *M. nigra*, and *M. rubra* distributed in Turkey. We also attempted to identify diagnostic anatomical and morphological properties that could contribute to the taxonomy of the genus.

Experimental

Herbarium specimens were used to determine morphological and anatomical properties. *M. alba* collected from Balıkesir [Herbarium of İstanbul University Faculty of Pharmacy (ISTE) 109772] and İstanbul (ISTE 116445), *M. nigra* collected from İstanbul (ISTE 80737), and *M. rubra* collected from Gaziantep (ISTE 40076) are stored in the ISTE. Collected data on each studied species are shown in Table 1. Morphological studies were carried out on herbarium materials. For anatomical studies, leaves were pretreated by immersion in warm water. Minimum 15 individual specimens were used. Hand sections were taken from samples with a razor blade then stained with chloral hydrate and Sartur solution.[27] Sections were examined using an Olympus BH2 light microscope, and detailed photos were taken using a Canon Power Shot A640 camera. Measurements of each samples were performed with KAMERAM© software, and the obtained data are given below. The stomatal index (SI) was calculated according to the following formula: SI: (S/S + E) x100, where S refers to the number of stomata per unit area, and E to the number of epidermal cells in the same unit area.[28] No further statistical analysis was used.

Table 1. Collection data of *Morus* taxa examined

Taxon	Locality, voucher number (ISTE)
Morus alba	B1 Balıkesir: Kepsut, Büyükkatrancı village, 800 m elevation, 30.05.2015, ISTE 109772
Morus nigra	A2 (E) İstanbul: Çatalca, İnceğiz gateway, field border, 50 m elevation, 25.05.2003, ISTE 80737
Morus rubra	C6 Gaziantep: Between Nizip-Gaziantep, Altındağ village, 650 m elevation, 30.05.1978, ISTE 40076

ISTE: Herbarium of İstanbul University Faculty of Pharmacy

RESULTS AND DISCUSSION

The lamina anatomical traits of the collected specimens were defined by examination of the lamina transverse and surficial sections.

Morus alba L.

The midrib is rich in collenchymatic elements with 4-5 layered collenchyma located under the lower epidermis, 2-3 layered collenchyma are located under the upper epidermis (Figure 1A-G). Between 5 and 6 layers of parenchyma cells exist between the collenchyma layers and the collateral vascular bundles (Figure 1A). The average leaf thickness at the midrib is 735.105 µm. Moreover, plenty of druse crystals of calcium oxalate were observed in the midrib and the mesophyll of the leaf (Figure 1C, F, I). Prismatic crystals were observed only in the midrib region. The density of the crystals is increased near the veins in the midrib (Figure 1C).

There is a single layered epidermis covered with a thin cuticle on both adaxial and abaxial surface of the lamina. The epidermis has polygonal cells with usually straight anticlinal

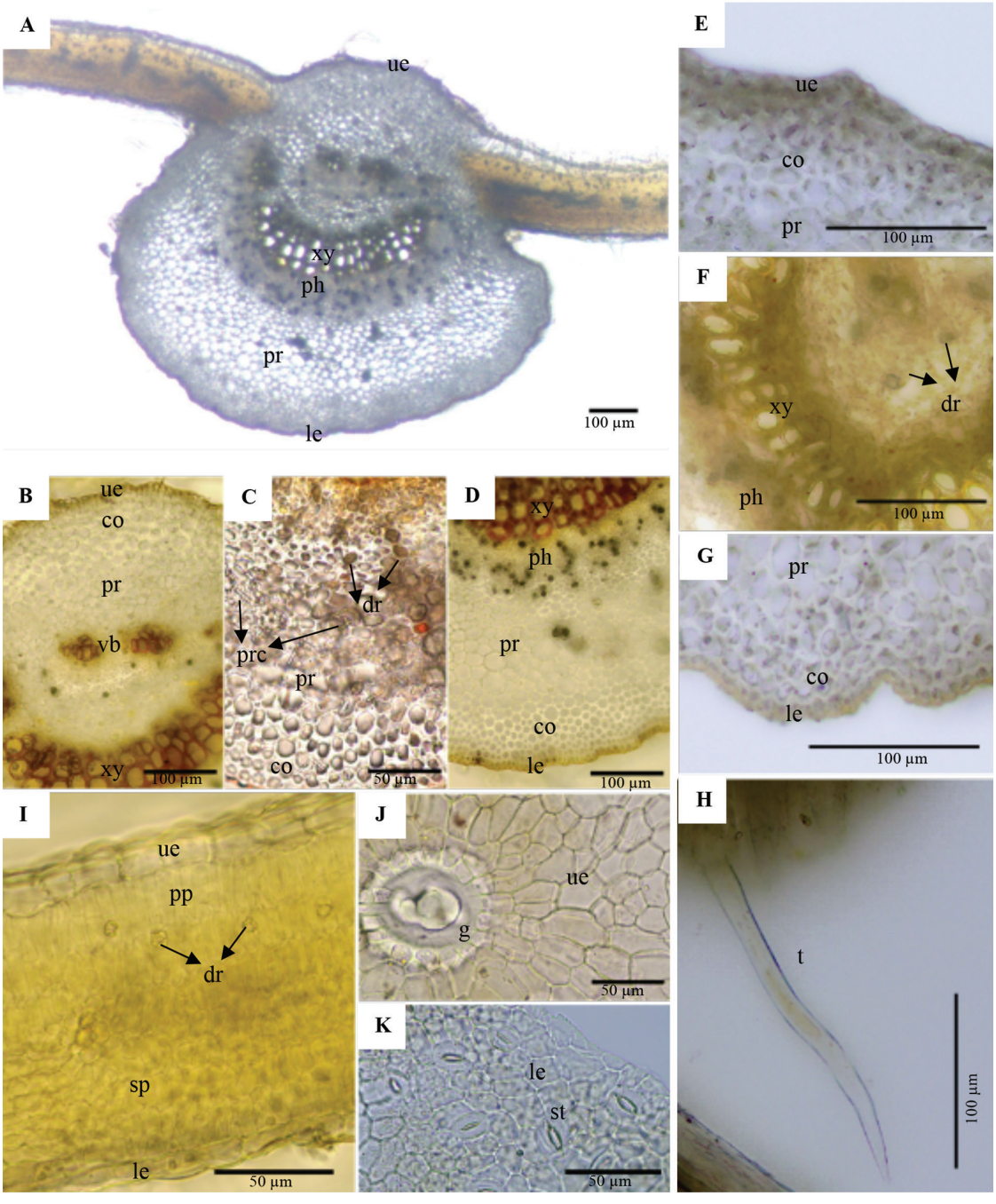

Figure 1. The transverse and surface sections of the leaf of *Morus alba*. The midrib region (A-H), mesophyll (I), adaxial surface (J), and abaxial surface (K)

co: Collenchyma, dr: Druse, g: Peltate gland, le: Lower epidermis, ph: Phloem, pp: Palisade parenchyma, pr: Parenchyma, prc: Prismatic crystal, sp: Spongy parenchyma, st: Stomata, t: Non-glandular trichome, ue: Upper epidermis, xy: Xylem, vb: Vascular bundle

Table 2. Measurements from leaf anatomical traits of *Morus alba*, *Morus nigra*, and *Morus rubra* (mean, min-max values)

	Morus alba	*Morus nigra*	*Morus rubra*
	Length x width (μm)	**Length x width (μm)**	**Length x width (μm)**
UEC	32.93 (23.62-44.06)x24.41 (18.34-33.63)	20.14 (15.55-28.67)x18.24 (15.21-19.87)	38.50 (37.81-40.42)x20.51 (19.15-22.38)
LEC	19.73 (13.13-29.40)x11.43 (8.00-12.94)	18.25 (16.03-22.41)x8.31 (8.05-8.64)	28.13 (26.95-30.18)x13.61 (13.05-14.86)
LS	18.99 (15.39-23.37)x14.22 (11.81-17.36)	19.12 (16.71-21.19)x13.97 (12.76-15.48)	34.22 (31.39-36.98)x23.44 (19.84-26.33)
PPL (thickness)	56.139	40.081	45.008
SPL (thickness)	86.254	72.685	71.222

UEC: Upper epidermis cell, LEC: Lower epidermis cell, LS: Stomata of abaxial epidermis, PPL: Palisade parenchyma layer, SPL: Spongy parenchyma layer, min: Minimum, max: Maximum

walls, and the upper epidermis cells are larger than the lower ones. The length and width of epidermis cells are presented in Table 2. Unicellular non-glandular trichomes, varying in length, are also present on the both leaf surfaces. The number of non-glandular trichomes is higher along the veins and the midrib (Figure 1A, H). Glandular trichomes with a unicellular stalk and multicellular head are sparse on the lower surface. The leaf is dorsiventral. The mesophyll is composed of two layers of palisade cells under the upper epidermis and 5-6 layers of spongy cells under the lower epidermis. Cylindrical palisade cells were found in the transverse section. Spongy parenchyma cells with wide intercellular spaces have ovoid or circular shapes (Figure 1I). The spongy parenchyma occupies about 60.57% of the mesophyll. Stomata cells were found only on the abaxial surface of the leaf (hypostomatic) (Figure 1J, K). The leaf blade thickness ranges from 159.096 to 175.017 μm, with a mean value of 169.112 μm.

On the abaxial surface, anomocytic stomata are oval shaped and vary in size. They are situated at the same level as the other epidermal cells (mesomorphic). Each stoma is surrounded by 5-6 subsidiary cells (Figure 1K). Lithocysts, a specific type of enlarged epidermal cells in which calcium carbonate is deposited, and peltate glands were detected on the upper surface of the leaf (Figure 1J). The SI for the lower surface of the lamina was calculated as 10.71.

Morus nigra L.

Regarding the midrib region, 2-3 layered collenchyma are presented on the lower surface and 1-2 layered collenchyma on the upper surface (Figure 2A-F). Between 5 and 6 layers of parenchyma cells fill the space between the collenchyma layers and the collateral vascular bundles (Figure 2A, B). The leaf thickness at the midrib is on average 516.083 μm. Many druse crystals of calcium oxalate were observed in the midrib and the mesophyll (Figure 2B, C, E, I, J). Several prismatic crystals were observed only in the midrib region. The crystals are abundant near the veins in the midrib (Figure 2B, C).

Both leaf surfaces have a single layered epidermis covered with a thin cuticle. Epidermis cells, which are polygonal in shape, usually have straight anticlinal walls. Their sizes are variable. The upper epidermal cells are larger than the lower ones (Table 2). Unicellular, non-glandular trichomes were observed on both leaf surfaces, and their number was higher on the lower surface (Figure 2A, D, G). Glandular trichomes with a unicellular stalk and multicellular head are scattered on both surfaces (Figure 2H). The mesophyll consists of two layers of palisade cells under the upper epidermis and 4-5 layers of spongy cells with wide intercellular spaces under the lower epidermis. Hence, the leaf is dorsiventral. While the palisade parenchyma cells were cylindrical, the spongy parenchyma cells were found to be ovoid-circular in transverse section (Figure 2I, J). The spongy parenchyma occupies approximately 64.46% of the mesophyll. The leaf is also hypostomatic and mesomorphic, stomata cells were found only on the lower surface of the leaf (Figure 2K-M). Leaf blade thickness ranges from 149.042 to 160.843 μm, with a mean value of 158.052 μm.

The stomata are anomocytic. They have an oval shape and vary in size. Each stoma is surrounded by 5-6 subsidiary cells (Figure 2L). Lithocysts and peltate glands were defined on the upper surface of the leaf (Figure 2I, K). The SI for the lower surface of the lamina was calculated as 13.26.

Morus rubra L.

In the midrib, while 2-3 layered collenchyma are located under the lower epidermis, 1-2 layered collenchyma are located under the upper epidermis (Figure 3A-G). Parenchyma cells form 4-5 layers. They are present between the collenchyma layers and the collateral vascular bundles. The leaf thickness at the midrib is on average 740.899 μm. Many druse crystals of calcium oxalate were observed in the midrib and also in the mesophyll (Figure 3B, C, F). Several prismatic crystals were observed only in the midrib region.

The epidermis cells, which are covered by a thin cuticula layer on both surfaces of the leaf, is single layered. Their shape is polygonal, and they vary in size (Table 2). However, the upper epidermis cells are larger than the lower epidermis cells. They usually have straight anticlinal walls. On both leaf surfaces, indumentum of unicellular non-glandular trichomes were noticed, but they were more numerous on the lower surface (Figure 3H, I). Glandular trichomes with a unicellular stalk and head were rare on both surfaces. The leaf is dorsiventral and

hypostomatic (Figures 3J-L). The mesophyll is differentiated into palisade and spongy parenchyma. It comprises one layer of palisade cells under the upper epidermis and 3-4 layers of spongy cells with wide intercellular spaces under the lower epidermis. Palisade parenchyma cells are cylindrical, and the spongy parenchyma cells are ovoid-circular (Figure 3J). The spongy parenchyma occupies about 61.28% of the mesophyll. Leaf blade thickness ranges from 125.705 to 133.690 µm, with a mean value of 130.398 µm.

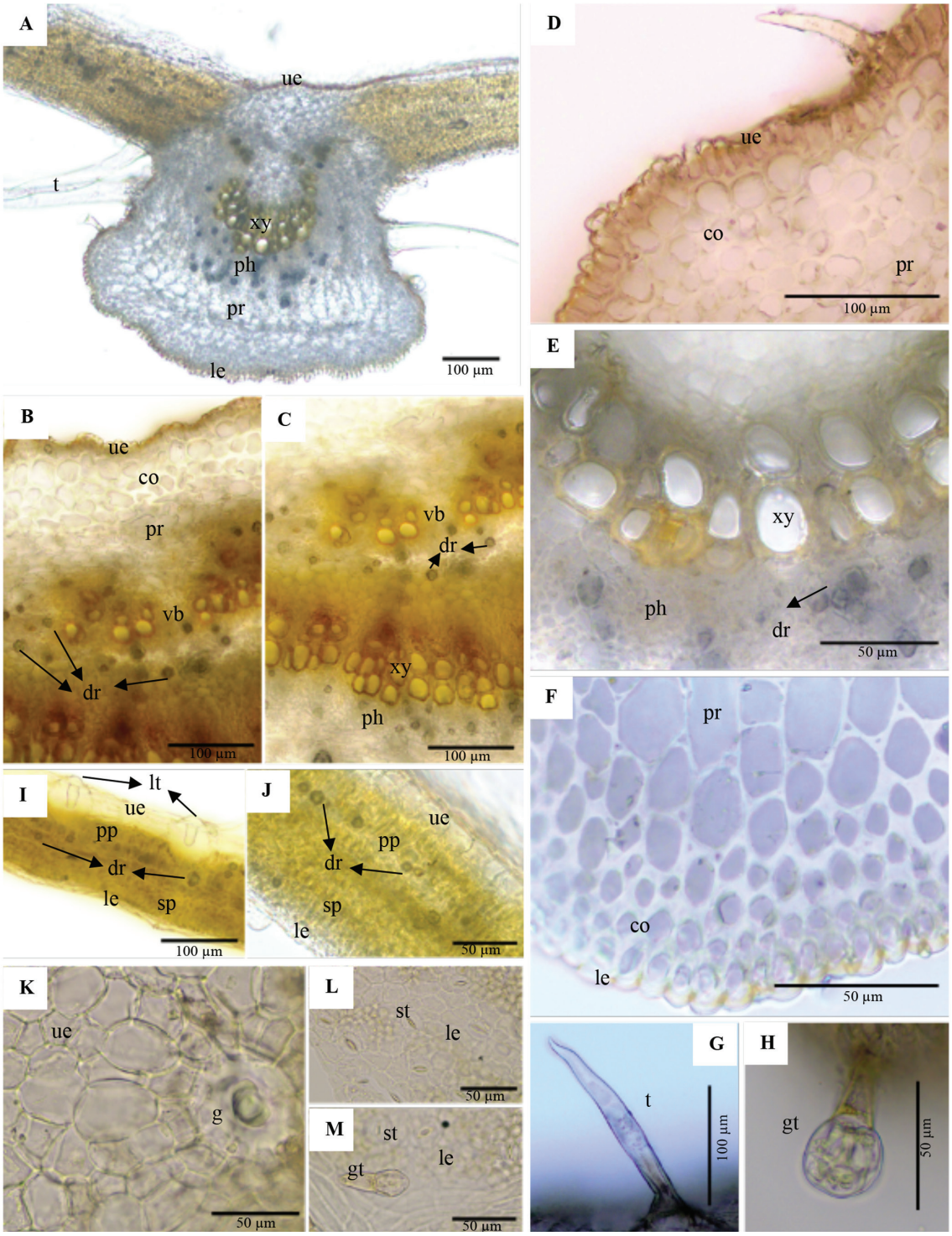

Figure 2. The transverse and surface sections of the leaf of *Morus nigra*. The midrib region (A-H), mesophyll (I), (J), adaxial surface (K), and abaxial surface (L, M)

co: Collenchyma, dr: Druse, g: Peltate gland, gt: Glandular trichome, le: Lower epidermis, ph: Phloem, pp: Palisade parenchyma, pr: Parenchyma, prc: Prismatic crystal, sp: Spongy parenchyma, st: Stomata, t: Non-glandular trichome, ue: Upper epidermis, xy: Xylem, vb: Vascular bundle

Oval shaped and different sized stomata are anomocytic. Each stoma is surrounded by 5-6 subsidiary cells, and the leaf is mesomorphic (Figure 3L). Lithocysts and peltate glands were found on the upper surface of the leaf (Figure 3K). The SI for the lower surface of the lamina was calculated as 11.11.

Morus species could vary in morphological appearance when the climate or habitat change. Hence, it is difficult to assign a taxonomic classification to these species.[23] Comparative morphological and anatomical studies are the basic tools of plant taxonomy, and they provide fundamental data which are helpful for a majority of classification systems.[29] Furthermore,

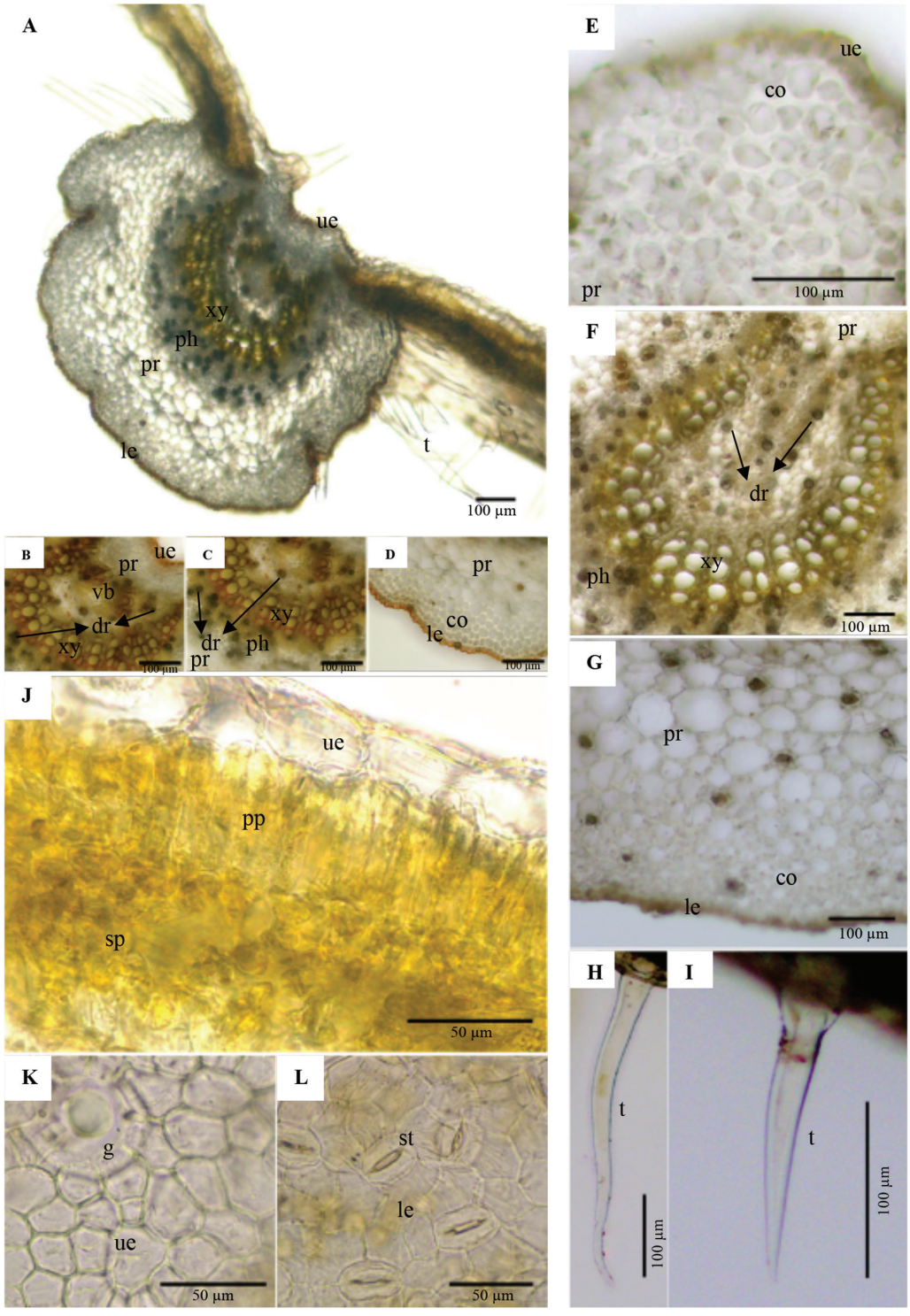

Figure 3. The transverse and surface sections of the leaf of *Morus rubra*. The midrib region (A-I), mesophyll (J), adaxial surface (K), and abaxial surface (L)
co: Collenchyma, dr: Druse, g: Peltate gland, le: Lower epidermis, ph: Phloem, pp: Palisade parenchyma, pr: Parenchyma, prc: Prismatic crystal, sp: Spongy parenchyma, st: Stomata, t: Non-glandular trichome, ue: Upper epidermis, xy: Xylem, vb: Vascular bundle

studies based on plant morphology and anatomy help us to understand the phylogeny of life.[30] In this study, *M. alba*, *M. nigra*, and *M. rubra* were examined and compared morphologically and anatomically. Differences as a result of the investigation are given in Table 3.

Certain morphological characteristics, such as leaf shape, size, base, margin, and indumentum were found to be useful for identifying the studied species (Figure 4). Moreover, the indumentum of the shoot and the peduncle and the size of the fruit, peduncle, and petiole differ in these species. The species with the broadest leaves was found to be *M. alba*. The difference in the color of the fruits of these three species is perhaps the most striking organoleptic feature. *M. alba* has white, pinkish, or purplish fruit, whereas *M. nigra* has blackish-violet or black fruit, and *M. rubra* has dark reddish-purple fruit. Moreover, there are some similarities in their morphological features, such as inflorecences of short, dense spikes, ellipsoid syncarps, and fleshy drupelets.

We also know that some anatomical traits are very diagnostic. Thus, they are frequently used in routine identification. Since the leaf is regarded as the most varied organ of the angiosperms, taxonomic studies of various taxa were carried out on the basis of leaf anatomy.[29,31-34] These studies present many anatomical characteristics of potential taxonomic significance.[29] The

Table 3. Morphological and anatomical comparison of the studied taxa

	Morus alba	*Morus nigra*	*Morus rubra*
Shoots	**Slender, glabrous**	**Stout, pubescent**	**Slender, pubescent**
Peduncle	Hairy	Hairy	Pubescent
Peduncle length	(1-) 2 cm, circa as long as syncarp	1-1.5 cm	0.5-1 cm, 1/2 as long as syncarp
Fruit length	(1-) 1.5-2.5 cm	(1.5-) 2-2.5 cm	(1.5-) 2-3 cm
Fruit color	White, pinkish, or purplish	Blackish-violet or black	Dark reddish-purple
Leaf shape	Ovate to broadly ovate	Broadly ovate	Broadly ovate to oblong-ovate
Leaf size	3-10 (-18)x2-12 cm	5-12 (-20)x(4) -5.5-13 cm	6-12 (-20)x4-10 cm
Leaf apex	Acute or shortly acuminate	Acute or shortly acuminate	Abruptly long-acuminate
Leaf base	Rounded or obliquely cordate	Deeply cordate	Truncate or subcordate
Leaf margin	Crenate-dentate	Serrate	Serrate
Indumentum of leaf	Upper surface glabrous/lower surface pubescent on the midrib and the veins	Upper surface scabrous/lower surface pubescent	Upper surface slightly scabrous/lower surface roughly hairy
Non-glandular trichomes	Unicellular trichomes on the both leaf surfaces (density is higher along the veins and the midrib)	Unicellular trichomes on the both leaf surfaces (density is higher on the lower surface)	Unicellular trichomes on the both leaf surfaces (density is higher on the lower surface)
Glandular trichomes	Trichomes with unicellular stalk and multicellular head on the lower surface (sparsely)	Trichomes with unicellular stalk and multicellular head on the both surface	Trichomes with unicellular stalk and head on the both surfaces (rarely)
Epidermal cells	Upper epidermis cells are larger than the lower ones	Upper epidermis cells are larger than the lower ones	Upper epidermis cells are larger than the lower ones
Mesophyll type	Dorsiventral	Dorsiventral	Dorsiventral
Mesophyll	38%-45% palisade parenchyma (2 layer)	35%-38% palisade parenchyma (2 layer)	35%-40% palisade parenchyma (one layer)
Location of stomata	Hypostomatic	Hypostomatic	Hypostomatic
Stomatal index	10.71	13.26	11.11
Collenchyma cell layers of midrib	4-5 layered on the lower surface, 2-3 layered on the upper surface	2-3 layered on the lower surface, 1-2 layered on the upper surface	2-3 layered on the lower surface, 1-2 layered on the upper surface
Thickness of leaf blade (average)	169.112 µm	158.052 µm	130.398 µm
Thickness of midrib (average)	735.105 µm	516.083 µm	740.899 µm
Petiole length	1-3.5 (-4) cm	1.5-3.5 cm	(1-) 1.5-3 cm

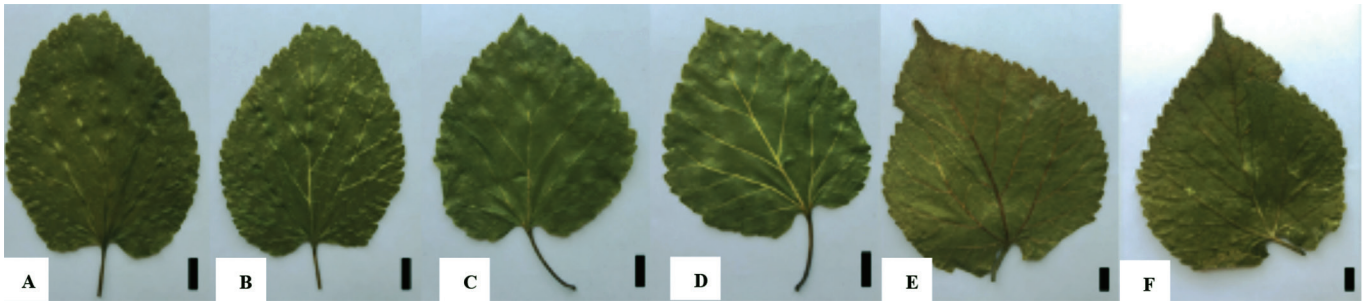

Figure 4. General view of the leaves of *Morus* species. Upper surface of the leaf of *Morus alba* (A), lower surface of the leaf of *M. alba* (B), upper surface of the leaf of *Morus nigra* (C), lower surface of the leaf of *M. nigra* (D), upper surface of the leaf of *Morus rubr*a (E), lower surface of the leaf of *M. rubra* (F), bar: 1 cm

results of our detailed anatomical study revealed that there were some differences among the leaf anatomy of these three taxa. Metcalfe and Chalk[35] have reported that the epidermis of the Moraceae generally comprises of a single layer of quadrangular or elongated anticlinal cells. Although the upper epidermis cells were found to be larger than the lower ones in all studied taxa, the length and width of epidermis cells on the two sides differed. Accordingly, the epidermis cells on the lower and upper surfaces of *M. nigra* were found to be smaller than those in the other two taxa. Since the stomatal size may change according to environmental conditions, some authors do not regard this as a diagnostic characteristic. However, the stomatal size is generally accepted because the size of the stomata is generally stable enough to be used as a diagnostic characteristic.[36,37] In the Moraceae family, stomata usually do not have special subsidiary cells.[38,39] Leaves of the three taxa were determined to be hypostomatic (stomata were only observed on the abaxial surface) with anomocytic-type stomata. However, concerning size, the width, and length of stomata were significantly different. The mean value of the stomata size of *M. rubra* was found to be the highest species. The term SI is used to define stomatal frequency, and the size of the epidermal cells is neglected. Since taxa from distinct localities have more or less constant SI values, the SI is considered as a significant taxonomic characteristic.[36,40] In the taxa studied, different values of the SI were calculated in this study.

Many studies have revealed the taxonomic value of trichomes in angiosperms.[41,42] Glandular and non-glandular trichomes are common in the Moraceae.[18,22,38] According to a previous study, while simple, unicellular, non-glandular trichomes and multicellular, capitate, glandular trichomes are common in *Morus* taxa, conical unicellular non-glandular trichomes and bicellular capitate glandular trichomes are rare.[38] Abbasi et al.[26] indicated that unicellular non-glandular and glandular trichomes and also hooked hairs are present on the leaf surfaces of *Morus* species. Moreover, multicellular glandular trichomes, unicellular non-glandular trichomes and cystolith trichomes were observed in *M. alba* and *M. nigra*.[22] In the present study, unicellular non-glandular trichomes of various sizes were found on both leaf surfaces of the studied taxa, but their densities were variable. Glandular trichomes with a unicellular stalk and multicellular head were detected in *M. alba*, *M. nigra*, and glandular trichomes with a unicellular stalk and head were observed in *M. rubra*. We also found peltate glands on the upper surfaces of all studied taxa, as in previous studies.[24,26,43]

In the Moraceae family, calcium oxalate and carbonate crystals are mostly present in the leaves.[18,25,33,44,45] Regarding calcium oxalate, two types of crystals (druse crystals in the cells of the mesophyll and bundle sheaths, prismatic crystals only in the cells of the bundle sheath) are located in the leaves of the Moraceae.[44] The most often seen calcium carbonate crystal type cystolith (a calcified body) are located in several families, such as Urticaceae, Ulmaceae, Moraceae, Cucurbitaceae, and Acanthaceae.[1,18,46] Many species of the Moraceae are recognized by the presence of cystolith. Cystolith is deposited in a specialized cell called a lithocyst, which is known as an excretory idioblast.[18,44,46-48] Lithocysts are very common in the Moraceae family and were observed in many anatomical studies on Moraceae. *Ficus* L. species mostly have lithocysts; moreover, they were reported on *Morus* leaves.[33,37,44,45] According to Esau[49] the presence and location of crystals may be distinctive and useful in taxonomic classification. In our study, while many druse crystals were found in the mesophyll and also in the midrib region, prismatic crystals were only found in the midrib. Furthermore, lithocysts were noticed only on the upper surface of the leaves of all investigated taxa. In contrast to our study, lithocysts were not observed in some studies on *Morus* taxa.[22,24,26]

As seen in transverse sections, spongy and palisade parenchyma cells can be distinguished easily from each other in the mesophyll. However, palisade parenchyma layers and their ratio of occupation vary. The leaves of the studied taxa are dorsiventral. The dorsiventral leaf is characteristic of some members of the Moraceae family and is therefore not useful for species identification.[18,35] Besides, in some works on the Moraceae family, dorsiventral and isobilateral leaves were reported.[33,37,50-52] In this study, collateral vascular bundles were seen in the midrib region. The differences were determined concerning collenchymatic elements located with various layers between epidermal and parenchyma cells on the midrib of the three taxa.

CONCLUSION

Some differences were determined in the morphological and anatomical properties of all studied taxa. It is obvious that certain characteristics, such as the size, shape, and indumentum of leaves, are helpful in the recognition of taxa. Furthermore, some anatomical characteristics of the leaves were found to be of diagnostic importance, such as the ratio of the density of the palisade parenchyma and collenchyma layers in the midrib region, type, and density of trichomes, length and width of stomata, and SI. All of these characteristics are environmentally influenced, and future studies analyzing plants from several localities are needed; nevertheless, they can be very useful in the delimitation of species.

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ORIGINAL ARTICLE

Microwave-assisted Preparation of Cross-linked Gelatin-Paracetamol Matrices: Optimization Using the D-optimal Design

Çapraz Bağlı Parasetamol Matrikslerinin Mikrodalga Yardımıyla Hazırlanması: D-optimal Tasarımı Kullanılarak Optimizasyon

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ABSTRACT

Objectives: This study was conducted to assess the effect of microwave heating on the preparation of paracetamol cross-linked gelatin matrices by using the design of experiment (DoE) approach and explore the influence of the duration of microwave irradiation, the concentrations of cross-linker, and the amount of sodium bicarbonate (salt) on paracetamol release. These parameters were also compared with those of the matrices prepared via conventional heating.

Materials and Methods: Twenty gel matrices were prepared with different durations of microwave irradiation, amounts of maize, and concentrations of sodium bicarbonate as suggested by Design Expert (DX®). The percentage drug release, the coefficient of variance (CV) in release, and the mean dissolution time (MDT) were the properties explored in the designed experimentation.

Results: Target responses were dependent on microwave irradiation time, cross-linker amount, and salt concentration. Classical and microwave heating did not demonstrate statistically significant difference in modifying the percentage of drug released from the matrices. However, the CVs of microwave-assisted formulations were lower than those of the gel matrices prepared via classical heating. Thus, microwave heating produced lesser variations in drug release. The optimized gel matrices demonstrated that the observed percentage of drug release, CV, and MDT were within the prediction interval generated by DX®. The release mechanism of the matrix formulations followed the Peppas-Korsmeyer anomalous transport model.

Conclusion: The DoE-supported microwave-assisted approach could be applied to optimize the critical factors of drug release with less variation.

Key words: Controlled release gel matrices, paracetamol, microwave heating, classical heating, experiment design

ÖZ

Amaç: Bu çalışma mikrodalga ısıtmasının parasetamol çapraz bağlı jelatin matriksleri üzerine etkisini değerlendirmek ve mikrodalga ışıma süresinin etkisini, çapraz bağlayıcıların konsantrasyonlarını ve parasetamol salımı üzerinde sodyum bikarbonat tuzunun miktarını bulmak üzere gerçekleştirilmiştir. Bu parametreler konvansiyonel ısıtma yöntemleriyle hazırlanan matrikslerle de karşılaştırılmıştır.

Gereç ve Yöntemler: Yirmi jel matriks Design Expert (DX®) tarafından önerilen farklı mikrodalga ışıma süresi, farklı miktarda mısır ve farklı konsantrasyonlarda sodyum bikarbonat kullanılarak hazırlanmıştır. İlaç salımının yüzdesi salımdaki varyans katsayısı (CV) ve ortalama dissolüsyon zamanı (MDT) tasarımlandırılmış deneylerle araştırılmıştır.

Bulgular: Hedef yanıtlar mikrodalga ışıma süresi, çapraz bağlayıcı miktarı ve tuz konsantrasyonuna bağlı bulunmuştur. Klasik ve mikrodalga ısıtma matrikslerden ilacın salım yüzdesi üzerine istatistiksel olarak anlamlı bir fark oluşturmamıştır. Ancak, mikrodalga ile oluşturulan formülasyonların CV'leri klasik ısıtma ile oluşturulan jel matrikslerden düşük bulunmuştur. Ayrıca, mikrodalga ısıtma ilaç salımında daha az varyasyon oluşturmuştur. Optimize jel matriksler gözlenen ilaç salımının yüzdesi, CV ve MDT DX® tarafından oluşturulan tahmini interval içinde bulunmuştur. Matriks formülasyonların salınım mekanizması Peppas-Korsmeyer kuraldışı taşınım modeline uymuştur.

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Sonuç: DoE ile desteklenen mikrodalga ile gerçekleştirilen yaklaşım ilaç salınımının kritik faktörlerini optimize etmekte daha düşük varyasyonu sağlamak için uygulanabilir.

Anahtar kelimeler: Kontrollü salımlı jel matriksler, parasetamol, mikrodalga ısıtma, klasik ısıtma, deney tasarımı

INTRODUCTION

Controlling the release of drugs from a polymeric matrix is challenging in the development of controlled release dosage forms because of the different release patterns and physicochemical properties of drugs.[1,2] Drug release can be delayed by cross-linking a drug with polymeric matrices.[3] Chemical cross-linking is associated with the production of chemical cross-linker residuals; nevertheless, this problem can be overcome by using biopolymers that can be cross-linked with natural and non-toxic cross-linkers, such as maize, because of their safety, biodegradability, and biocompatibility. Microwave-assisted methods can also be used for cross-linking and developing dosage forms.[4-6] Microwave emits electromagnetic radiations with a frequency of 300 GHz to 300 MHz. Most commercial microwave ovens produce a microwave wavelength of 12.25 cm, which is equivalent to 2.45 GHz[6]. Microwave heating can modify the state of molecular interactions between polymer chains to control physicochemical properties and drug release.[1] Microwave energy is absorbed by materials, converted into molecular kinetic energy, and dissipated by molecules because of the inertial, elastic, and frictional forces of the surroundings. Its specific heating causes dipolar polarization and promotes cross-linking without using harsh solvents.[7,8] Carbohydrate polymers, such as alginate, gelatin, and cellulose, have been incorporated in controlled release drug delivery systems.[3,4] Sodium alginate is a water-soluble biopolymer, which is cross-linked in the presence of multivalent cations in aqueous media. It may form a hydrogel upon cross-linking.[3] Calcium alginate-coated matrices significantly reduce the percentage of drugs released by the main matrix structure.[1] Gelatin is a natural polymer rarely used alone, because of its low intensity and high brittleness unless it is modified by several methods, such as cross-linking, grafting, and blending. Traditional experimentation for optimizing multiple factors of formulations is time consuming and does not reveal factor interactions, which may be synergistic or antagonistic. Conversely, the design of experiment (DoE) determines the relationship between factors and responses, reveals factor interactions, and facilitates the development of optimized formulations with lesser time, cost, and material consumption.[2,9] The factors that may affect microwave-assisted cross-linking include microwave power, microwave exposure time, and temperature. The concentrations of macromolecules, cross-linkers, and salts and the nature of drugs may be the other factors that should be considered in designing a gel formulation.

This study was performed to assess the effect of microwave heating in the presence of a cross-linker and salt on cross-linking and drug release characteristics of gelatin matrices. This study was also conducted to optimize the above conditions for the release of a drug via the DoE approach. The model drug used in this study was paracetamol, a water-soluble drug, because of its availability and lack of risks in handling by being an over-the-counter drug, which can be rigorously tested in a study.[10]

MATERIALS AND METHODS

Chemicals and reagents

Paracetamol BP (Zulat Pharmacy, China), gelatin (R&M Chemicals), maize (National Starch & Chemical), potassium dihydrogen orthophosphate (KH_2PO_4; Fisher Scientific, UK), and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$; Merck, Germany) were used. Sodium hydroxide (NaOH, A. R. grade, purity 99%, MW 40; Batch no. 020606; JRL, System®) was utilized to prepare 0.1 M NaOH solution. All the other materials and reagents were of analytical grade.

Preparation of gelatin-paracetamol matrices

Microwave power from 10% to 100% and irradiation time from 10 s to 60 s were varied to generate a temperature of 60°C.[11] The maximum amount of paracetamol to be added was determined on the basis of its weight (grams) dissolved in a predefined volume of water with or without using a sonicator. The time of drug dissolution was also noted.

The amount of maize (as fructose) was changed within 0.5-2.5 g, sodium bicarbonate within 2-4 g, and duration of irradiation from 20 s to 50 s by using the D-optimal design implanted in Design Expert (DX®) version 12 to generate an experimental matrix (Table 1).[12] The amounts of paracetamol (0.4 g) and gelatin (9.3 g), microwave irradiation power (30%), and stirring time (30 s) were fixed. All the above ingredients were dissolved with stirring for 30 s in 22.2-26.2 mL of water to keep the total number of parts at 38.4. The resultant mass was exposed to 30% microwave power (input: 1.60 kW, output: 1100 W, and frequency: 2450 MHz; Sharp equipped with a magnetron emitter) for 50 s to achieve 60˚C and maintain drug stability. Twenty matrices were prepared and incubated (230 V, 3.9 A, 50/60 Hz, and 900 W; Memmert type BE 500, Germany) at 37˚C for 17 h.

In vitro drug release study

Paracetamol-gelatin matrices were subjected to an *in vitro* drug release study with Franz diffusion cells (PermeGear, Inc., USA; Figure 1a) fitted with a thermostat (CC1, Huber, D77656, Figure 1a) in phosphate buffer (pH=5.8) set at 37°C±2°C by utilizing a Visking membrane (VM) with a thickness of 0.0145 cm and a pore size of 0.45 µm (Whatman® International Ltd., Maidstone, England). Before the experimentation, the VM was heated in 2% sodium bicarbonate and 1 mM EDTA at 80°C for 30 min and rinsed with dissolution media.[13,14] The receiver compartment was loaded with a dissolution medium and a magnetic stirrer. The membrane was mounted between the donor and receptor

compartments and clamped (Figure 1b). The conditions for the release study (pH, membrane type, membrane treatment, and rpm of magnetic stirrer) were set for the maximum release after screening experimentations.

The donor compartment was filled with a test sample and covered with parafilm to prevent sample leakage. The magnetic stirrer was rotated at 300 rpm. The sample (1 mL) was withdrawn initially at 5, 15, 30, and 45 min and subsequently at 1, 2, 3, 5, and 8 h from the receiver compartment and replenished with fresh dissolution media.[15] The paracetamol concentration was

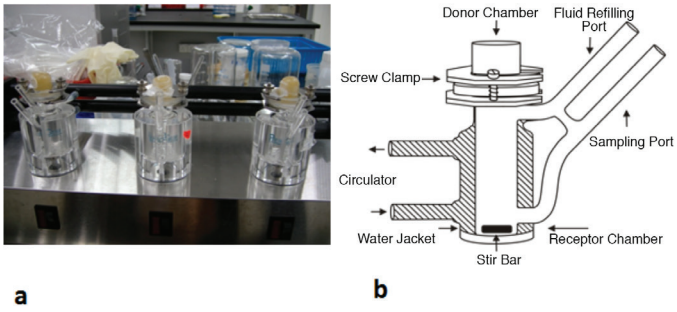


Figure 1. (a, b) Franz diffusion cells

Table 1. Experimental layout of the production of the optimized paracetamol matrices

Matrix	Time of irradiation (s)	Maize (g)	Na bicarbonate (g)	Water (mL)
1	20	1.5	4.0	23.2
2	20	0.5	2.0	26.2
3	35	0.5	3.0	25.2
4	50	0.5	4.0	24.2
5	20	0.5	4.0	24.2
6	50	2.5	4.0	22.2
7	50	1.5	3.0	24.2
8	50	0.5	4.0	24.2
9	50	0.5	2.0	26.2
10	50	2.5	2.0	24.2
11	35	2.0	3.5	23.2
12	50	0.5	2.0	26.2
13	20	0.5	2.0	26.2
14	20	2.5	2.0	24.2
15	20	2.5	3.0	23.2
16	35	1.5	2.0	25.2
17	50	2.5	2.0	24.2
18	35	0.5	2.0	26.2
19	50	2.5	4.0	22.2
20	20	2.5	4.0	22.2

determined with a ultraviolet spectrophotometer (Shimadzu, model 1240, Japan) at 244 nm by using calibration curves in the media.[10]

Preparation of optimized and control gelatin-paracetamol matrices

The percentage of drug release, coefficient of variance (CV), and mean dissolution time (MDT) were calculated, and the release mechanism was noted with PCP Disso v3. All the outputs were entered in the generated template and analyzed using DX®. The best mathematical model for each response was selected on the basis of the statistical parameters. The optimized formulation based on the best levels of the factors given by DoE was prepared and labeled as the validation formulation, and drug release was studied.[16] Control gelatin matrices were prepared with the optimized factor levels but were exposed to classical heat with a hot plate equipped with a digital magnetic stirrer (MSH-20D, Daihan Labtech Co. Ltd., WiseStir®) instead of microwave heating. The target temperature was 60˚C.[10]

Thermal analysis of the control and optimized matrices

Differential scanning calorimetry [(DSC), Mettle Toledo, Switzerland] was performed to thermally analyze the control and optimized matrices. The samples of weight of 5-10 mg were crimpled in a standard aluminum pan by using a crucible sealing press. Hermetically sealed aluminum pans were heated from 20˚C to 200˚C at a rate of 10˚C/min under constant nitrogen flow at 20 mL/min to record DSC thermograms.

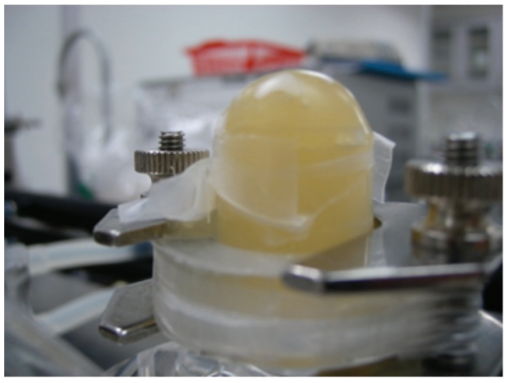
Statistical analysis

The release data after classical heating (control) and microwave heating were compared via Mann-Whitney U test statistics in SPSS at a significance level of $p<0.05$.

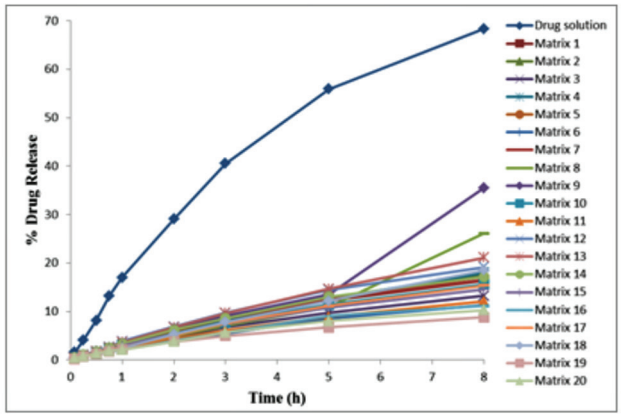
RESULTS

In this study, a smooth and swellable paracetamol gel matrix was obtained from 0.4 g of paracetamol dissolved in 24 mL of water in a bath sonicator for 45 min under varying cross-linker concentrations, microwave power, and radiation exposure (Figure 2a). A minimum of 30% microwave power upon exposure to microwave energy for 50 s helped achieve 60˚C.

Figure 2b shows that the pure paracetamol solution achieved 68.35% drug release (permeation) until the last time point (8 h). This value was higher than that from all the cross-linked matrices (8.82%-35.51%). The drug release of cross-linked matrices 8 and 9 was higher after 6 h as compared to the rest of the matrices. The CV of the drug release for the majority of matrices at 0.083 h was comparatively higher than that at the other time points. However, the value of this parameter was lesser at the later time intervals. MDT was increased from 0.04 h at 1 h to 5.05 h after 8 h. The maximum coefficient of relationship indicated that paracetamol released from microwave-treated cross-linked matrices followed the Peppas model except matrix 18, which was fitted to the first-order kinetics. The percentage of drug release at 1 h slightly decreased when the irradiation time was extended from 20 s to 35 s at low cross-linker concentrations (Figure 3a-e). MDT at 1, 3, and 8 h (Figure 4a-d)



a



b

Figure 2. (a) Paracetamol swellable gel matrices. (b) Drug release (%) of paracetamol solution and cross-linked matrices 1-20 designed by DX® for 8 h

DX®: Design Expert

were lower in matrices that had high concentrations of salt and cross-linker and were exposed to microwave energy for 50 s as compared to the matrices having lower amounts of salt and cross-linker. The response plot showed a saddle-shaped surface for MDT at 1 and 5 h. The same decreasing trend was observed in MDT until 8 h. The CV was increased from 3.51 to 14.83 as the duration of microwave exposure increased from 20 s to 50 s at 3 h (Figure 5a-d). It was also increased in the presence of moderately high levels of sodium bicarbonate and prolonged microwave exposure. Conversely, it was not affected by the increased maize levels.

The DoE-predicted combinations of maize, sodium bicarbonate, and radiation exposure time for the desired properties of outputs, namely, lower release, least CV, and lower variations in drug release, were 2.50 g, 2 g, and 45 s, respectively. The amount of gelatin, drug, and water were fixed at 9.30 g, 0.40 g, and 24.2 mL, respectively. Based on the composition of matrices and conditions of their preparation, the optimized and control matrices showed responses, such as percent release, CV of release and MDT as given in Table 2.

Three control matrices were prepared using the same optimized factor levels via classical heating at 60°C instead of microwave heat exposure. The percentages of drug release from the optimized and control formulations were almost similar and best described with the Peppas model (Figure 6a). For the majority of the release time points, the Mann-Whitney U test revealed no difference between classical heating (control) and microwave heating ($p>0.05$). Only the percentage of drug release at 5 h, CV at 5 and 8 h, and MDT at 0.75 h significantly differed between the optimized and control formulations ($p<0.05$). However, the standard deviation of the matrices exposed to classical heat

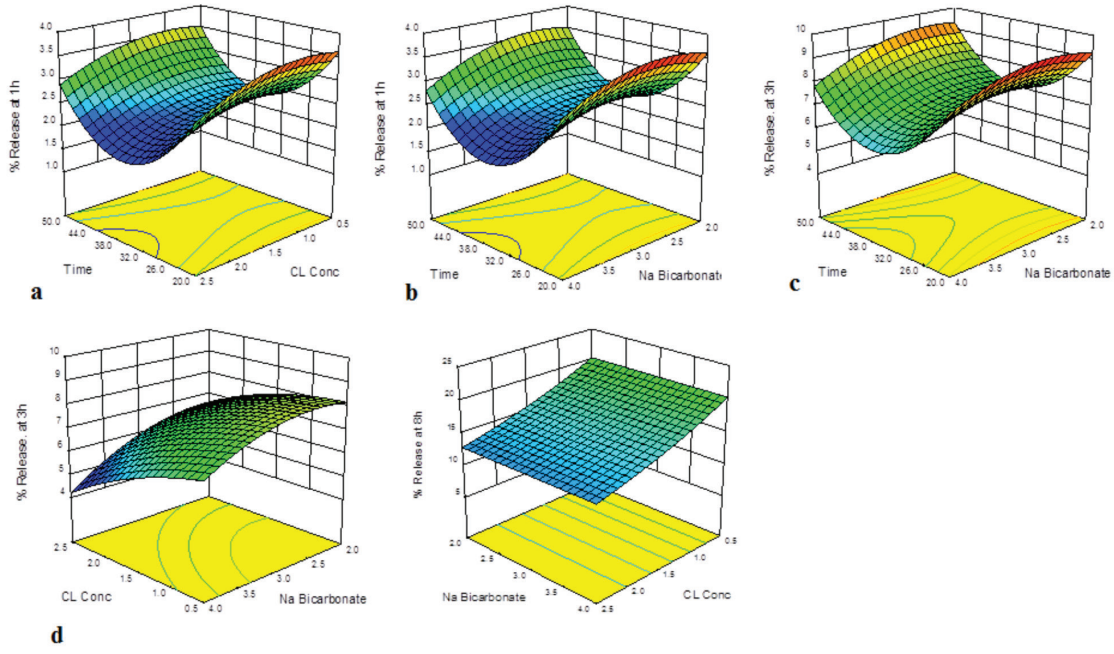


Figure 3. Combined effects of (a) irradiation time and cross-linker concentration, (b) irradiation time and sodium bicarbonate at 1 h, (c) salt and irradiation time, (d) salt and cross-linker on drug release at 3 h, and (e) cross-linker and salt at 8 h

was higher than that of the microwave-treated matrices. Figure 6b illustrates the interaction of the formulation ingredients and the drug in the optimized and control formulations. The peak temperature of paracetamol shifted from 169.79°C to 120°C-160°C as other components were added to the formulation. Generally, the optimized formulations had two major peaks at 69.65°C-91.89°C and 118.81°C-155.63°C. The control formulation had one major peak 126.57°C-133.28°C. The combined thermogram of all the optimized samples revealed some differences in peak temperature.

Table 2. Responses of the optimized and control formulations

Time (h)	Mean ± SD					
	Release (%)		Coefficient of variance		Mean dissolution time	
	Validation formulations	Control formulations	Validation formulations	Control formulations	Validation formulations	Control formulations
0.083	0.259±0.0566	0.403±0.1159	34.325±28.9560	27.993±21.8610	0.040±0.0000	0.040±0.0000
0.25	0.597±0.0715	0.780±0.1562	22.813±15.9570	17.630±16.9270	0.113±0.0058	0.103±0.0115
0.5	1.108±0.0953	1.287±0.1888	17.240±9.4518	20.037±9.1173	0.237±0.0115	0.210±0.0200
0.75	1.577±0.0904	1.770±0.2816	13.897±6.3350	17.317±9.5348	0.350±0.0173	0.323±0.0115
1	1.866±0.1543	2.073±0.2272	12.830±6.2367	16.820±10.5052	0.430±0.0100	0.403±0.0321
2	3.639±0.2180	3.923±0.5237	8.360±2.6254	17.857±11.9501	0.953±0.0252	0.923±0.0153
3	5.102±0.3239	5.793±0.4143	6.730±1.2869	17.673±10.7575	1.397±0.0115	1.433±0.0666
5	7.658±0.6042	8.513±0.4153	7.865±2.6375	18.960±7.9269	2.267±0.0503	2.257±0.1002
8	10.993±1.1533	11.957±0.7836	7.233±0.9347	18.350±6.1724	3.533±0.1350	3.460±0.1493

SD: Standard deviation

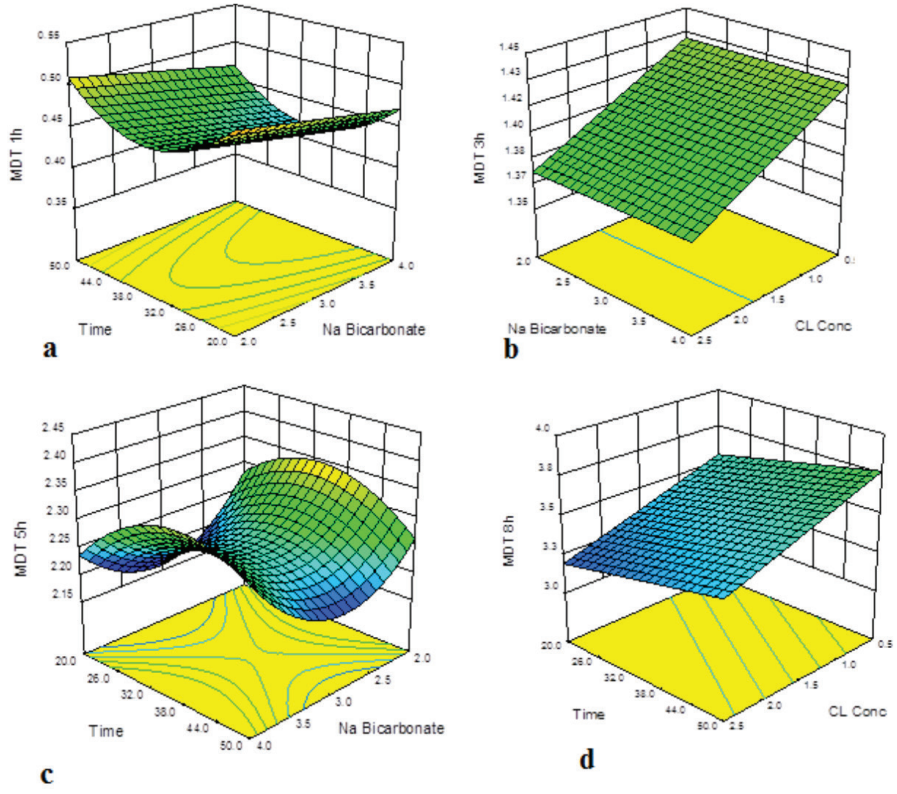


Figure 4. Combined effects of (a) irradiation time and cross-linker concentration on MDT at 1 h, (b) irradiation time and sodium bicarbonate at 3 h, (c) salt and irradiation time at 5 h, and (d) irradiation time and cross-linker on MDT at 8 h

MDT: Mean dissolution time

DISCUSSION

The maximum effective temperature that could induce cross-linking was 60°C, which was obtained at the minimum microwave energy of 30% in a short duration of 50 s. The percentage of paracetamol released from matrices 8 and 9 was higher after 6 h. By comparison, 35.51% paracetamol release was observed in the other matrices at 8 h. This difference might be ascribed to variations in drug diffusivity behavior and possible structural damage of gel matrices during dissolution.[17] Using a cross-linker and microwave energy reduced the percentage of drug release, which seemed to be an optimal extended release pattern from the resulting cross-linked swellable gelatin matrices. The high CV in the drug release of the majority of matrices at the initial time interval was decreased at terminal time intervals possibly because of a decrease in porosity, leading to compact cross-linking in matrices. An increase in MDT after 8 h suggested that the dissolution of paracetamol from the gel matrices was considerably slower than that of paracetamol from the pure solution. The drug released from most of the microwave-treated cross-linked matrices was best described by the Peppas model. Conversely, the drug released from matrix 18 followed the first-order kinetics. The diffusional exponent, n between 0.5 and 1 supported the combined involvement of Fickian diffusion and macromolecule chain relaxation (anomalous or non-Fickian transport) during drug dissolution.[18] However, only response surface plots exhibiting the prominent effects on drug release are shown here. Drug release was considerably controlled by the duration of microwave exposure and the concentrations of maize and sodium bicarbonate. This phenomenon was caused by the cross-linking of gelatin under a suitable combination of factors, as described in an earlier report,[10] which demonstrated that a cross-linked gelatin product requires the presence of suitable combinations of all components (gelatin, sugar, and salt) to effectuate cross-linking.[10] In the present study, a low percentage of paracetamol release, at 1 h was achieved at the middle levels of irradiation time, coupled with any amount of cross-linker or salt (Figure 3a, b, respectively). At 2 h, a low drug release was accomplished with the middle levels of exposure time, complemented with the higher amount of salt (Figure 3c) and higher concentrations, both of cross-linker and salt (Figure 3d). At 8 h, the higher amount of cross-linker coupled with all the concentrations of salt resulted in a lower percent of paracetamol release (Figure 3e).

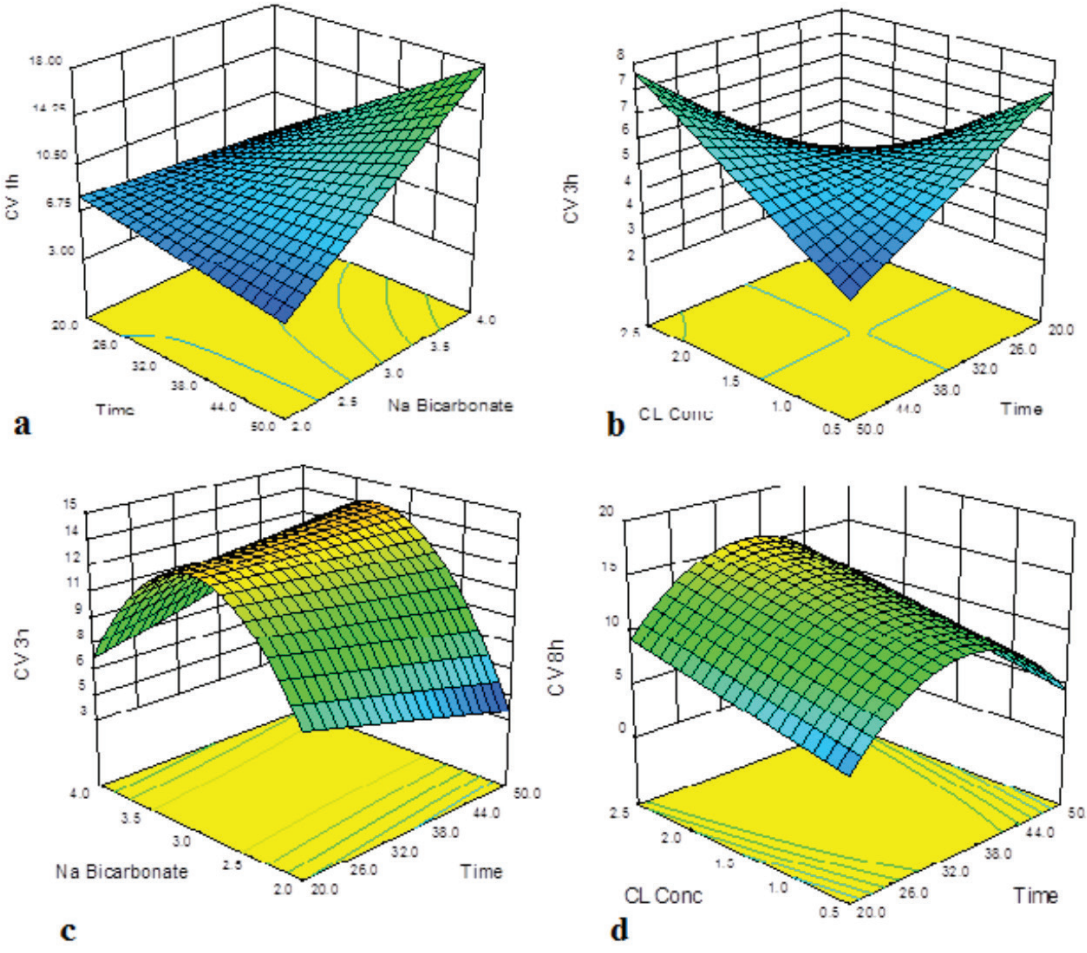


Figure 5. Combined effects of (a) irradiation time and sodium bicarbonate on CV at 1 h, (b) cross-linker concentration and time, (c) salt and irradiation time at 3 h, and (d) cross-linker and time on CV at 8 h

CV: Coefficient of variance

The long irradiation time alone failed to reduce the percentage of drug release at 8 h when a lesser amount of the cross-linker and salt were present in the matrices. Our findings were consistent with previous report,[6] which showed that the brief duration of microwave irradiation favored the sustained release characteristics of ketoprofen matrix beads. By contrast, long irradiation conferred the immediate release features possibly because of the effects of irradiation time on matrix porosity and the solid state of the drug in the gel matrix. MDTs at 1, 3, and 8 h were low at high salt and cross-linker concentrations and long microwave exposure. The saddle-shaped response plot of MDT at 1 and 5 h revealed the non-linear effect of the above factors on MDT. The duration of microwave exposure increased CV, whereas moderately high sodium bicarbonate concentrations could increase CV only at high microwave exposure. Thus, low CV was related to a comparatively shorter regime of microwave irradiations, suggesting high precision and reliability under microwave heating conditions. Controlling and limiting variations in outputs show the consistent quality of products and are critically required in research and industrial settings.[19,20] A low percentage of drug release, a low CV, and a high MDT were the desired characteristics for the optimized matrices. A high MDT indicates a good release-retarding ability of a cross-linked system.[21] In our study, three control matrices were prepared by classical heating at 60°C. The controlled and optimized formulations had almost similar percentage of drug release and followed the Peppas model. Heating by either classical or microwave facilitated the cross-linking of macromolecular (gelatin) chains in the presence of the cross-linker and salt. The decrease in the percentage of drug release from the gelatin matrices prepared via exposure to 330 W of microwave energy for 20-60 s was an indirect evidence of cross-linking. However, microwave exposure in this study was shorter than that in another study (10 min) that generated a temperature range of 150°C-250°C in the preparation of gelatin microspheres. Gelatin is sensitive to and is modulated at low temperatures,[4,22] particularly in the presence of cross-linker. The same finding was observed in the present study. An appropriate concentration of cross-linkers (genipin and glutaraldehyde) improves the mechanical strength and thermal stability of gelatin films.[23] In the present study, cross-linking occurred via a chemical interaction between sugar (maize) and gelatin under controlled heating. Cortesi et al.[24] demonstrated that sugars react with gelatin via two main reactions. One reaction is called Amadori rearrangement through which the gelatin amino group (lysyl ε-amino group) reacts with the aldehyde group of sugar to produce a cationic imine that rearranges to form a methylene link between 2 ε-amino groups of lysine. As a result, a cross-linked structure is formed. In the other reaction, the carbonyl group of an open-chain sugar form reacts with free amino groups of gelatin. After a number of tautomerizations, a ketose sugar is produced. This carbonyl adduct can further associate with another amine group, thereby forming a cross-linked structure.[24] The DSC thermograms supported the cross-linking (Figure 6b). Consistent with previous findings,[25] our results revealed that pure paracetamol showed a sharp melting endotherm at 169.79°C because of the crystalline nature of the drug. The DSC curves of maize and gelatin had shallow endotherms because of their poor thermal conductivity,[26] illustrating a gradual structure loss over time. The DSC curves of the microwave heat-treated (V1, V2, and V3) formulations and the classical heat-treated formulations (C1, C2, and C3) exhibited a curve with variable endothermic peak positions. Overall, the peak broadened as the melting temperatures decreased from 126.57°C to 133.28°C probably because of a decreased drug crystallinity caused by drug-polymer cross-linking. The findings also demonstrated different levels of cross-linking with different types of heat treatments of the samples as depicted by varied peak positions. Controlling various parameters, such as heating rate, placement of sample, and amount of sample either in classical or microwave heating methods, probably influenced the thermal behavior of the matrices. As expected, relatively better standard deviation, i.e., lesser varied peak positions, were observed in microwave heating than in classical heating.

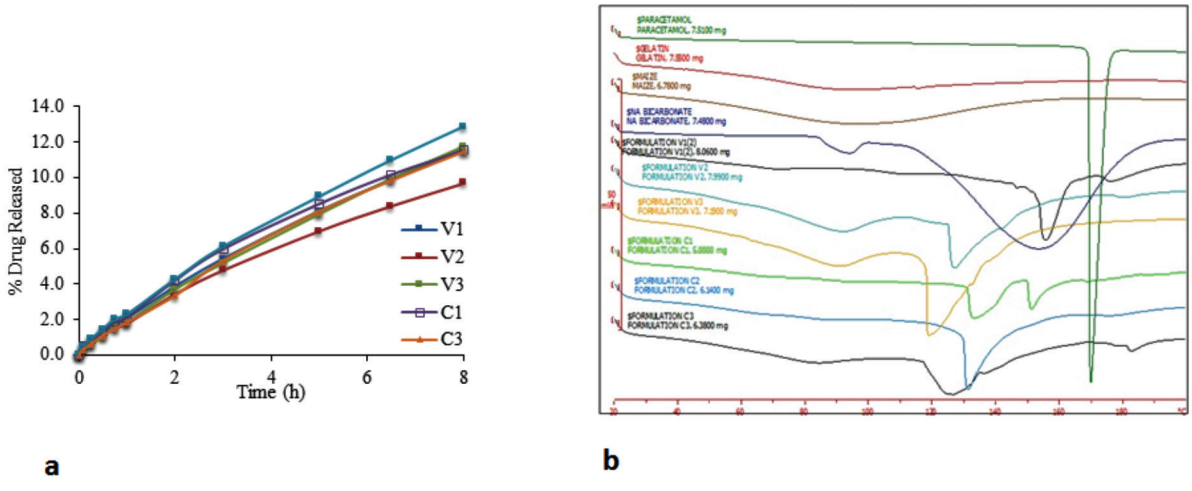


Figure 6. (a) Drug release (%) of the validation and control formulations. (b) Combination of ingredients and optimized and control formulation thermograms

The matrices prepared via classical heating (control) and microwave did not differ. However, the optimized and control matrices varied in the percentage of drug release at 5 h, CV at 5 h, CV at 8 h, and MDT at 0.75 h. Interestingly, the output of microwave exposure was having low variations (indicated by low standard deviation) and consistent quality obtained within a short period. The consistent quality of a sustained release carrier could ensure a consistent drug delivery in terms of correct timing and accurate dosage. This discriminative effect of microwave irradiation was probably attributed to its ability to penetrate a specimen with a uniform amount of heat, thereby facilitating an appropriate aldehyde cross-linking with an amino group of gelatin.[4] Thermal analysis via DSC indicated the interaction between paracetamol and other ingredients, as shown by the left peak shift. A previous study reported the chemical stability of a drug embedded in a matrix generated via microwave irradiation. Hence, paracetamol in this study was expected to sustain its stability.[27,28] Although the combined thermograms of all the optimized samples showed some differences in peak temperature, endothermic enthalpy may be caused by the storage duration of samples.[29] Nevertheless, the microwave may be an effective approach for the preparation of sustained release matrices with better reproducibility (outputs lower variation) as compared to the conventional heating approach.

Study limitations

The time for the complete release of paracetamol was not determined. The non-thermal effects of microwave irradiation were also not assessed. These parameters should be explored in future studies.

CONCLUSION

Cross-linked gelatin treated with microwave could sustain the release of paracetamol compared to that of the paracetamol solution alone. As indicated by the relative standard deviation in the formulations, the microwave-treated cross-linked gelatin formulations yielded consistent release and lower variations in the release data compared with those prepared via classical heating. DoE could be used as a tool to optimize these conditions. Therefore, microwave heating could be applied to produce a consistent quality of pharmaceutical products.

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ORIGINAL ARTICLE

Development of a Structured Communication and Counseling Skills Course for Pharmacy Students: A Simulation-based Approach

Eczacılık Fakültesi Öğrencilerine Yönelik İletişim ve Danışmanlık Becerileri Eğitim Programı Geliştirme Çalışması: Simülasyona Dayalı Bir Yaklaşım

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ABSTRACT

Objectives: We aimed to develop a structured communication and counseling education program to improve pharmacy students' skills. Then, we objectively assessed this program by using simulated patients. The program aims to improve pharmacy students' communication and counseling skills by using a patient-centered approach.

Materials and Methods: The study was conducted in three stages. First, a "Pharmacist-Patient Communication and Counseling Skills" education program was developed. Second, this program was implemented for pharmacy students. Third, the program was tested on volunteer students and evaluated for its effectiveness.

Results: The education program had a very large effect (Cohen's d_z: 6.074) on improving students' communication and counseling skills, especially their empathy skills.

Conclusion: The education program achieved its goals. After demonstrating the program's success, a course was added to the pharmacy curriculum, and a communication skills laboratory was established in the school.

Key words: Health communication, social skills, teaching methods, professional competence, problem-based learning

ÖZ

Amaç: Bu çalışma ile eczacılık öğrencilerinin iletişim ve danışmanlık becerilerini geliştirmeye yönelik yapılandırılmış bir eğitim programı geliştirmeyi amaçladık. Sonrasında programı objektif olarak standart hastalar kullanılarak değerlendirdik. Program, eczacılık öğrencilerinin iletişim ve danışmanlık becerilerini hasta odaklı bir yaklaşımı kullanarak geliştirmeyi hedeflemektedir.

Gereç ve Yöntemler: Çalışma üç aşamada yürütülmüştür. İlk önce "Eczacı-Hasta İletişim ve Danışmanlık Becerileri" eğitim programı geliştirilmiştir. İkinci aşamada, bu eğitim programı gönüllü eczacılık öğrencileri için uyarlanmıştır. Üçüncü aşamada, program gönüllü öğrenciler üzerinde denenmiş ve etkinliği değerlendirilmiştir.

Bulgular: Eğitim programının, öğrencilerin iletişim ve danışmanlık becerilerini, özellikle empati becerilerini geliştirmede çok büyük bir etkisi (Cohen d_z: 6,074) olduğu görülmüştür.

Sonuç: Eğitim programı hedeflerine ulaşmıştır. Programının başarısı kanıtlandıktan sonra, eczacılık lisans programına bir ders olarak eklenmiş ve okulda iletişim becerileri laboratuvarı kurulmuştur.

Anahtar kelimeler: Sağlık iletişimi, sosyal beceriler, öğretim yöntemleri, mesleki yeterlilik, probleme dayalı öğrenme

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INTRODUCTION

The role of pharmacists has evolved over time. Today, pharmacists should be able to establish effective therapeutic relationships with patients.[1,2] When pharmacists are involved in patient care, patients have improved clinical outcomes and quality of life.[1,3-7] To provide optimal patient care and establish a good relationship with patients, pharmacists must be able to communicate effectively.[8,9] Communication skills can be improved through both practice and education.[2]

Training in patient communication skills has taken on a growing role in the pharmacy curriculum.[10,11] Pharmacy schools now regard communication as a core clinical skill rather than an optional curricular component.[12] Since the content of communication skills courses can vary from one culture to another, different guidelines can arise for what pharmacy students are expected to learn. That said, patient counseling is considered as a crucial component of virtually all pharmacy curriculums.[13] Nevertheless, cultural differences have led to inconsistent communication-based learning outcomes and teaching modalities.[14]

Communication courses in pharmacy schools have generally covered the following: (1) Key communication skills (active listening, empathy, and assertiveness); (2) counseling skills (initiating the interview, establishing trust, eliciting information, giving information and educating the patient, shared decision making/involving the patient, and verifying patient understanding); (3) ability to handle difficult situations and develop conflict management skills; (4) ability to communicate with diverse patients; and (5) ability to employ behavior modification strategies.[13,15] While prior studies have described the content and outcomes of courses, there remains a need to develop a structured education program that demonstrates the optimal order or combination of course content.[14]

With the increasing awareness of the value of communication skills in pharmacy practice, developing effective methods for teaching and assessing communication skills has become important for educators. The simulated patients (SPs) method is commonly used both as a teaching Tool and a standalone assessment technique.[16] Yet, even though SPs are used as an assessment method, there is little published research on the validity and reliability of standardized assessments of pharmacy student communication skills.[15]

Several theories have been proposed to explain why and how communication skills might be developed in pharmacy education. According to the structured training theory, communication skills can be developed by structured training.[14] Based on that theory, this study developed a structured "Pharmacist-Patient Communication and Counseling Skills Education Program (PPCCE)" to improve pharmacy students' skills. Further, the program was objectively assessed using SPs. This work evaluates the impact of the PPCCE on students, not the effect of SPs. We hypothesized that the students in this class would show improved communication and counseling skills.

MATERIALS AND METHODS

PPCCE development involved three major phases, following Eng et al.[17] model for developing and evaluating health communication programs. The study has three stages. First, the PPCCE was developed. Second, the PPCCE was implemented with pharmacy students. Third, the program was tested on volunteer students and its effectiveness evaluated.

Ethics statement

The Ankara University Ethics Committee on Non-clinical Research on Human Beings approved this study (date: 03.12.2015, number of decision: 328). Written informed consent was obtained from each participant.

Conceptualization and design

Content

For the content of PPCCE, topics were identified based on the literature.[18-24] In addition, ACPE guidelines were reviewed regarding pharmacist-patient communication and counseling.[25] Table 1 presents the goals, objectives, and content of the sessions.

PPCCE development also used Miller's pyramid of clinical competence.[26] The program focused on practical approaches and a learner-centered format. After each theoretical lesson, one practical session with SPs was added to the curriculum. Table 2 shows the content of the PPCCE and the sequence of actions. Five scenarios were written adapted from real life for the sessions with SPs (Table 3). The scenarios used in pre- and post-tests were the same.

Evaluation of the education program

Statistical analysis

A mixed method was used to evaluate the PPCCE. First, a quasi-experimental, pre- test and post-test design was executed. SPs were used as an assessment method for the pre- and post-tests. Additionally, SPs were used as a teaching method during the PPCCE. Before the PPCCE was implemented, the students interviewed with SPs as a baseline. After training, students interviewed with SPs to determine the improvement (Table 2). Each SP interview was rated by one rater (GG). To eliminate experimenter bias, all tapes were blinded in terms of whether they were pre- or post-test. A second method was employed in the form of a feedback form administered to students. The learning experience, which is a personal insight, was not assessed in the study based on the SP interviews. The form consisted of several statements based on a five-point Likert scale and open-ended questions. The instrument was developed from the literature.[15]

Students' tapes were assessed by the trainer (GG) using a modified version of the patient-centered communication tool (PaCT). Recent research has shown that the PaCT is a valid, reliable, and appropriate grading instrument for assessing pharmacy students' communication skills.[27] In addition, the PaCT focuses on the learning objectives the researchers had targeted, it directs the observer toward important skills, and it can be utilized for performance-improvement tracking.[28]

Adaptation to the Turkish language was conducted through a pilot study. Some PaCT items were modified to remove ambiguity and to fit the research design. Six health communication experts were asked for their opinions on the content of the PaCT based on specific evaluation criteria. The expert panel also reviewed the consultations with SPs by using the instrument and concluded that it was highly relevant to pharmacy-patient communication skills. On the basis of the expert panel's guidance, planning the visit agenda was deemed not suitable for pharmacist-patient encounters in Turkey due to a lack of sufficient time and a suitable backdrop to negotiate with patients before the consultation. Therefore, one item was omitted from the PaCT. The modified instrument has 22 items (skills) gathered under five tools: Tool A: Establish a connection (opening the session); Tool B: Explore and integrate the patient's perspective (asking question); Tool C: Demonstrate interest and empathy (acknowledge emotions, respond to emotions appropriately); Tool D: Collaborate and educate (shared decision making, plan, educate, and complete the visit); Tool E: Communicate with Finesse (verbal and non-verbal skills, effective questioning, organization, professionalism). The students could earn up to five points for each item. The modified instrument was tested on fifth-year students (n=15) to calculate inter-rater reliability. Using video recordings of the encounters, raters (n=2) assessed student performance using the modified instrument. Rating data were fully crossed (i.e., all raters evaluated all learners on all behaviors). Ratings were analyzed using generalizability theory.[29-31] We hypothesized that rating by one rater would be as reliable as rating by two raters. As a result, the generalizability coefficient was found to be 0.76 for one rater and 0.789 for two raters. The coefficients were considered as acceptable.[32] Increasing the number of raters led to a very small increase in the G coefficient, and such an increase would not be practical in cases where it is difficult to find competent raters. Therefore, assessing the program by using the PaCT with one rater would produce reliable results.

Learning activities

To ensure uniform teaching, a detailed training protocol was written for each session. It contained the goals and objectives of each session, preparatory materials (student, trainer, and SP guides), and scenarios. Lectures were organized using active-learning techniques to engage students (e.g., group discussion, brainstorming, video-based skills demonstration, small group role-playing practice, interviews with SPs). Objectives and goals were set at the beginning of the lectures. The lectures then continued with interactive discussions. The PaCT was introduced and defined by reviewing the instrument; students were asked to give examples demonstrating such skills during an interview. Students made suggestions, and the trainer provided supporting insight. To provide individual learning experiences, each student privately completed a single-case SP encounter at the end of each session. In practical sessions, SPs were used as a teaching technique.

Interactive exercises, pharmacy-patient consultation scenarios for role-play, and appropriate readings were included as student guides. Trainers' guides were designed on the basis of facilitators' needs. The processes for both lectures and SP

Table 1. Objectives of the education program

Students will be able to:
Distinguish the different barriers of interpersonal communication between pharmacists, patients, and other health professionals
Use active listening skills; elicit the needs, requests, and capabilities of the patient; and provide information using effective non-verbal, explanatory, and questioning skills (reflection, picking up patient's cues, paraphrasing, summarizing, open, closed, and focused questioning, etc.)
Encourage the patient to express their own ideas, concerns, expectations, and feelings and accept the legitimacy of the patient's views and feelings
Adapt their own communication to the level of understanding and language of the patient, avoiding jargon
Show awareness of the patient's non-verbal communication (e.g., eye contact, gestures, facial expressions, and posture) and respond to them appropriately
Involve the patient in decision making and adapt the plan/intervention to patient's needs and capabilities
Organize a conversation from beginning to end with regard to structure
Display appropriate professionalism
Give information to the patient (oral and written) in a timely, comprehensive, and meaningful manner
Use education methods appropriate for the patient and give the appropriate amount of information
Verify the patient's understanding of new information provided and suggest changes to be initiated
Recognize difficult situations and communication challenges (e.g., crying, strong emotional feelings, interruptions, aggression, anger, anxiety, embarrassing or sensitive issues) and manage the challenging patient sensitively and constructively
Use adequate strategies to solve conflicts
Discuss with the patient for follow up interview
Promote adherence to appropriate therapy

practice, along with a chronological description of steps to be taken, were explained in detail. SP guides also included the written scenario, feedback rules for SPs, topics to be addressed during feedback, and objectives of the practice session.

Approach to grading

The three main grading criteria were the final examination (post-test interviews with SPs; 50%), class participation (10%), and students' efforts to apply the new skills (40%).

Implementation

All students enrolled in the PPCCE of the undergraduate pharmacy program at the Ankara University Faculty of Pharmacy in the first semester of 2016 were asked to participate. Of the 26 students, 5 declined to participate, leaving a total of 21 participants. The students were advised of the goals of the study and assured that the data would be kept confidential. Only students who did not consent to participate were excluded. The

Table 2. Description of the education program with the sequence of actions

Component		Content	Domain	Addressed tools in the PaCT	Duration
Session 1	Pretest	Simulated patient consultation interviews for all students, evaluated by one independent observer using an instrument, without feedback given	-	Whole PaCT	3 hrs/1 week
	Lecture 1 by the trainer	Description of effective communication skills, fundamental techniques for the process of key communication in everyday life, such as behaving assertively, showing empathy to others, listening actively, etc.	Cognitive affective "knows how"	C and E	4 hrs/2 weeks
	Interview 1 with SP	Role-play for one scenario by students with a SP. Feedback facilitated by trainer	Cognitive and affective psychomotor "shows how"	C and E	4 hrs/1 week
Session 2	Lecture 2 by the trainer	Description of effective pharmacist-patient communication dimensions and fundamental techniques for the process of pharmacist communication, such as avoiding the use of medical and pharmaceutical jargon, educating the patient, shared decision making with patient and responding to a patient's concerns, organizing the interview with regard to structure	Cognitive affective "knows how"	A and D	6 hrs/3 weeks
	Interview 2 with SP	Role-play for one scenario by students with a SP. Feedback facilitated by trainer	Cognitive and affective psychomotor "shows how"	A and D	4 hrs/1 week
Session 3	Lecture 3 by the trainer	Description of causes of conflict, expressing conflict management strategies, and steps in problem-solving, such as identifying all possible solutions, shared decision making, caring all parties to agreeing on an acceptable plan	Cognitive affective "knows how"	B	4 hrs/2 weeks
	Interview 3 with SP	Role-play for one scenario by students with a SP. Feedback facilitated by trainer	Cognitive and affective psychomotor "shows how"	B	4 hrs/1 week
Session 4	Lecture 4 by the trainer	Description of adherence, causes of non-adherence, and patient follow up strategies for a pharmacist, such as understanding the patient's reluctance to use medicine or willingness to change a behavior	Cognitive affective "knows how"	D	2 hrs/1 week
	Interview 4 with SP	Role-play for one scenario by students with a SP. Feedback facilitated by trainer	Cognitive and affective psychomotor "shows how"	D	4 hrs/1 week
	Post-test	Simulated patient consultation interviews for all students, evaluated by one independent observer using an instrument, without feedback given	-	Whole PaCT	3 hrs/1 week

PaCT: Patient-centered communication tool, SP: Simulated patient

class met once a week for 14 weeks for two hours of lectures, and three hours of SP practice. The PPCCE specifically targeted fifth-year students since, they had already met different types of patients during their internship and had sufficient knowledge of medicines, side effects, their proper doses, and so on. Targeting fifth-year students also allowed students to easily be involved in real-world pharmacy settings without forgetting their acquired skills.

Thirteen SPs participated in the study; they had prior experience in role-playing with the students. The SPs attended eight hours of training. They were paid 30₺ per hour during the training and when working with students. SPs were expected to practice their roles in the scenario in advance. To provide efficient learning, SPs also had to give the students constructive feedback after the consultations regarding their experiences as a patient.

The outcomes and what was expected of the students during the interviews were all described in advance. Advance notice about medications to be counseled helped students acquire the knowledge they would need during the consultation. This helped to reduce, students' anxieties so they could focus on their communication skills rather than the clinical content. Every student had five minutes to re-read the relevant information before interviewing the SPs.

RESULTS

Evaluation and assessment

Information was collected on the participants' age and gender. They ranged in age from 20 to 22 years and were mostly female (n=16, 76%).

Table 4 shows the mean scores on the tools and the total scores. An increase can be observed in the students' mean scores after the program's implementation. The baseline and outcome data were compared using paired-sample t-tests (Table 4). Comparisons between pre- and post-test scores indicated that the students received significantly higher scores on all tools ($p<0.001$). Figure 1 shows the changes in the pre- and post-test scores.

Table 4 shows that the greatest improvements were in tool C (demonstrate interest and empathy). Meanwhile, tool E showed the least improvement (mean deviation: 2.23), with higher pretest scores (mean: 2.51) than on all other tools.

In addition to pre- and post-test scores, we calculated the effect sizes (ES) for both variables to estimate the relevance of possible effects of the program. We used the Cohen's d_z formula[33] as follows:

Cohen's d_z: $t\sqrt{n}$

The ES for communication and counseling skills was 6.074; in terms of Cohen's d_z, this should be considered as a very large effect. Combining the tests for significance, we may firmly conclude that the education program had an effect on the students' skill development.

The answers obtained from the feedback form supported the value of the PPCCE for improving students' communication skills. Eighteen of the 21 participants filled out the form (response rate: 86%). Most students enjoyed the PPCCE and

Figure 1. Changes in the pre- and post-test scores

Table 3. Content and objectives of the scenarios used in the education program

Scenarios	Content	Objectives
Interview 1 with SP-scenario 1	A patient with type-2 diabetes who had to give himself insulin injections fears giving injections to himself. He requests that the pharmacists give him the insulin injection every day instead	Being assertive, using effective questions, displaying suitable body language, demonstrating empathy, and listening actively to the patient
Interview 2 with SP-scenario 2	A patient with osteoporosis comes to pharmacy. She had some concerns about the side effects of the drugs when hearing from the pharmacist	Creating rapport, integrating the patient's perspective, using effective questions, organizing the consultation, educating the patient, and completing the visit
Interview 3 with SP-scenario 3	An insistent patient wanting the pharmacist to persuade her daughter to use a food supplement although she does not want to	Recognizing the difficult situations and communication challenges and managing the challenging patient sensitively and constructively
Interview 4 with SP-scenario 4	The patient with osteoporosis who had come to the pharmacist a few weeks before (in scenario 2). She has some difficulties using the medication and decided to stop taking it	Promoting adherence to appropriate therapy. Involving the patient in decision making and adapting the plan/intervention to the patient's needs and capabilities

SP: Simulated patient

believed it improved their problem-solving skills (Table 5).[15] However, students also expressed their wish to have had more interviews during the PPCCE. Moreover, they felt that their fifth-year was too late to learn these skills and that the education program should be earlier in the curriculum.

Some SPs also commented on the education program during the laboratory practices. They said they were not used to seeing a pharmacist behave this way in real life. They expressed the view that pharmacists tended to be more interested in paperwork and that patients communicated more with technicians than with pharmacists. The SPs said they hoped this education program could help produce pharmacists who were well-educated and empathic.

DISCUSSION

The PPCCE had a very large effect on improving the communication and counseling skills of students. At the end of the course, development was seen in all five tools, though the most development was seen in tool C (interest and empathy). When designing the PPCCE, the basic philosophy was determined on the basis of patient-centeredness. Mead and Bower[33,34] described patient-centeredness as "the physician tries to enter the patient's world, to see the illness through the patient's eyes". In this way, patients play a more active role in the treatment process, and respecting their needs is crucial. Hence, the main theme in all sessions was to understand and care for the patients' needs. The improvement in tool C could have resulted from the concept of patient-centeredness being adopted during the development of the PPCCE.

The most challenging part of the PPCCE was tool D (collaborate and educate). Students mentioned difficulties performing these skills, and they regarded the skills in tool D as complex. Figure 1 shows the low level of development revealed for tool D. Surprisingly, the students were not aware that providing a plan and contributing to patient adherence are among pharmacists' responsibility. This could have to do with Turkish pharmacists paying more attention to business issues and paperwork than to counseling patients. In a recent study, Turkish pharmacists

Table 4. Comparison of pharmacy students' scores on various tools of the modified version of PaCT at pretest and post-test (n=21)

Tools of PaCT	M (SD) (pre)	M (SD) (post)	MD (SD) (pre-post)	t-test*	p value
A (establish a connection)	1.57 (0.53)	4.63 (0.35)	3.06 (0.56)	24.9	<0.001
B (explore and integrate patient's perspective)	1.29 (0.54)	4.69 (0.60)	3.40 (0.68)	4.1	<0.001
C (demonstrate interest and empathy)	1.07 (0.24)	4.74 (0.41)	3.67 (0.43)	4.1	<0.001
D (collaborate and educate)	1.66 (0.58)	4.52 (0.46)	2.86 (0.57)	23.0	<0.001
E (communicate with Finesse)	2.51 (0.49)	4.74 (0.37)	2.23 (0.56)	18.3	<0.001
T (total)	1.87 (0.41)	4.65 (0.35)	2.78 (0.46)	27.8	<0.001

*For tool B and tool C, a Wilcoxon signed-rank test was performed. For tools A, C, D, E, and for total score a paired-sample t-test was conducted. PaCT: Patient-centered communication tool, M: Mean, SD: Standard deviation, MD: Mean deviation

Table 5. Students' answers obtained from the feedback form

Item	M (SD)
The SPs were the key component of the education program	4.7 (0.4)
I was happy to get feedback from the SPs because the SPs' feedback contributed to my improvement	4.6 (0.6)
I was happy to get feedback from the trainer because the trainers' feedback contributed to my improvement	4.9 (0.3)
The themes of the scenarios reflected what was covered in the lectures	4.7 (0.6)
The practices with SPs improved my problem-solving skills	4.7 (0.5)
I enjoyed the practices with SPs	4.8 (0.4)
Practicing with SPs played an essential role in preparing me for "real life" counseling situations	4.7 (0.5)
I believe my communication and counseling skills were improved at the end of the education program	4.9 (0.3)
I felt confident using my theoretical knowledge during the laboratory practices with SPs	4.3 (0.7)
I enjoyed working in a small group sessions	4.8 (0.4)
I am of the opinion that the effectiveness of the education program mostly depends on the performance of the trainer	3.2 (0.8)
I wish I could practice with SPs more during the education program	3.1 (0.5)
I wish I could have taken this course earlier in my pharmacy education	4.6 (0.8)

SP: Simulated patient, M: Mean, SD: Standard deviation

expressed their feelings about the pharmacist-patient relationship. They reported that the pressure to distribute medications to keep their businesses alive caused problems with patient communication; they also felt that they were not performing their roles as consultants.[35,36] Pharmacists in Turkey thus experience conflicts between their roles as business people and as healthcare advisors.[37] While the low development in tool D could be attributable to the structure of the community pharmacy practice in Turkey, it might also have to do with role models. According to Rogers[38], empathy can be learned from empathic persons. Students during placements in community pharmacies observe the pharmacists' consultations with patients. As a consequence, by observing insufficient consultation styles negative informal messages conveyed to the students might explain the results for tool D. Since tool D is considered complex, more practice may be required to improve students' skills.[27]

As shown in Figure 1, students had the highest pretest scores for tool E. It can be said, therefore, that prior to the PPCCE, students were strong in the areas of verbal skills, non-verbal skills, effective questioning, organization, and professionalism. The high pretest scores could be attributable to students' awareness of being watched and scored during the interviews and their corresponding efforts to show professionalism. Such unnatural attitudes highlight the insufficiencies of the role-playing technique. Many studies have suggested that role-playing cannot mimic nature.[39,40]

Communicating effectively with patients while projecting a professional image is considered a distinct and important professional skill.[41] In the free comments, students expressed different opinions on how to behave as professionals during the opening session (tool A). Figure 1 shows that the PPCCE had an impact on developing a professional attitude at the beginning of the interview. Using in-class activities, such as discussions with the trainer and the other students, appeared to be effective and could have contributed to this development. The role of trainers is to help students gain the proper skills using their own insight.[42] The students participated actively and took at least some responsibility for their own learning.[43] Additionally, recognizing exemplars of professionalism through discussion and observation could help to provide a better example.[44]

Study limitations

Despite the demonstrated improvement in communication and counseling skills, as well as the positive feedback from students, there were some limitations to this study. First, the quasi-experimental design could have made it difficult to clearly ascertain the causes of improvements in skills. In future studies, control groups and some comparison groups including different teaching methods could be employed to determine the elements providing the improvements. Second, to explore the validity and reliability of PaCT and the feedback form in Turkish, detailed and comprehensive studies should be designed to demonstrate their psychometric properties. Third, as the education program was being implemented, the students continued their placements at community pharmacies which, as discussed above, could have influenced them. A fourth limitation concerns the feedback from the SPs. Some students said the feedback from the SPs was unnatural. Since the SPs in the present study had been working as SPs for many years, routinization may have developed. It can be suggested, therefore, that changing SPs or educating them periodically might be necessary to contribute to outcomes. A fifth important limitation concerns the total length of the education program. Longitudinal education programs that aim to improve skills or attitudes should be designed to cover more than one semester. A spiral curriculum might be a better way to develop such skills. Finally, it should be noted that the results may not be generalizable to other universities or students.

After the study was completed, the PPCCE was added to the undergraduate curriculum of the Faculty of Pharmacy at Ankara University. This will help to guarantee the acquisition of such skills during undergraduate education for all students.

CONCLUSION

The communication and counseling skills education program met its goals of introducing students to the application of these skills with patients. In the future, the authors plan to develop this education program for greater effectiveness. Expanding the SP encounters and feedback sessions should be considered on the basis of students' evaluations. Peer assessment will be used for students to observe each other's performance and to improve feedback skills. In addition to adding this course to its curriculum, the Faculty of Pharmacy, Ankara University, has established a communication skills laboratory within the school.

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ORIGINAL ARTICLE

Investigation of Physiological Effects Induced by Dehydroepiandrosterone in Human Endothelial Cells and Ovarian Cancer Cell Line

İnsan Endotel Hücreleri ve Ovaryum Kanseri Hücre Hattında Dehidroepiandrosteronun Neden Olduğu Fizyolojik Etkilerin Araştırılması

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ABSTRACT

Objectives: Dehydroepiandrosterone (DHEA) is an endogenous hormone that acts as a ligand for several cellular receptors. An age-dependent decline in circulating levels of DHEA is linked to changes in various physiological functions. In gynecological clinical practice, DHEA is commonly prescribed to induce ovulation. Some clinical studies report a positive association between high serum concentrations of DHEA and an increased risk of developing ovarian cancer. However, the *in vitro* physiological effects of DHEA on ovarian cancerous cells have not been explored thus far. In this study, we aimed to investigate the physiological effects of DHEA treatment (0-200 µM, 24-72 hours) on MDAH-2774 human ovarian cancer cell line and primary HuVeC human endothelial cells.

Materials and Methods: The physiological effects of DHEA treatment (0-200 µM, 24-72 hours) on MDAH-2774 human ovarian cancer cell line and primary HuVeC human endothelial cells were investigated with the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, acridine orange/ethidium bromide staining, and scratch assay.

Results: DHEA treatment promoted proliferation of the MDAH-2774 cancer cell line in a dose-dependent manner ($r=0.6906$, $p<0.0001$, for 24 hours) ($r=0.6802$, $p<0.0001$, for 48 hours) ($r=0.7969$, $p<0.0001$, for 72 hours). In contrast, DHEA inhibited proliferation of the primary HuVeC cells ($r=0.9490$, $p<0.0001$, for 24 hours) ($r=0.9533$, $p<0.0001$, for 48 hours) ($r=0.9584$, $p<0.0001$, for 72 hours). In agreement with these observations, DHEA treatment resulted in a dose-dependent increase in the number of necrotic cells in the primary HuVeC cells ($r=0.97$, $p<0.0001$). However, the number of necrotic or apoptotic cells did not change significantly when the MDAH-2774 cells was exposed to DHEA. Moreover, we found that DHEA treatment reduced the migration rate of HuVeC cells in a dose-dependent manner ($r=0.9868$, $p<0.0001$), whereas only a slight increase was observed in the MDAH-2774 ovarian cancer cell line ($r=0.8938$, $p<0.05$).

Conclusion: Our findings suggest that DHEA promotes the proliferation of ovarian cancer cells in a dose-dependent manner *in vitro*. Moreover, DHEA induced necrosis and inhibited proliferation in endothelial cells. Although mechanistic evidence is required, our preliminary findings imply that exposure to high doses of DHEA may be associated with an increased risk of developing ovarian cancer.

Key words: Dehydroepiandrosterone, MDAH-2774, HuVeC, ovarian cancer

ÖZ

Amaç: Dehidroepiandrosteron (DHEA), bir dizi hücresel reseptör için ligand olan bir endojen bir hormondur. Fizyolojik işlevlerdeki değişiklikler, dolaşımdaki DHEA seviyelerinde yaşa bağlı gerçekleşen düşüşe bağlıdır. Jinekolojik klinik uygulamada DHEA genellikle ovulasyon indüksiyonu için reçetelenmektedir. Bazı klinik çalışmalar, yüksek serum DHEA konsantrasyonları ile ovaryum kanseri gelişme riskinde artış arasında pozitif bir ilişki olduğunu bildirmiştir. Ancak, DHEA'nın over kanseri hücreleri üzerindeki *in vitro* fizyolojik etkileri şimdiye kadar araştırılmamıştır. Bu çalışmada, DHEA inkübasyonunun (0-200 µM, 24-72 saat) MDAH-2774 insan over kanseri hücre hattı ve primer HuVeC insan endotel hücreleri üzerindeki fizyolojik etkileri araştırılmıştır.

Gereç ve Yöntemler: DHEA inkübasyonunun (0-200 µM, 24-72 saat) MDAH-2774 insan over kanseri hücre hattı ve primer HuVeC insan endotel hücreleri üzerindeki fizyolojik etkileri (3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür) testi, akridin turuncusu/etidyum bromür boyaması ve yara iyileşme testi ile araştırılmıştır.

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Bulgular: DHEA inkübasyonu, doza bağlı bir şekilde MDAH-2774 kanser hücre hattının proliferasyonunu indüklemiştir (r=0,6906, 24 saat boyunca, p<0,0001) (r=0,6802, 48 saat boyunca p<0,0001) (r=0,7969, p<0,0001, 72 saat). Tam tersine, DHEA primer HuVeC hücrelerinin proliferasyonunu ise inhibe etmiştir (r=0,9490, 24 saat için p<0,0001) (r=0,9533, p<0,0001, 48 saat için) (r=0,9584, p<0,0001, 72 saat için). Bu sonuçları destekler şekilde, DHEA uygulaması HuVeC hücrelerinde nekrotik hücre sayısını doz bağımlı bir şekilde artırmıştır (r=0,97, p<0,0001). Ancak, DHEA'ya maruz kalan MDAH-2774 hücre hattında nekrotik veya apoptotik hücre sayısı önemli ölçüde değişmemiştir. Ayrıca, DHEA inkübasyonunun HuVeC hücrelerinin migrasyon oranını doz bağımlı bir şekilde azaltırken (r=0,9868, p<0,0001), MDAH-2774 over kanseri hücre hattında az bir oranda artırmıştır (r=0,8938, p<0,05).

Sonuç: Bulgularımız DHEA'nın *in vitro* olarak over kanseri hücrelerinin doz bağımlı bir şekilde çoğalmasını artırdığını göstermektedir. Ayrıca, DHEA endotelyal hücrelerinde nekrozu indüklemiş ve proliferasyonu inhibe etmiştir. Her ne kadar mekanistik veri gerekse de, ön bulgularımız, yüksek dozlardaki DHEA maruziyetinin over kanseri geliştirme riskiyle ilişkili olabileceğini göstermektedir.

Anahtar kelimeler: Dehidroepiandrosteron, MDAH-2774, HuVeC, ovaryum kanseri

INTRODUCTION

Dehydroepiandrosterone (DHEA) is one of the most abundant circulating steroid hormones produced by adrenal glands[1] and it can also serve as a precursor of other steroids in the brain.[2] DHEA is involved in the biosynthesis of sex steroids, and it may act as a ligand for several nuclear receptors as well as G-protein-coupled receptors. Physiological levels of DHEA in human serum range between 1 and 1000 nm,[3] whereas prescribed pharmacological doses range between 10 and 100 μM.[4] An age-dependent decline in circulating levels of DHEA can induce changes in cardiovascular tissues,[5] female fertility,[6] and metabolic and neuronal/central nervous system functions.[7]

Clinical findings show that DHEA administration can increase serum androgen levels, improve mood, improve sexual function, and decrease fatigue.[8] DHEA is also widely used to induce ovulation in gynecological clinical practice. However, data also suggest that high doses or prolonged use of DHEA is associated with an increased risk of developing ovarian cancer.[9,10] Cameron and Braunstein[8] have reported that DHEA activates estrogen receptors[11] in breast cancer and endothelial cell lines.[4,12,13] In line with these observations, clinical findings also implicate DHEA as a risk factor for developing breast cancer.[14] In contrast, *in vitro* findings suggest an inhibitory effect of DHEA on the proliferation of breast, myeloma, hepatoma, prostate, human colon adenocarcinoma, cervical, and leukemia cancer cell lines.[15-21] Thus, our understanding of the role of DHEA as a risk factor for developing cancer is limited.

At present, the *in vitro* effects of DHEA on normal and ovarian cancer cell lines have not been compared. Therefore, we aimed to investigate the physiological effects of DHEA on MDAH-2774 human ovarian cancer and non-cancerous HuVeC human endothelial cell lines. A better understanding of the differential effects of DHEA on normal and cancer cell lines can have important implications in clinical research.

MATERIALS AND METHODS

Cell culture and chemicals

Human ovarian carcinoma epithelial cell [MDAH-2774 (ATCC® CRLM10303™)] and human umbilical vein endothelial [HUV-EC-C (HuVeC) ATCC® CRL-1730™)] cell lines were used. The cell lines were cultured in high-glucose Dulbeccos modified eagle medium (DMEM) (Sigma, 5546) supplemented with 1% 2 mM L-glutamine (Biological Industries, BI03-020-1B), P/S (50 U/mL penicillin and 50 μg/mL streptomycin; Biological Industries, 03-031-1B), 10% fetal bovine serum (Biowest, S1810-500). Each cell line was seeded into 10 cm plates ($1.5x10^6$ cells/plate) and Biotest divided 72 hours later. DHEA tablets [approximately 0.6-1.2 g (2-3 pieces)] were dissolved in 50 mL of absolute ethanol as stock solutions. A 0.45-μm membrane filter was used for sterilization and the solution prepared fresh for every test.[4] As a negative control, cells were treated with ethanol (the solvent of DHEA) containing DMEM.

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay

12 mM stock solution of MTT (Neofrox 3580 MTT) was prepared as described by Mosmann.[22] Cells were seeded into each well of a 96-well plate (approximately 10^4 cells/well) in a volume of 100 μL. The MTT assay was conducted as follows: 10 μL of 12 mM MTT stock solution was added to each well and incubated at 37°C for 4 hours. Cell-free wells containing only 100 μL of medium were included as negative controls. After incubation with MTT for 4 hours, 75 μL of medium was removed from the wells, and the formazan crystals were then dissolved in 50 μL of DMSO by mixing the wells with a pipette. After a further 10 minutes of incubation at 37°C, the samples were mixed briefly again and the absorbance recorded at 540 nm.

Acridine orange/ethidium bromide (AO/EtBr) dual staining

The AO/EtBr dual staining technique was performed as described by Liu et al.[23] Briefly, the cells were seeded in a 96-well plate at a density of approximately 10^4 cells per well. After 48 hours of incubation with DHEA, the cells were trypsinized, and 10-25 μl of the cell suspension was transferred to glass slides. One microliter of AO/EtBr staining (dye mixture containing 100 μg/mL AO and 100 μg/mL EtBr) solution was added to the cell suspension, and then the sample was covered with a cover glass. Within 20 minutes after addition of AO/EtBr dye, the cell morphology was checked under a fluorescence microscope (Carl-Zeiss/Axio Observer 3., Zen 2.3 Blue Edition software). For statistical analysis, at least 200 cells were counted and the results expressed as the average of at least three independent experiments. Both live and dead cells stained with AO, while only the dead cells that had lost membrane integrity stained with ethidium bromide. Living cells appeared uniformly green, while cells that were in the early apoptotic stage showed green dots in their nuclei. Late apoptotic cells stained orange and showed nuclear condensation and/or frequent fragmentation. Necrotic

cells stained orange, and their nuclear morphology was similar to that of living cells, but without concentrated chromatin.[23]

In vitro scratch assay

The *in vitro* scratch assay was carried out to evaluate the migration rates according to the protocol described by Liang et al.[24] Briefly, the surface of the wells in a 6-well plates was scratched with a 10 µL sterile pipette tip. After gentle washing with culture medium, images of the scratches at 0-24 hours were taken under a microscope with a magnification of 10x (Carl-Zeiss/Axio observer 3). By using ImageJ software, the gap size was analyzed and the cell migration rate calculated by comparing the cell-free areas of scratches at 24 hours after injury and the scratched areas at 0 hours. The results are expressed as the average of three repeated experiments.

Statistical analysis

Statistical analysis was performed using GraphPad (Prism 5) software. The statistical significance of differences between groups was assessed by One-Way ANOVA and Tukey's post-hoc test (n=3).

RESULTS

First, we investigated the effects of DHEA on cell viability by comparing the changes in proliferation rates of MDAH-2774 (human ovarian carcinoma epithelial cell) and HuVeC (human non-cancerous umbilical vein endothelial) cell lines. For this purpose, we used the MTT assay, which is a relatively simple and well-established procedure for the evaluation of cell viability and proliferation.[25] We tested the effects of four different concentrations (10 µM, 50 µM, 100 µM, and 200 µM) of DHEA for 24, 48, and 72 hours. The concentration range was determined according to literature data.[4]

Data analysis obtained from the MTT assay showed that DHEA treatment (10-200 µM) promoted the proliferation of the MDAH-2774 cell line in a dose-dependent manner ($r=0.6906$, $p<0.0001$, for 24 hours; $r=0.6802$, $p<0.0001$, for 48 hours; $r=0.7969$, $p<0.0001$, for 72 hours). In contrast to the MDAH-2774 ovarian cancer cell line, DHEA treatment inhibited proliferation of the non-cancerous HuVeC cells. Although, 10 µM (24 hours) DHEA treatment did not appear to affect the rate of cell proliferation, higher doses of DHEA (≥50 µM for 24, 48, and 72 hours) significantly inhibited proliferation of HuVeC cells in a dose-dependent fashion ($r=0.9490$, $p<0.0001$, for 24 hours; $r=0.9533$, $p<0.0001$, for 48 hours; $r=0.9584$, $p<0.0001$, for 72 hours) (Figure 1b, d, f).

As seen in Figure 1, 48 and 72 hours of DHEA exposure had the greatest effect on cell proliferation. In this context, to avoid the possible toxic effects that might arise due to prolonged drug exposure, we selected 48 hours as the optimum exposure time. Thus, subsequent experiments were carried out by exposing the cells to 10 µM, 50 µM, 100 µM, and 200 µM DHEA for 48 hours.

Next, we used the AO/EtBr dual staining protocol to compare changes in the ratio of apoptotic/necrotic cells. Dual AO/EB staining is a reliable and inexpensive technique to detect apoptotic and necrotic cells in cell culture experiments.[23] The changes in the ratio of apoptotic and necrotic cells were evaluated upon exposure to DHEA (10-200 µM for 48 hours). Our findings demonstrated that DHEA treatment did not induce apoptosis or necrosis in MDAH-2774 cells (Figure 2a, b). On the other hand, we detected a dose-dependent increase in the number of necrotic cells when HuVeC cells were treated with DHEA (48 hours, by 5% ± 1% for 10 µM, 14.33% ± 6% for 50 µM, 32% ± 3% for 100 µM, 54.33% ± 5% for 200 µM, $p<0.05$). Suggesting that DHEA treatment induces necrotic death in the HuVeC cell line. Representative microscope images from AO/EtBr-stained samples are shown in Figure 3.

Then, we investigated whether or not DHEA treatment could cause changes in cell migration rates. To this end, we

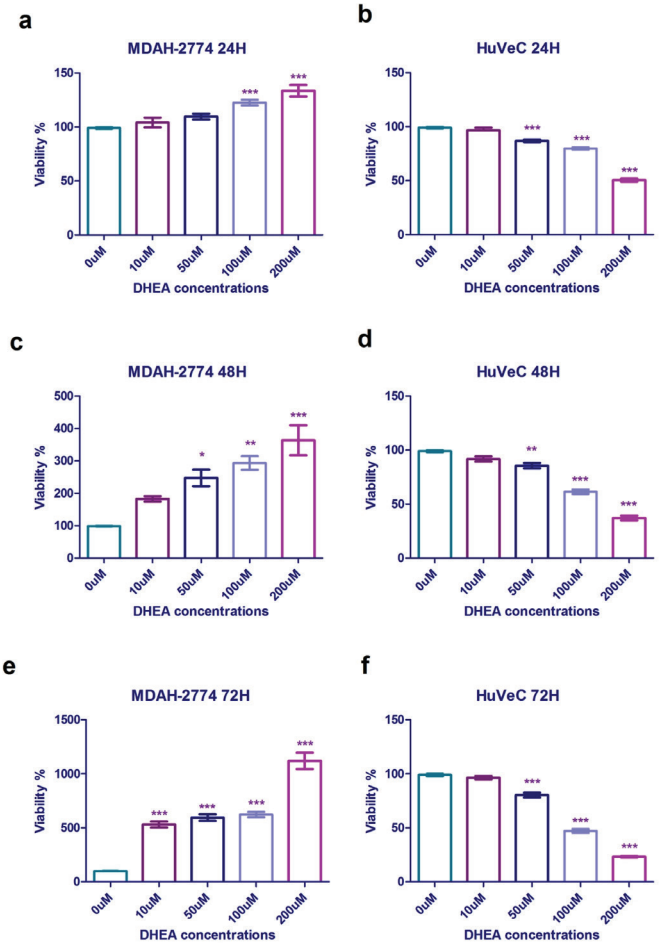

Figure 1. The effect of DHEA on cell proliferation. a, c, e) MDAH-2774 ovarian cancer and b, d, f) HuVeC endothelial cell lines were treated with dehydroepiandrosterone for 24, 48, and 72 hours in an incubator. MTT assays were performed 24, 48, and 72 hours after treatment with the indicated doses of DHEA relative % changes in proliferation rates were compared against the non-treated control group (0 µM), and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple-comparison test (*, **, *** $p<0.0001$, n=6). The correlation between DHEA dose and its effect on proliferation was analyzed by linear regression (GraphPad Prism program). MDAH: $r=0.6906$, $p<0.0001$, for 24 hours; $r=0.6802$, $p<0.0001$, for 48 hours; and $r=0,7969$, $p<0.0001$ for 72 hours. HuVeC: $r=0.9490$, $p<0.0001$ for 24 hours; $r=0.9533$, $p<0.0001$, for 48 hours; and $r=0,9584$, $p<0.0001$ for 72 hours. DHEA: Dehydroepiandrosterone, MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

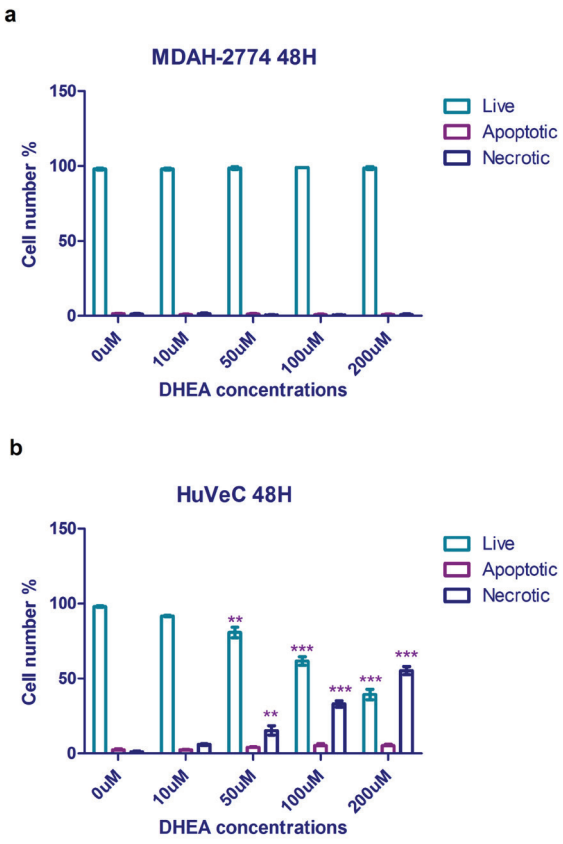

Figure 2. DHEA induces necrosis at high concentrations. a) MDAH-2774, and b) HuVeC endothelial cell lines were treated with 10-200 μM DHEA for 48 hours in an incubator. AO/EtBr double staining was performed 48 hours after treatment with the indicated doses of DHEA. Percent changes in the ratio of necrotic cells were compared against the non-treated control group (0 μM), and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple-comparisons test. **, ***: $p<0.05$, n=3, DHEA: Dehydroepiandrosterone, AO/EtBr: Acridine orange/ethidium bromide

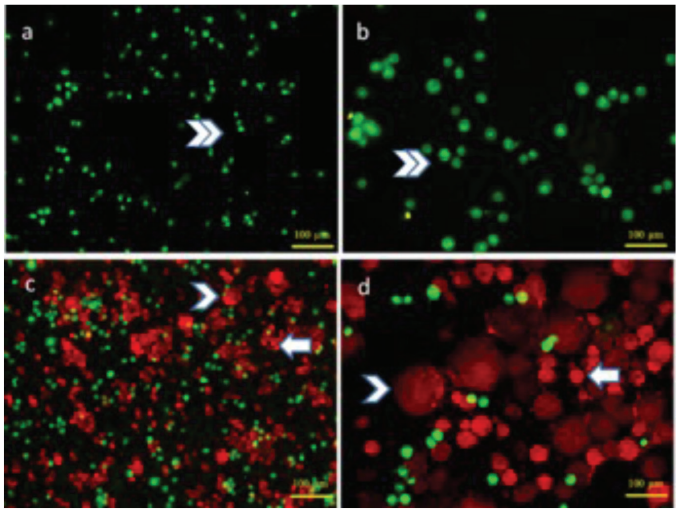

Figure 3. Representative microscope images from AO/EtBr double staining. a) Magnification: 10x, MDAH-2774 cells (control: 0 μM DHEA), b) magnification: 40x, MDAH-2774 cells (100 μM DHEA), c) magnification: 10x, HuVeC cells (100 μM DHEA), d) magnification: 40x, HuVeC cells (100 μM DHEA). Arrows point to apoptotic cells, arrow heads point to necrotic cells, and double arrow heads point to live cells. AO/EtBr: Acridine orange/ethidium bromide

performed the scratch assay. This is a simple *in vitro* method that serves as a powerful tool for analyzing cell migration rates in two dimensions.[26] In this context, we tested the effect of 10-200 μM DHEA (48 hours) treatment on cell migration rates by the *in vitro* scratch assay protocol. Our findings indicated that DHEA treatment triggered a slight increase in the migration rate of MDAH-2774 ovarian cancer cells (48 hours, by 7% ± 1% for 10 μM, 8% ± 1% for 50 μM, 9% ± 1% for 100 μM, 10% ± 1% for 200 μM, $p<0.05$) (Figure 4a). In contrast, DHEA reduced the migration rate of non-cancerous HuVeC cells in a dose-dependent manner (48 hours, by 5% ± 3% for 10 μM, 14% ± 1% for 50 μM, 31% ± 3% for 100 μM, 52% ± 4% for 200 μM, $p<0.05$) (Figure 4b). Representative microscope images from scratch assay experiments are shown in Figure 5.

DISCUSSION

DHEA is one of the most widespread adrenal steroids in humans. DHEA is the precursor of bioactive steroids, such as estrogen and testosterone.[27] Although DHEA is shown to be capable of activating estrogen receptors,[11] it can also act independently

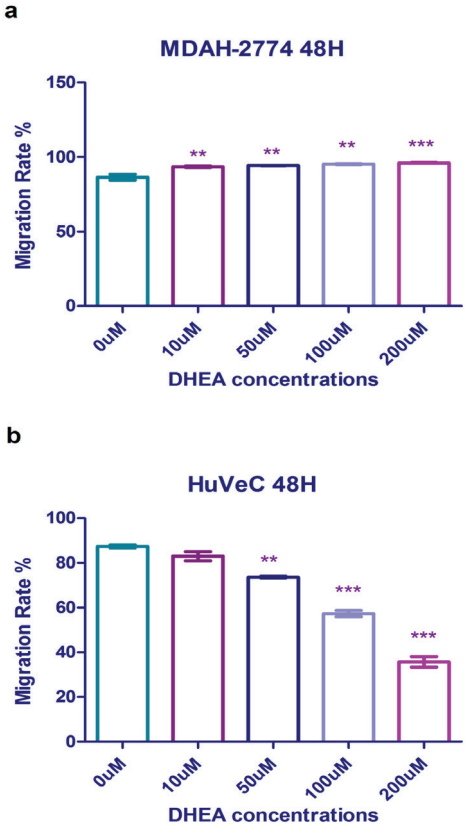

Figure 4. DHEA reduces the cell migration rate at high concentrations in HuVeC and increases the migration rate in MDAH-2774. a) MDAH-2774, b) HuVeC endothelial cell lines were treated with 10-200 μM DHEA for 48 hours in an incubator. A scratch assay was performed 48 hours after treatment with the indicated doses of DHEA. The rate of migration (how soon the gap closes) in 48 hours was calculated by measuring the gap at 0 and 48 hours after scratching the plates. Percent changes in the migration rates were compared against the non-treated control group (0 μM), and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple-comparison test. *, **, ***: $p<0.05$, n=3, DHEA: Dehydroepiandrosterone

of estrogen and androgen receptors in endothelial and breast cancer cells.[4,12,13] In gynecological clinical practice, DHEA is also prescribed to induce ovulation. There are conflicting findings as to the role of DHEA as a risk factor for developing cancer. Higher levels of DHEA have been correlated with an increased risk of developing breast cancer.[28] On the other hand, Yoshida et al.[29] proposed that DHEA can reduce the risk of developing breast cancer by blocking estrogen receptors. For the most part, DHEA appears to inhibit the proliferation of cancer cells, according to *in vitro* findings.[15-21] Several different groups have demonstrated that DHEA inhibits the proliferation of hepatoma, breast, myeloma, prostate, human colon adenocarcinoma, leukemia, and cervical cancer cell lines.[15-21] On the other hand, clinical data also suggests that DHEA may be positively associated with breast cancer.[14] For example, Michels et al.[30] have identified an increased risk of endometrial cancer with high levels of adrenal androgens and estrogens. A review of the literature revealed that sufficient data were not available regarding the *in vitro* physiological effects of DHEA on ovarian cancer cell lines.

MDAH-2774 is a widely used ovarian cancer cell line[31] with a high mean tumor volume and is accepted as a suitable model for human ovarian cancer.[32] Endothelial cells are one of the key players in maintaining physiological hemostasis[33] and are involved in many important processes, including regulation of cell migration, proliferation, and apoptosis.[34] Therefore, we have chosen a well-established endothelial cell model, the HUVEC cell line, as a non-cancerous cell model. In this context, we investigated the physiological effects of DHEA on MDAH-2774 human ovarian cancer cells in comparison with the non-cancerous HuVeC human endothelial cell line.

We have assessed the effect of DHEA on cell proliferation via the MTT assay, which is a relatively simple and well-established procedure for evaluating cell viability.[25] Our findings revealed that DHEA inhibits proliferation in the non-cancerous HuVeC cells in a dose-dependent manner (Figure 1). In line with our observation, Liu et al.[35] showed that DHEA inhibited cell proliferation of non-cancerous primary rat Leydig cells. They suggested that this cell type-specific response could be due to the differences in the way DHEA was processed or metabolized inside the cell. Surprisingly, we found that DHEA promoted proliferation of MDAH-2774 cancer cells in a dose-dependent manner (Figure 1). This finding supports the notion that DHEA may exert its effect in a cell type-specific manner. Similarly, Joshi et al.[36] reported cell type-specific differential effects of DHEA between mouse and human melanoma cell lines.

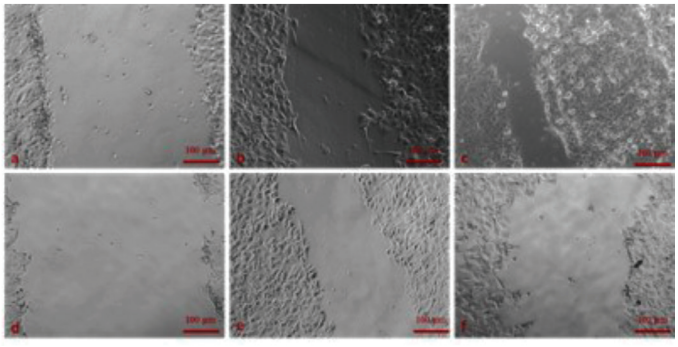

Figure 5. Representative microscope images from *in vitro* scratch assay (magnification 10x). a) 0 hour MDAH-2774 cells, b) 48 hour 0 µM DHEA, MDAH-2774 cells, c) 48 hour 100 µM DHEA, MDAH-2774 cells, d) 0 hour HuVeC cells, e) 48 hour 0 µM DHEA, HuVeC cells, f) 24 hour 100 µM DHEA, HuVeC cells. DHEA: Dehydroepiandrosterone

We have employed dual AO/EB staining technique to assess the DHEA-induced effects on apoptotic activity in cells.[23] We did not observe a significant change in the number of necrotic or apoptotic cells in the ovarian cancer cell line MDAH-2774 (Figure 2). However, our findings revealed that DHEA can induce necrotic cell death in the non-cancerous HuVeC cell line. Girón et al.[19] previously reported that DHEA treatment induced both apoptotic and necrotic cell death in cervical cancer cell lines. Interestingly, supplementation of the medium with DHEA was reported to increase the number of primordial follicles by inhibiting apoptotic activity in mice.[37] In contrast, Kim et al.[38] demonstrated that DHEA administration can induce apoptosis in the mouse ovaries. More recent evidence also suggests that DHEA supplementation can protect ovarian cells in rats rather than inducing apoptotic activity.[39] Similarly, DHEA was found to inhibit H_2O_2-induced oxidative stress damage and apoptosis in Leydig cells.[40] The presence of contradictory findings further supports the idea that DHEA can exert its effects in a cell-type-specific manner.

Available data on the signaling mechanisms involved in DHEA-mediated physiological changes are also scarce. Girón et al.[19] suggested that DHEA can exert its effects through the mitogen-activated protein kinase signaling pathway, independently of either estrogen receptor or androgen receptor. Jimenez et al.[41] showed that DHEA may inhibit the pentose phosphate pathway and thereby alter oocyte lipid metabolism in mice. DHEA is shown to be able to directly act on plasma membrane receptors, such as the G-protein-coupled receptor in endothelial cells and aminobutyric-acid-type A in neurons. DHEA also binds androgen and estrogen receptors and can inhibit voltage-gated T-type calcium channels.[42] Nevertheless, molecular and mechanistic approaches are necessary to dissect the mechanisms involved in this cell type-specific action of DHEA.

Previously, the migration of cervical cancer cell lines was found to be suppressed by DHEA.[43] In line with these reports, López-Marure et al.[20] proposed that DHEA inhibited the proliferation of all breast cancer cell lines. Consistently, we found that DHEA treatment can reduce the migration rate of non-cancerous HuVeC cells in a dose-dependent manner (Figure 4). Curiously, however, we detected a slight increase in the migration rate of the MDAH-2774 ovarian cancer cell line (Figure 4). This observation suggests that DHEA may induce the migration of ovarian cancer cells. In agreement with our observations, Montt-Guevara et al.[44] showed that DHEA stimulates cell invasion via moesin activation in the T47D breast cancer cell line.

Study limitations

In this study, we presented reproducible data to support our observations as to the physiological effects of DHEA on ovarian

cancer and non-cancerous cells. However, we were not able to provide insight into the underlying molecular mechanisms and pathways due to a lack of molecular evidence. We were not able to provide mechanistic evidence to support our descriptive findings.

CONCLUSION

Although DHEA inhibited proliferation of the non-cancerous HuVeC cells, DHEA treatment was able to promote the proliferation of the MDAH-2774 cancer cell line in a dose-dependent manner. We detected a dose-dependent increase in the number of necrotic cells in the non-cancerous HuVeC cell line in response to DHEA treatment. However, the number of necrotic or apoptotic cells did not change significantly in the MDAH-2774 cell line. DHEA treatment reduced the migration rate of non-cancerous HuVeC cells, whereas only a slight increase was observed in the MDAH-2774 ovarian cancer cell line. Our findings suggest that DHEA promotes the proliferation of ovarian cancer cells in a dose-dependent manner *in vitro*. Moreover, we found that DHEA induced necrosis and inhibited proliferation in a non-cancerous endothelial cell line. Although molecular and mechanistic evidence is required, our preliminary findings imply that exposure to high doses of DHEA may be associated with an increased risk of developing ovarian cancer.

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ORIGINAL ARTICLE

Ethnopharmacological Knowledge for Management of Oral Mucositis in Zahedan, Southeast Iran

Zahedan, Güney İran'da Oral Mukositle Mücadele İçin Etnofarmakolojik Bilgi

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ABSTRACT

Objectives: Oral mucositis is among the complications of cancer therapy that affects quality of life and imposes remarkable financial costs for patients with cancer. This study aimed to explore, preserve, and scientifically investigate the ethnomedicinal knowledge of traditional healers for treatment of oral mucositis in Zahedan, Iran.

Materials and Methods: Field surveys were performed from September 2018 to October 2018 in Zahedan. Data was collected using a structured questionnaire in Persian. All species recorded for the treatment of oral mucositis were sampled. Samples were identified by a botanist and a voucher specimen of them was deposited in the Herbarium Center of the Faculty of Pharmacy in Kerman, Iran. Information, such as scientific name, family, local name, parts used, and preparation method, were also provided. Literature review on available data on effect of the addressed plant species on mucositis and other relative pharmacological actions, such as wound healing and anti-inflammatory properties, was performed.

Results: A total of 29 informants (attars) were interviewed and 18 medicaments were recommended, of which three samples were of synthesis or mineral origin and 15 samples were of herbal origin. Drugs were administered both topically and orally. According to recent studies, two herbs were evaluated for their direct effect on mucositis. Some pharmacological properties related to mucositis treatment by the other 11 samples have been confirmed.

Conclusion: This study provides information on the characteristics of medicinal plants from Zahedan, Iran based on their ethnopharmacological knowledge and pharmacological properties for mucositis treatment.

Key words: Mucositis, ethnopharmacology, traditional medicine, medicinal plants, Zahedan

ÖZ

Amaç: Oral mukosit hayat kalitesini etkileyen ve kanserli hastalarda belirgin ekonomik giderlere yol açan kanser terapisinin komplikasyonlarından biridir. Bu çalışma Zahedan, İran'da oral mukositin tedavisi için geleneksel tedavi edicilerin etnomedisinal bilgilerini bilimsel olarak araştırmak, saklamak ve incelemeyi amaçlamıştır.

Gereç ve Yöntemler: Alan çalışmaları Zahedan'da Eylül 2018 ve Ekim 2018 arasında gerçekleştirilmiştir. Veriler Perslerden yapılandırılmış bir anket kullanarak toplanmıştır. Oral mukositin tedavisi için tüm türler örneklendirilmiştir. Örnekler bir botanikçi tarafından tanımlanmıştır ve örneklerden alınan bir kısım Kerman İran'da bulunan Eczacılık Fakültesi Herbaryum Merkezi'nde saklanmıştır. Bilimsel isim, aile, yerel isim, kullanılan kısımlar ve hazırlama yöntemleri de sağlanmıştır. Mukosit üzerine kullanılan bitki türleri üzerine var olan veriler ve bitkilerin yara iyileştirme ve antiinflamatuvar özellikleri gibi diğer farmakolojik etkileri ile ilgili literatür değerlendirmeleri gerçekleştirilmiştir.

Bulgular: Toplamda 29 bilgi verici (aktar) ile görüşülmüş ve 3 tanesi mineral kökenli ve 15 tanesi herbal kökenli olmak üzere 18 ilaç önerilmiştir. İlaçlar hem topikal hem de oral uygulanmıştır. Son çalışmalara göre, 2 bitki mukosit üzerindeki doğrudan etkileri için değerlendirilmiştir. Bunların diğer 11 örnekle birlikte mukosit ile ilgili diğer farmakolojik özellikleri onaylanmıştır.

Sonuç: Bu çalışma, Zehedan, İran'daki medisinal bitkilerin mukosit tedavisinde etenofarmakolojik bilgilere ve farmakolojik özelliklerine göre karakteristikleri ile ilgili bilgi sağlamaktadır.

Anahtar kelimeler: Mukosit, etenofarmakoloji, geleneksel tıp, medisinal bitkiler, Zahedan

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INTRODUCTION

Oral mucositis is among the serious complications that are secondary to cancer therapy.[1] Approximately 20%-40% of patients who underwent conventional chemotherapy, 80% of patients undergoing high dose chemotherapy due to hematopoietic stem cell transplantation, and nearly all patients receiving radiotherapy due to head and neck cancer may present oral mucositis.[2-4] Mucositis is described as the inflammation of the mucosa, which results from mucotoxic cancer therapy either via chemotherapy or radiation. It is known as erythema and/or ulceration of mucosa, which may be induced by trauma or secondary infections.[1,5] Mucositis not only affects the quality of life of patients with cancer but also imposes remarkable financial costs. More than 75% of patients receiving head and neck radiotherapy usually experience severe pain and burning sensation in their mouths, leading to the difficulty in chewing and swallowing and ultimately causing several problems in their dieting.[6] Additionally, oral diseases are expensive to treat and sometimes inaccessible.[7]

According to the World Health Organization (WHO), most people in developing countries tend to use medicinal plant resources due to their accessibility, effectiveness, and fewer complications. Iran is an ancient Asian country with a great history of medicine thousands of years ago.[8] Ancient Iranian medicine based on humoral theory was a global medical paradigm during the medieval times.[9] Despite the replacement of traditional Persian medicine with modern medicine in academia from the 19th century, ethnomedicine with its potent traditional history is still very common among Iranian people.[8,10] For instance, a study demonstrated that 62.5% of the urban population in Isfahan utilize at least one of the traditional and complementary medicine methods.[11] People in different parts of Iran use medicinal plants for the management of diseases based on their ethnic culture and ethno-knowledge. For instance, a study on the ethnobotany of Khabr and Rouchon region in Kerman province, Iran showed that the native people utilize 50 medicinal plant species for the alleviation of different disorders, especially gastrointestinal problems.[12] It is reported that more than 77 medicinal plant species are used by the elderly in Sirjan city, Iran, of which the plants with therapeutic effects on the respiratory tract have been more considered.[13] Traditional healers, named "Attar", who work in traditional herbal shops, named "Attari", are the most common consultants and practitioners of ethnomedicine services in Iran.[8,10] Attars are individuals who prescribe and sell medicinal herbs and natural drugs, whose (most of them) information on herbal medicine is inculcated from older generations (verbally), personal experiences, and traditional medicine cultures. These resources can potentially form the basis for the use of medicinal herbs in new drug discovery after scientific research. Recording the ethno-knowledge and techniques of these traditional healers can help prevent the loss of such non-written information due to death.[14,15]

Several studies on ethnopharmacological knowledge of Iranians population have been published;[8,14] however, to the best of our knowledge, no report in this regard has been found in Zahedan. In contrast, there is a need to explore and preserve ethno-knowledge by documenting the herbs and natural products that have been traditionally applied in folkloric medicine. In this regard, this ethnobotanical study was designed to collect natural products and herbs that are practically used for the treatment of oral mucositis in Zahedan, Southeast Iran and to evaluate them by applying current medical concept and recent scientific studies. We also aimed to highlight weaknesses in current knowledge and suggest future studies.

MATERIALS AND METHODS

Study area

Zahedan is the capital city of Sistan and Baluchestan (SB) province, the widest province of Iran, located in the Southeastern region of the country. It has a common international border (187,502 km^2) with Afghanistan and Pakistan at the East and Southeast region, respectively, and also a common maritime boundary in the Northern coast of Oman Sea. Kerman and Hormozgan provinces are located in the West and South, respectively. Khorasan province is located at the North of SB (Figure 1).

SB province consists of two distinct regions that are naturally different from each other and have a varied herbal flora: 1- Baluchestan is located in the Southern part of the province with diverse climates tied to the Oman Sea. 2- The Northern part of the Province is named Sistan, which is characterized by the Hirmand River, and Hamun, which is a large freshwater lake.[16]

Shahr-e sukhteh is an archaeological site "[Burnt city (BC)" from the third millennium BC and is located 154 kilometers far from Zahedan, with a considerable evidence on the advanced ancient medicine, which can be regarded as an honored record of this area of Iran.[17,18]

The climatic diversity resulting in unique vegetation areas and trade relations with Afghanistan, Pakistan, and India (through the sea), the ancient history of medicine, and great traditional physicians, such as Hakim Azam Khan (Nazim Jahan) in the 19th century[19,20] have made this region rich and noteworthy in traditional medicine and ethnomedicine.

Zahedan, similar to many other capitals, has its attractions compared with other cities in the province. As a result, several

Figure 1. Geographic position of study area (Zahedan)

immigrants from other cities of the province have gathered in this city. Traditional medicine is a common among people living in Zahedan and it takes the advantages of both native and nonnative herbs for treating diseases. This city (31250 km^2 area) is located between latitude 29°29'46.68"N and longitude 60°51'46.44"E. It mostly enjoys a warm and dry weather throughout the year. It has hot days and very low-temperature nights in summer. The average annual rainfall is 120 mm. It has an altitude of 1385 m and is comprised of ~672,589 people.

Ethnopharmacological investigation and data collection

The protocol of this research has been approved by the Ethics Committee of Kerman University of Medical Sciences (code: IR.KMU.REC.1399.023). This study was conducted from September to October 2018. Face to face interview with traditional healers was conducted and structured questionnaires were filled. First, personal information of traditional healers (attar), including age, sex, education, and source of their information, was taken. The traditional healers were asked to explain which traditional remedies can help patients with "oral mucosa inflammation (relatively characterized by erythema and pain of mucosa) with/without ulcer". All needed information, including local name, part(s) used, preparation, and administration methods of the remedies, were collected.

Identification

A sample of all reported traditional drugs was collected from their habitat and transferred to the Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences and a voucher code was assigned for each sample as mentioned in the result section.

The information is systematically shown in Table 1.

Data analysis and literature review survey

The next step was to investigate studies on the intended plants, especially those associated with mucositis and the relative pharmacological properties published in Scopus and Pubmed databases (Table 2). The scientific name of plants and the following keywords were used for the literature search:

1- Mucositis

2- Antibacterial, antimicrobial

3- Antifungal

4- Wound, ulcer wound healing, ulcer protection

5- Inflammation, antiinflammatory

6- Pain, analgesia, antinociceptive, antinociceptive, analgesic

In this research, no specific statistical method was used (except for the cases expressed as percentage).

RESULTS

Information from herbal practitioners

Of the 36 traditional herbal stores, 29 attars volunteered to be interviewed for the study. All healers were male, with the age range of 23-68 years, and 55% of them were younger than 40 years of age. Approximately 48% of the participants had a below diploma degree, 24% had a high school diploma, and 28% had an academic education. 62% of the healers reported that they have obtained information via older generations, 20% obtained theirs by reading traditional medicine and herbal remedies books, 38% had their own experiences, and 17% obtained theirs via the internet (some of the interviewees had more than one source of information).

Information about traditional remedies

A total of 18 medicaments were introduced for the management of oral mucositis (Table 1), of which 15 samples had herbal origin and three samples had synthesis or mineral origin. *Alcea digitata* Alef (11 attars), *Cotoneaster discolor* Pojark (10 attars), *Johare ghermez*, which has mineral origin (10 attars), and *Rhazya stricta* Decne (9 attars) were the most recommended species (Figure 2). Approximately 83% of the drugs were native to Iran and the others were transferred from India or Afghanistan to Iran. Three medicaments were used both topically and orally, 12 medicaments were used topically, and four medicaments were used orally. The preparation methods were mostly decoction, dissolving in water, extraction, distillation, maceration, oil and hydrocolloid produced in water (loab), and powder.

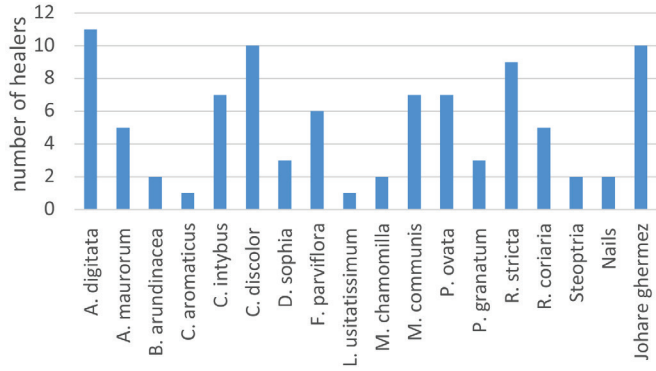

Figure 2. Number of traditional healers that mentioned the use of each remedy for the treatment of oral mucositis

Information obtained via the literature search in various databases reveal the effect of only two herbs, including *Matricaria chamomilla* L. and *Alcea digitata* Alef, on mucositis. These two studies respectively demonstrated that these herbs are effective against mucositis. Different studies on the other 11 herbs indicated some related pharmacological activities for the management of mucositis, such as anti-inflammatory, antibacterial, antifungal, and wound healing effects. No study was found to prove the effect of *Cotoneaster discolor* Pojark and *Bambusa arundinacea* Willd on mucositis, as well as their relative pharmacological effects (Table 2).

Table 1. Different remedies for mucositis recommended by traditional healers in Zahedan with their related information

Local name	Voucher number	N	Scientific name	Family	Part (s) used	Habitant	Administration	Preparation
Khatmi	KF 1325	11	*Alcea digitata* Alef	Malvaceae	Flower	NI	T O	Decoction
Toranjabin	KF1261	5	*Alhagi maurorum* Medik.	Papilionaceae	Manna	SB	O	Dissolved in water
Tabasheer	KF 1347	2	*Bambusa arundinacea* Willd.	Gramineae	Manna	NN	T	Powder
Mikhak	KF3124	1	*Caryophyllus aromaticus* L.	Myrtaceae	Bud	NN	T	Extract
Kasni	KF1157	7	*Cichorium intybus* L.	Asteraceae	Leave Seed Root	SB	O	Distillate
Shirkhesht	KF1821	10	*Cotoneaster discolor* Pojark	Rosaceae	Manna	NI	T O	Dissolved in water
Khakshir	KF1012	3	*Descurainia sophia* (L.) Webb ex Prantl	Cruciferae	Seed	NI	O	Maceration
Shahtare	KF1235	6	*Fumaria parviflora* Lam.	Fumariaceae	Aerial part	SB	O	Distillate
Katan	KF1253	1	*Linum usitatissimum* L.	Linaceae	Seed	NI	T	Oil
Babune	KF1151	2	*Matricaria chamomilla* L. Syn. Chamomilla recutita (L.) Rauschert	Asteraceae	Flower Aerial part	NI	T	Extract
Murd	KF1356	7	*Myrtus communis* L.	Myrtaceae	Leave	SB	T	Distillate Powder
Esfarze	KF1312	7	*Plantago ovata* Forssk.	Plantaginaceae	Seed-Husk	SB	T O	Hydrocloid obtain from maceration in water (loab)
Anar	KF1027	3	*Punica granatum* L.	Punicaceae	Flower Peel of Fruit	SB	T	Powder Decoction
Ishrak	KF1167	9	*Rhazya stricta* Decne.	Apocynaceae	Leave	SB	T	Powder
Somagh	KF0931	5	*Rhus coriaria* L.	Anacardiaceae	Fruit	NI	T	Powder
				Origin				
Zaje sefid	KF1281	2	Steoptria	Synthesis		SB	T	Powder
Nile abi	KF1282	2	Nails	Synthesis		SB	T	Powder
Johare ghermez	KF1297	10	Not found any scientific information	Mineral		NN	T	Powder

N: Number of citation, SB: Native to Sistan and Baluchestan province, NI: Native to Iran, but not to Sistan and Balouchestan province, NN: Non-native to Iran, T: Topical, O: Oral

Table 2. Reported pharmacological properties relevant to ethnomedicinal use of plants for management of mucositis in Zahedan

No	Medicinal plants	Plant part preparation	Study design	Main related outcome	References
1	*Alcea digitata* Alef	Flower powder	Human study triple-blind parallel two-armed randomized clinical trial evaluating the effectiveness of *Alcea digitata* Alef and *Malva sylvestris* L. from the beginning of radiotherapy to 2 weeks after the completion of the treatment	↓Mucositis	Rezaeipour et al.[21]
		Ethanolic extract	*In vitro* Evaluating the effectiveness against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus agalactiae*	Antibacterial activity	Zareii et al.[22]
2	*Alhagi maurorum* Medik.	Alcoholic extracts	An animal study (rat) antiinflammatory activity: Using carrageenan-induced rat paw edema method antinociceptive activity: Peripherally and centrally using the writhing and the hot-plate test	↓Inflammation Algesic property	Awaad et al.[23]
		Aqueous extract	An animal study (rat) evaluating the effectiveness after 21 days treatment of wound site	↑Wound healing	Pourali and Yahyaei[24]
		Butanol, ethyl acetate, chloroform, methanol and water extract	*In vitro* Evaluating the effectiveness against seven bacterial strains and one fungal specie (*Candida albicans*) using disk diffusion susceptibility assay	Antibacterial activity Antifungal activity	Bakht et al.[25]
3	*Bambusa arundinacea* Willd.	None	None	None	None
4	*Caryophyllus aromaticus* L.	Essential oil	An animal study (mice) Evaluating the effectiveness on tongue edema and acute inflammation induced by Dieffenbachia picta Schott	↓Inflammation	Dip et al.[26]
		Flower bud	An animal study (rat) by evaluating central and peripheral analgesic activity by formalin test	Analgesic property	Mathiazhagan et al.[27]
		Essential oil	*In vitro* Evaluating its effectiveness on bacterial strains isolated from clinical human specimens and foods	Antibacterial activity	Barbosa et al.[28]
		Essential oil	*In vitro* Evaluating the effectiveness against different *Candida* species isolated from urine samples	Antifungal activity	Khosravi et al.[29]
5	*Cichorium intybus* L.	Aqueous seed extract	An animal study (rat) by evaluating expression of hepatic NF-κB and IKKβ and serum TNF-α in streptozotocin and streptozotocin + niacinamide-induced diabetes in rats	↓Inflammation	Rezagholizadeh et al.[30]
		Lactucin and some lactucin-like guaianolides derived from leaves and roots	An animal study (rat) Evaluating the effectiveness using the hot-plate test and tail-flick test	Analgesic property	Wesołowska et al.[31]
		Whole plant and root methanolic extract, its subextracts, and fractions	An animal study (rat) Evaluating the effectiveness using *in vivo* linear incision and circular excision wound models and assessment the hydroxyproline content of the tissues treated with test ointments	↑Wound healing	Süntar et al.[32]

Table 2. Continued

No	Medicinal plants	Plant part preparation	Study design	Main related outcome	References
5		Ethanolic and methanolic extracts of leaves and roots	*In vitro* Evaluating the effectiveness by agar well diffusion assay against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *A. niger* and, *Penicillium expansum*	Antibacterial activity	Khalaf et al.[33]
		Crude extract and its different solvent soluble fractions (Water a- ethyl acetate-chloroform)	*In vitro* Evaluating the effectiveness on six bacterial strains and four fungal strains: *Aspergillus flavus*, *Fusarium solani*, *Aspergillus fumigatus* and *Aspergillus niger*	Antibacterial activity Antifungal activity	Rehman et al.[34]
6	*Cotoneaster discolor* Pojark	None	None	None	None
7	*Descurainia sophia* (L.) Webb ex Prantl	Ethanol extract of seeds	An animal study (rat) using multi-omics analysis for assessment the epigenetic effects	↓Inflammation	Baek et al.[35]
8	*Fumaria parviflora* Lam.	Methanolic extract	An animal study (mice) Evaluating the effectiveness using acute thermal (hot plate) and persistent chemical (formalin) pain stimuli	Analgesic property	Heidari et al.[36]
		N-octacosan 7β ol compound from methanolic extract of the whole plant	*In vitro* Evaluating the effectiveness against *Leishmania donovani* promastigotes, *Staphylococcus epidermidis*, *Escherichia coli*, *Candida albicans* and, *Aspergillus niger*	Antibacterial activity Antifungal activity	Jameel et al.[37]
9	*Linum usitatissimum* L.	Dried powder from ethanoic extract of leaves	An animal study (mice) antiinflammatory activity: By Xylene test Antinociceptive activity: Using the hot-plate test	↓Inflammation Analgesic property	Rafieian-kopaei et al.[38]
		Gel A mixture of seed oil with Carbomer	Human study Randomized clinical trial Evaluating the effectiveness of gel on symptoms of carpal tunnel syndrome compared with split	↓Inflammation Analgesic property	Setayesh et al.[39]
		Seed powder (in combination with som other seeds)	An animal study (mice) Evaluating the effectiveness using tail-flick, hot-plate, and formalin tests	Analgesic property	Sheibani et al.[40]
		Oil from seeds and then preparation gel form	Animal study (rat) Evaluating the effect of topical gel on the wound healing process, according to histomorphometrical, and stereological parameters	↑Wound healing	Rafiee et al.[41]
		Dried crude (methanol) extract from seeds and also fractionation with different solvents	*In vitro* Evaluating the effectiveness against *Bacillus cereus*, *Candida albicans*, *Erwinia carotovora*, *Escherichia coli*, *Kleibsiella pneumonia*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	Antibacterial activity Antifungal activity	Bakht et al.[42]
10	*Matricaria chamomilla* L. Syn. *Chamomilla recutita* (L.) Rauschert	Mouthwash containing a liquid extract	Human study Randomized, controlled, phase II clinical trial for evaluating the effectiveness on prevention and treatment of oral mucositis in patients undergoing hematopoietic stem cell transplantation	↓Mucositis	Braga et al.[43]
		Apigetrin (isolated flavonoid)	*In vitro* Investigating the inhibitory effects of apigetrin on neuroinflammation using the BV-2 microglia cell line	↓Inflammation	Lim et al.[44]

Table 2. Continued

No	Medicinal plants	Plant part preparation	Study design	Main related outcome	References
10		Extract in sesame oil	Human study A randomized, double-blind, placebo-controlled, crossover study Evaluating the effect of topical chamomile oleogel in migraine without aura	Analgesic property	Zargaran et al.[45]
		Fluid extract ointment 10%	An animal study (rat) Evaluating the effect of ointment on wounds inflicted on the rats tongue	↑Wound healing	Duarte et al.[46]
		Essential oil and methanol extract	*In vitro* Evaluating the effectiveness against bacterial and fungal strains using a broth microdilution method	Antibacterial activity Antifungal activity	Abdoul-Latif et al.[47]
11	*Myrtus communis* L.	Essential oil from Aerial parts	An animal study (mice) evaluating the effectiveness by the carrageenan-induced paw edema test	↓Inflammation	Touaibia[48]
		Essential oil of leaves	An animal study (mice) Evaluating the effectiveness using acetic acid-induced writhing test	Analgesic property	Mubarak et al.[49]
		Ethanol extract of leaves	*In vitro* Description of some molecular mechanisms involved in the angiogenic and wound healing process	↑Wound healing	Raeiszadeh et al.[50]
		Ethanolic extract of seed	An animal study (rat) Evaluating the effectiveness on the oral ulcer recovery process	↑Wound healing	Hashemipour et al.[51]
		Essential oil of leaves	*In vitro* Evaluating the effectiveness against *Bacillus subtilis*, *Staphylococcus aureus* and, *Candida albicans* using a disc diffusion assay	Antibacterial activity Antifungal activity	Anwar et al.[52]
		Methanolic extract of leaves	*In vitro* Evaluating the effectiveness against *Enterococcus faecalis*	Antibacterial activity	Nourzadeh et al.[53]
12	*Plantago ovata* Forssk.	Seed	An animal study (rat)/*in vitro* evaluating the effectiveness on the colonic inflammatory status, both histologically and biochemically in HLA-B27 transgenic rats fed a fiber-supplemented diet/ testing the interaction between two SCFA (butyrate and propionate) as inhibitors of cytokine production in THP-1 cells	↓Inflammation	Rodríguez-Cabezas et al.[54]
		Bulk agent, *Plantago ovata*	Human study Randomized clinical trial to determine the usefulness of the bulk agent in reducing postoperative pain and tenesmus after open hemorrhoidectomy	Analgesic property	Kecmanovic et al.[55]
		Aqueous extract of seed	An animal study (rat) Evaluating the effectiveness on microscopic and macroscopic ulcer index in peptic ulcer induced by indomethacin	↑Wound healing	Bagheri et al.[56]
		Ethanolic and methanolic extracts of seed husk	*In vitro* Evaluating the effectiveness against six Gram-negative and eight Gram-positive bacteria by disc diffusion method. *Staphylococcus epidermidis* and *Staphylococcus aureus* were the most sensitive species	Antibacterial activity	Motamedi et al.[57]

Table 2. Continued

No	Medicinal plants	Plant part preparation	Study design	Main related outcome	References
13	*Punica granatum* L.	Ethanol extract of flower	*In vitro* Evaluating antiinflammatory effect in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages	↓Inflammation	Xu et al.[58]
		Hydro-alcohol fruit extracts	Animal study (rat) Evaluating the effectiveness using thermal stimulus assays (hot plate and tail immersion) and, chemically-induced writhing test	Analgesic property	Nadia et al.[59]
		Flower extract	An animal study (Wistar rats) Evaluating the effectiveness on wound area, healing time, percentage wound contraction and histopathological characteristics in thermal burn injuries	↑Wound healing	Nasiri et al.[60]
		Peel ethanolic extracts, ethanolic extract 80% and aqueous extract	*In vitro* evaluating the effectiveness by disk method against *Escherichia coli*, *Pseudomonas aerogenosa* and *Staphylococcus aureus*	Antibacterial activity	Mohamed et al.[61]
		Peel extract	An animal study (rat) against oral candidiasis	Antifungal activity	Bassiri-Jahromi et al.[62]
14	*Rhazya stricta* Decne.	Crude extract	An animal study (mice) Evaluating the effectiveness on dermatitis via intensity score and then histological observations	↓Inflammation Analgesic property	Ahmad et al.[63]
		Aqueous alkaloid, aqueous non-alkaloid, organic alkaloid, organic non-alkaloid and whole aqueous extracts derived from leaves	*In vitro* Evaluating the effectiveness against several multidrug-resistant, human-pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* and extended-spectrum beta-lactamase-positive *Escherichia coli*	Antibacterial activity	Khan et al.[64]
		Monoterpene indole alkaloids isolated from the plant	*In vitro* Evaluating the effectiveness against six Candida strains	Antifungal activity	Ahmed et al.[65]
15	*Rhus coriaria* L.	Ethanolic extract	An animal study (mice) Evaluating the effectiveness on retinal ischemia induced by optic nerve crush injury using fluorescence molecular tomography for monitoring	↓Inflammation	Khalilpour et al.[66]
		Fruit juice	Human study Evaluating the effectiveness on reducing muscle pain during aerobic exercise in healthy volunteers	Analgesic property	Alghadir and Gabr[67]
		Crude ethanolic extract	*In vitro* Evaluating the effectiveness against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus niger*	Antibacterial activity Antifungal activity	Ertürk[68]
		Essential oil	*In vitro* Evaluating the effectiveness against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*	Antibacterial activity	Zhaleh et al.[69]

DISCUSSION

Oral mucositis has been described as erythema or/and ulcer of the oral cavity mucosa. The proposed pathobiology of mucositis is a complex pathway that involves five phases. Inflammation is among the most important and effective factors in the process of mucositis and it causes the thinning of the epithelial layer and it inclines the development of ulcers. Through progression of the damage from the epithelium into the submucosa, ulceration and oral bacterial colonization can occur. Due to this superimposed infection, the condition may get worse. The lesions of oral mucositis are typically very painful, thus analgesic agents, especially opioids are required. Healing phase is the last phase of mucositis. This phase begins with signaling from extracellular matrix of submucosa and eventuates to migration, proliferation, and differentiation epithelial cells at the border of the mucosal ulcers.[70,71] Accordingly, reducing inflammation as an initiator factor plays an important role in the control of mucositis. Additionally, antibacterial and antifungal agents are effective in mucositis treatment, since they prevent or treat secondary infections. Pain control can also lead to a sense of well-being in patients and enhance their quality of life. Speeding up the wound healing process by shortening the duration of mucositis can decline mucositis complications.

This study provided the first ethnopharmacological survey, focusing on oral mucositis. The traditional healers applied various preparation methods for different remedies. Maceration is among the common specific methods for plant extraction, where heat is not normally used. Some of the examples cited in the sources or deduced from traditional stores of medicinal plants only mentioned the extract method, but failed to provide details of the extraction method. Thus, extraction is a generic term and it involves decoction, infusion, and maceration, among other methods. In distillate method, the plant is heated in water so that the essential oil of the plant enters the water in a few amounts and gives a weak odor to the water.[72] In maceration method, as mentioned above, extraction is done without the use of heat.[73] For oil isolation, hydro-distillation method is done using Clevenger apparatus,[74] while for powder preparation, the plant is milled and passed through a sieve with definite mesh. Hydrocolloid is extracted by floating the plant in water and, after a definite time, the extract is filtered and dried.[75]

The literature review demonstrates that, among the 15 recommended herbs, the effectiveness of *Matricaria chamomilla* L. and *Alcea digitata* Alef have been directly evaluated. In a pilot study, the effectiveness of a combination of *Alcea digitata* Alef and *Malva sylvestris* L. was evaluated for prevention of head and neck radiotherapy-induced oral mucositis. A total of 23 patients were divided into intervention and placebo groups that received the drug for 7 weeks. The WHO scale was used for evaluation of severity of oral mucositis symptoms weekly. The results indicated that patients in the placebo group experienced more severe mucositis from the second week, which was significantly different from the herbal drug-treated group ($p<0.0001$).[21] A randomized-controlled phase II clinical trial has been conducted on the effectiveness of liquid extract of *Chamomilla recutita* at the dosages of 0.5%, 1%, or 2% in prevention and treatment of oral mucositis in patients undergoing hematopoietic stem cell transplantation. Patients who received the standard care plus mouthwash of *C. recutita* at 1% dosage showed less incidence, intensity, and duration of oral mucositis when compared with the control group.[43]

The search throughout scientific databases revealed that several remedies used by traditional healers in Zahedan for mucositis treatment have approved pharmacological properties. In this study, we aimed to categorize the mechanism of actions according to recent scientific studies as follows:

Plants with antiinflammatory activities (*Alhagi maurorum* Medik.,[23] *Caryophyllus aromaticus* L.,[26] *Cichorium intybus* L.,[30] *Descurainia sophia* (L.) Webb ex Prantl,[35] *Linum usitatissimum* L.,[38] *Matricaria chamomilla* L.,[44] *Myrtus communis* L.,[48] *Plantago ovata* Forssk.,[54] *Punica granatum* L.,[58] *Rhazya stricta* Decne.,[63] and *Rhus coriaria* L.[66]); plants with wound healing properties (*Alhagi maurorum* Medik.,[24] *Cichorium intybus* L.,[32] *Linum usitatissimum* L.,[41] *Matricaria chamomilla* L.,[46] *Myrtus communis* L.,[50] *Plantago ovata* Forssk.,[56] and *Punica granatum* L.[60]); plants with antimicrobial/antifungal effects (*Alcea digitata* Alef.,[22] *Alhagi maurorum* Medik.,[25] *Caryophyllus aromaticus* L.,[28,29] *Cichorium intybus* L.,[34] *Fumaria parviflora* Lam.,[37] *Linum usitatissimum* L.,[42] *Matricaria chamomilla* L.,[47] *Myrtus communis* L.,[52] *Plantago ovata* Forssk.,[57] *Punica granatum* L.,[61,62] *Rhazya stricta* Decne.,[64,65] and *Rhus coriaria* L.[68]); and plants with antinociceptive properties (*Alhagi maurorum* Medik.,[23] *Caryophyllus aromaticus* L.,[27] *Cichorium intybus* L.,[31] *Fumaria parviflora* Lam.,[36] *Linum usitatissimum* L.,[40] *Matricaria chamomilla* L.,[45] *Myrtus communis* L.,[49] *Plantago ovata* Forssk.,[55] *Punica granatum* L.,[59] *Rhazya stricta* Decne.,[63] and *Rhus coriaria* L.).[67]

Utilization of traditional medicine among Iranian people has a wide range of 10-75%, depending on diversity of populations.[76-79] Considering that the application of traditional medicine in patients with cancer is associated with potential advantages and possible risks, the necessity for further studies on herbal remedies has become more pertinent. For instance, although the antineoplastic properties of many herbs have been approved, the safety of some other herbs is uncertain. Administration of aqueous extracts of *Dioscorea opposita* and *Cistanche deserticola* in both estrogen receptor negative (SKBR3 and MDA-MB-231) and estrogen receptor positive (MDA-MB-361 and MCF-7) breast cancer cells can lead to stimulation of cell viability. However, patients with breast cancer in some parts of the world use these two herbs to relieve the adverse effects of cancer treatment.[80] Therefore, designing accurate scientific studies on herbal medicines to provide evidence to advice or forbid the mentioned remedies are indispensable.

CONCLUSION

Among the 18 medicaments used as ethnomedicine to alleviate mucositis in Zahedan, three of them had synthesis or mineral origin. Only two herbs were evaluated for their direct efficacy against mucositis, while the others have not yet been tested. Scientific studies have approved the related pharmacological effects of 11 medicaments. Accordingly, they can be regarded as

appropriate candidates for future studies on the determination of their probable influences on mucositis, followed by the discovery of new pharmacologic agents. However, the fact that the application of traditional medicine may be associated with potential risks instigates more scientific investigations.

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ORIGINAL ARTICLE

In Vitro Enzyme Inhibitory Properties, Antioxidant Activities, and Phytochemical Profiles of *Moltkia aurea* and *Moltkia coerulea*

Moltkia aurea ve *Moltkia coerulea*'nın *İn Vitro* Enzim İnhibitör Özellikleri, Antioksidan Aktiviteleri ve Fitokimyasal Profilleri

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ABSTRACT

Objectives: In Turkey, the genus *Moltkia* Lehm. is represented by two species, namely *Moltkia aurea* Boiss. and *M. coerulea* (Willd.) Lehm., which are used as both food and for medicinal purposes. This study aimed to evaluate the antidiabetic and antioxidant potential and phytochemical profiles of leaf, flower, and root extracts of *Moltkia* species.

Materials and Methods: α-Glucosidase and α-amylase inhibitory activities, antioxidant effects, and total phenol and flavonoid contents of *Moltkia* extracts were evaluated. High-performance liquid chromatography was performed for identifying and quantifying phenolic compounds, which are responsible for various activities of these extracts.

Results: Among the investigated phenolic compounds, caffeic and rosmarinic acids and rutin were determined and quantified in methanol extracts. Rutin was the major compound in the flower extract of *M. aurea*. Rutin and rosmarinic acid were the major compounds in the leaf extract of *M. aurea*. The flowers, leaves and roots of *M. coerulea* were also rich in rosmarinic acid. The antioxidant and antidiabetic potential of these extracts may be attributable to their rutin and rosmarinic acid content.

Conclusion: *Moltkia* species can be used as natural sources of antioxidants. Notably, *M. aurea* extracts can be used for the development of herbal products with antidiabetic potential.

Key words: *Moltkia*, Boraginaceae, antidiabetic, antioxidant, HPLC, phenolic compounds

ÖZ

Amaç: *Moltkia* Lehm. genusu Türkiye'de iki tür ile temsil edilmektedir: *Moltkia aurea* Boiss. ve *M. coerulea* (Willd.) Lehm. Her iki bitki de hem besin olarak hem de tıbbi amaçla kullanılmaktadır. Bu çalışma, *Moltkia* türlerinden elde edilen yaprak, çiçek ve kök ekstrelerinin antidiyabetik ve antioksidan potansiyellerini ve fitokimyasal profillerini incelemeyi amaçlamaktadır.

Gereç ve Yöntemler: *Moltkia* ekstrelerinin α-glukozidaz ve α-amilaz enzim inhibitör aktiviteleri, antioksidan etkileri, total fenol ve flavonoit içerikleri değerlendirilmiştir. Bu ekstrelerin farklı aktivitelerinden sorumlu olabileceği düşünülen fenolik bileşiklerin belirlenmesi ve miktar tayini için yüksek performanslı sıvı kromatografisi yöntemi kullanılmıştır.

Bulgular: İncelenen fenolik bileşikler arasında, kafeik asit, rozmarinik asit ve rutin metanol ekstrelerinde tespit edilmiş ve miktarları tayin edilmiştir. Rutin, *M. aurea* çiçek ekstresinin ana bileşeni olarak belirlenmiştir. Rutin ve rosmarinik asit *M. aurea* yaprak ekstresinin ana bileşenleri olarak belirlenmiştir. *M. coerulea* çiçek, yaprak ve köklerinin ise rozmarinik asit açısından da zengin olduğu tespit edilmiştir. Ekstrelerin antioksidan ve antidiyabetik etkileri rutin ve rozmarinik asit içeriklerine bağlı olabilir.

Sonuç: *Moltkia* türleri potansiyel doğal antioksidan kaynağı olarak kullanılabilir. Özellikle *M. aurea* ekstreleri antidiyabetik potansiyele sahip bitkisel ürün geliştirilmesinde kullanılabilir.

Anahtar kelimeler: *Moltkia*, Boraginaceae, antidiyabetik, antioksidan, YPSK, fenolik bileşikler

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INTRODUCTION

The Boraginaceae family is distributed in tropical, subtropical, and temperate areas of the world and comprises approximately 2500 species.[1] *Moltkia*, *Heliotropium*, *Cordia*, *Arnebia*, *Echium*, and *Onosma* are some of the important genera of Boraginaceae. Most members of this family are medicinally important plants that contain secondary metabolites, including flavonoids, terpenoids, alkaloids, fatty acids, glycosides, and phytosterols, and various proteins.[2,3]

Moltkia comprises five species, all of which grow in the Eastern part of the Mediterranean region. *Moltkia coerulea* (Willd.) Lehm. is endemic to Anatolia, Lebanon, and the Crimea; *M. aurea* Boiss. is endemic to Anatolia.[4] *Moltkia* species of Turkey, known as "emzik cicegi, sancı out, sormuk, sarı kesen", are traditionally used for various health problems, such as kidney disorders, diarrhea, and abdominal pain.[5,6] In Sivas, leaves of *M. coerulea* are consumed as food; in Nigde, the sweet flowers of *M. coerulea* are eaten by children.[7] Studies on *Moltkia* species of Turkey have revealed that these plants possess antioxidant, antibacterial, and cytotoxic activities.[3,5]

Inhibition of α-glucosidase in the bowel slows oligosaccharide degradation, thereby reducing the glucose level in the circulatory system. α-amylase degrades long-chain carbohydrates. Inhibition of such enzymes has been an important approach to decrease blood glucose levels and diabetes-related complications. Oxidative stress is an important determinant of diabetes-related complications, and the overproduction of free radicals is related to hyperglycemia.[8] Although studies comparing anatomical and morphological features of *M. aurea* and *M. coerulea* growing in Turkey[1] are available, only a few studies have examined the biological activity and phytochemistry of both species together.[5,9] Therefore, we investigated the antidiabetic and antioxidant potential of flowers, leaves, and roots of Turkish *Moltkia* species and compared their phytochemical profiles.

MATERIALS AND METHODS

Plant material

Plants from *Moltkia* species were collected in the flowering stage from Eryaman-Ankara, Turkey in May, 2015. Plants were collected by N. Orhan and Ç. Orhan and identified by Assoc. Prof. Dr. N. Orhan. Voucher specimens GUEF 3239 and GUEF 3240 (*M. aurea*) and GUEF 3241 and GUEF 3242 (*M. coerulea*) were deposited at Gazi University's Faculty of Pharmacy.

Extract preparation

Water extract: Dried and ground plant parts (leaf, flower, and root) were extracted with 50 mL hot water (4% w/v) on a heating-magnetic stirrer for 6 h and filtered. The residues were treated with 50 mL water using the same procedure. Filtered aqueous extracts were combined and freeze-dried.

Ethyl acetate and methanol (MeOH) extracts: Dried and powdered plant parts (leaf, flower, and root) were treated with 200 mL MeOH and ethyl acetate (2.5% w/v) on a shaker for 18 h at 25°C and filtered. This procedure was repeated two times; extracts were pooled and solvents were removed using a rotary evaporator. Extract yields are presented in Table 1.

Enzyme inhibitory activity

α-Amylase inhibitory activity

α-Amylase (porcine pancreatic, EC 3.2.1.1, Sigma) was dissolved in distilled water and added to plant extracts. Incubation was performed at 37°C for 3 min. Then, potato starch solution (0.5% w/v) was added to the mix and incubated at 37°C for 5 min. Subsequently, 3,5-dinitrosalicylic acid solution was added to the mix and incubated in an 85°C heater. Finally, distilled water was added, and the tubes were allowed to cool. Absorbance values were recorded at 540 nm. Acarbose was run as the reference.[10] Absorbance (*A*) due to maltose formation was estimated according to the formula: $A_{\text{Control or sample}} = A_{\text{Test}} - A_{\text{Blank}}$.

The quantity of maltose formation was assessed by using the maltose standard calibration curve (0-0.1% w/v) and the gained net absorbance value. Inhibition ratio was estimated as:

$$\text{Inhibition \%} = [(\text{Maltose}_{\text{Control}} - \text{Maltose}_{\text{Sample}}) / \text{Maltose}_{\text{Control}})] \times 100.$$

Table 1. Yield percentages and total phenol and flavonoid contents in *Moltkia* extracts

Plant	Part	Extract	Yield % (w/w)	Total phenol content (mean ± SD)	Total flavonoid content (mean ± SD)
Moltkia aurea	Leaf	Water	25.71	232.94±9.37	38.38±1.61
		MeOH	14.96	149.72±12.74	20.60±1.58
		EA	1.89	122.18±3.37	34.86±1.40
	Flower	Water	30.23	219.07±28.26	49.12±1.90
		MeOH	24.94	57.52±11.43	58.80±2.61
		EA	1.57	20.19±0.00	51.05±5.89
	Root	Water	9.47	376.53±34.19	30.46±1.33
		MeOH	5.05	369.40±34.30	32.39±0.61
		EA	0.53	125.85±28.81	66.55±1.90
Moltkia coerulea	Leaf	Water	22.68	201.32±13.59	36.09±3.52
		MeOH	9.72	18.97±0.00	36.62±1.22
		EA	1.00	77.71±27.54	127.46±4.33
	Flower	Water	22.17	224.17±17.18	29.75±5.29
		MeOH	12.55	204.79±29.68	30.63±1.40
		EA	0.84	25.09±3.06	62.67±3.23
	Root	Water	15.39	252.52±19.05	26.06±0.81
		MeOH	4.60	136.87±27.87	29.05±0.53
		EA	0.49	18.77±4.09	73.06±2.13

Total flavonoid content was expressed as mg quercetin equivalent/g extract and total phenol content was expressed as mg gallic acid equivalent/g extract
MeOH: Methanol, EA: Ethyl acetate, SD: Standard deviation

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory potential of *Moltkia* samples was evaluated using the assay of Lam et al.[11] α-Glucosidase (Sigma Co., St. Louis, USA) obtained from *Bacillus stearothermophilus* was dissolved in phosphate buffer (0.5 M) at pH 6.5. Extracts and enzyme solution were suspended in hydroalcohol (80%) and preincubated at 37°C. Then, 20 mM p-nitrophenyl-α-D-glucopyranoside (NPG, Sigma) was added as the substrate. Samples were incubated for nearly 30 min at 37°C. The difference in absorbance at 405 nm, due to the the hydrolysis of NPG by the enzyme, was quantified. The α-glucosidase inhibitor acarbose (Bayer Group, Turkey) was chosen as a reference. The inhibition ratio (%) was estimated using the following equation:

Inhibition % $=[(A_{Control} - A_{Sample}) / A_{Control}] \times 100$

Antioxidant activity

Radical [1.1-diphenyl-2-picrylhydrazy (DPPH)] scavenging activity

A total of 40 µL DPPH solution was vortexed with 160 µL of the extract and incubated in the dark. Then, absorbance was measured at 520 nm.[12] All calculations were performed using the Softmax PRO 4.3.2.LS software. Butylated hydroxytoluene was used as a reference at 0.1, 0.3, and 1 mg/mL.

Superoxide anion scavenging activity

Superoxide radicals were generated in a nonenzymatic system. The reaction mixture, including 25-2000 µg/mL of the test fraction in 70°C ethanol, 1 mL 468 mmol/L β-NADH, 1 mL 60 mmol/L phenazine methosulphate (PMS), and 1 mL 150 mmol/L nitro blue tetrazolium chloride in phosphate buffer (0.1 mol/L, pH 7.4), was incubated. Absorbance was recorded at 560 nm against blank samples, which were free of PMS. Activity was estimated as scavenging activity (%) $=[(A_{Control} - A_{Sample}) / A_{Control}] \times 100$, where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance of the extract.[13,14] Quercetin was chosen as the reference at 0.1, 0.3, and 1 mg/mL.

Ferric-reducing antioxidant power

The extract or ascorbic acid was incubated with 0.2 mol/L phosphate buffer (pH 6.6) and $K_3Fe(CN)_6$. Trichloroacetic acid was added to the mixture, vortexed, and centrifuged. Water and ferric chloride were added to the supernatant and absorbance was measured at 700 nm.[15] Ascorbic acid was used as reference.

Metal-chelating capacity

Extracts were incubated with $FeCl_2$ (2 mM). The reaction was initiated after the addition of 0.2 mL of ferrozine (5 mM). After a while, absorbance was recorded at 562 nm.[16] Ethylenediaminetetraacetic acid was used as a reference and $FeCl_2$ and ferrozine as controls. Inhibition ratio of ferrozine-Fe^{+2} complex generation was estimated using the following formula: Metal-chelating activity (%) $= [(A_{Control-}A_{Sample}) / A_{Control}] \times 100$.

Total antioxidant capacity

Molybdate reagent, water, and extracts were mixed, vortexed, incubated for 90 min at 95°C in test-tubes. Then, tubes were cooled and absorbance was recorded at 695 nm. Outcomes were shown as ascorbic acid equivalent in the extract (mg/g).[17]

Phytochemical content

Estimation of total phenol content

Extracts were mixed and incubated with the Folin-Ciocalteu reagent. Then, sodium carbonate solution was added to the mixture and immediately vortexed. Absorbance was measured at 735 nm after 30 min in the dark. Total phenol content of extracts in mg of gallic acid equivalent (GAE)[18] was calculated using the following equation:

Absorbance=1.6342 × (conc.) + 1.417, $r^2 = 0.9986$.

Determination of total flavonoid content

Extracts (1 mg/mL) were suspended in 80% ethanol. Sodium acetate, aluminum chloride solution, ethyl alcohol, and water were mixed and incubated with extracts for 30 min. Absorbance was measured at 415 nm. Outcomes in mg quercetin equivalent (QE)/g extract[19] were calculated using the following equation: Absorbance=1.8934 × (conc.) - 0.025, and r^2 value was 0.9996.

Qualitative and quantitative analyses of phenolic compounds using reverse phase-high-performance liquid chromatography (RP-HPLC)-photo diode array (PDA)

HPLC analyses were performed as described[20,21] using the HP Agilent 1260 series LC system and ACE column (5 µm, 250 mm × 4.6 mm) at 30°C. The flow rate of the gradient elution was 0.8 mL/min. The mobile phase was a mixture of water (solution A), MeOH (solution B), and acetonitrile (solution C), each containing 0.1% trifluoroacetic acid. Caffeic and rosmarinic acids were analyzed at 330 nm and rutin at 360 nm by external standardization.

Statistical analysis

Experiments were performed in triplicate and mean values were obtained. All values are given as the mean ± standard deviation. Computations were performed using GraphPad InStat and Microsoft Excel.

RESULTS

Results of the total phenol content assay revealed that lyophilized water and MeOH extracts of all investigated parts of both *Moltkia* species were rich in total phenolics. Water and MeOH extracts of *M. aurea* roots had the highest total phenolic content (376.53±34.19 and 369.40±34.30 mg GAE/g extract, respectively). The highest amount of total flavonoid was recorded in the ethyl acetate extract of *M. coerulea* leaves (127.46±4.33 mg QE/g extract). In addition, amount of total flavonoid in the MeOH extract of *M. aurea* flowers was high (58.80±2.61 mg QE/g extract), which was consistent with results from HPLC analysis, indicating high rutin content. Extract yields and obtained results for total phenol and flavonoid content are presented in Table 1.

One approach for managing diabetes includes inhibiting α-amylase and α-glucosidase activity. In this study, *Moltkia* species displayed mild enzyme inhibitory activity compared

to the reference acarbose (Table 2). Ethyl acetate and water extracts of *M. aurea* leaves inhibited α-amylase (24.07%±2.59% and 14.11%±2.59%, respectively) together with ethyl acetate extracts of *M. coerulea* flowers (18.89%±2.43%) at 3 mg/mL. MeOH and ethyl acetate extracts inhibited α-glucosidase activity more strongly than water extracts (Table 2).

Plant extracts have excellent antioxidant activity.[22] To evaluate the antioxidant potential of *Moltkia* species, various *in vitro* tests were performed. Analysis of total antioxidant capacity revealed that ethyl acetate extracts displayed significant antioxidant potential compared with extracts prepared using other solvents. Water, and especially MeOH, extracts exhibited significant ferric-reducing power compared to ethyl acetate extracts. All water extracts exhibited significant metal-chelating activity (Table 3). Although the superoxide-scavenging activity of water, MeOH, and ethyl acetate extracts of roots of both species were found to be promising, MeOH extracts of all samples scavenged the DPPH free radical significantly (Table 4).

HPLC analyses revealed that among the investigated phenolics (Rt chlorogenic acid: 8.9 min, Rt caffeic acid: 12.3 min, Rt ferulic acid: 20.25 min, Rt rutin: 23.1 min, Rt rosmarinic acid: 30.6 min, Rt quercetin: 36.5 min, Rt luteolin: 37.3 min, and Rt apigenin: 40.3 min) only caffeic acid, rutin, and rosmarinic acid were detected and quantified in methanolic extracts of flowers, leaves, and roots (Figures 1-6). Chromatograms of standard compounds are shown in Figures 7-8. Ultraviolet spectra of quantified compounds, which were overlaid with those of standard compounds, are shown in Figures 9-11.

Rutin a flavonoid glycoside, occurs naturally in many fruits and vegetables and has several biological activities, such as antioxidant, antibacterial, antifungal, and antiinflammatory effects.[23] As seen in the chromatograms, a significant amount of rutin was present in flower extracts of *M. aurea* (6.198%±0.271%; mean peak area: 3290), and MeOH extract of *M. coerulea* flowers contained less amount rutin (0.099%±0.002%; mean peak area: 81.23). We believe our findings reveal significant phytochemical differences between these two *Moltkia* taxa endemic to Turkey. Rosmarinic acid, a phenolic compound derived from hydroxycinnamic acid and mostly found in Lamiaceae and Boraginaceae, has many biological activities, including antioxidant, antimicrobial, antiinflammatory, antiproliferative, and chemopreventive effects.[24] As seen from the chromatograms, root extracts of both species were rich in rosmarinic acid (3.459%±0.005% and 2.028%±0.012%, respectively). Moreover, remarkable

Table 2. Enzyme inhibitory effects of *Moltkia* extracts

Plant	Part	Extract	α-Glucosidase inhibition % ± SD			α-Amylase inhibition % ± SD		
			3 mg/mL	1 mg/mL	0.3 mg/mL	3 mg/mL		
Moltkia aurea	Leaf	Water	52.77±7.26	29.29±1.71	3.31±1.74	14.11±2.59		
		MeOH	51.87±2.41	28.31±3.50	13.47±2.06	-		
		EA	86.02±1.23	76.17±0.22	59.41±1.23	24.07±2.59		
	Flower	Water	48.10±7.15	30.49±3.85	19.42±3.00	12.86±3.80		
		MeOH	61.01±4.07	47.86±2.98	25.26±2.54	-		
		EA	78.74±1.18	63.92±1.46	46.55±3.98	8.43±5.84		
	Root	Water	85.67±2.76	67.66±0.66	25.42±0.59	-		
		MeOH	77.68±3.85	68.35±0.72	31.04±4.81	8.28±1.10		
		EA	84.93±2.09	69.12±1.39	33.43±3.48	9.47±1.58		
Moltkia coerulea	Leaf	Water	49.84±0.99	33.64±3.31	36.78±6.07	-		
		MeOH	66.84±2.54	48.25±4.24	37.44±3.92	3.68±9.65		
		EA	66.26±2.58	64.69±2.12	41.08±5.87	-		
	Flower	Water	44.12±5.43	36.89±4.06	13.70±0.91	-		
		MeOH	60.04±0.65	41.28±0.97	16.64±2.20	-		
		EA	84.11±1.79	73.24±1.05	53.95±3.91	18.89±2.43		
	Root	Water	46.44±3.10	35.03±1.94	5.68±1.43	-		
		MeOH	74.65±1.54	48.66±1.83	19.00±4.25	-		
		EA	71.56±1.00	62.12±0.30	42.75±3.10	-		
Reference	Acarbose		1 mg/mL	0.3 mg/mL	0.1 mg/mL	1 mg/mL	0.3 mg/mL	0.1 mg/mL
	-		98.88±0.07	97.98±0.03	96.37±0.56	44.72±2.76	31.51±3.18	26.26±5.74

-: No activity, SD: Standard deviation, MeOH: Methanol, EA: Ethyl acetate

amount of rosmarinic acid was detected in the other investigated parts (Table 5).

DISCUSSION

Based on our comprehensive literature survey, only a few studies on pharmacological activities and chemical profiles of related *Moltkia* species have been preformed. Harput et al.[5] investigated the cytotoxic effect of the aerial parts of *M. aurea* on three cell lines and determined antioxidant activity. Moreover, dose-dependent cytotoxic activity against cancer cell lines as well as promising antioxidant activity have been reported. Additionally, some phenolic compounds were obtained from

Table 3. Metal-chelating activity, ferric-reducing power, and total antioxidant capacity of *Moltkia* extracts

Plant	Part	Extract	Metal-chelating activity % ± SD			Ferric-reducing power absorbance ± SD			Total antioxidant capacity*
			3 mg/mL	1 mg/mL	0.3 mg/mL	3 mg/mL	1 mg/mL	0.3 mg/mL	
Moltkia aurea	Leaf	Water	>100	90.33±4.19	48.96±5.72	1.5307±0.0370	0.7410±0.0605	0.2343±0.0090	-
		MeOH	22.42±3.58	-	-	2.3673±0.0393	1.9194±0.0615	0.1960±0.0149	-
		EA	-	-	-	0.7040±0.0867	0.2143±0.0084	0.0477±0.0042	66.30±9.08
	Flower	Water	>100	98.11±5.66	33.25±6.17	2.0137±0.1373	1.3583±0.0741	0.4033±0.0086	-
		MeOH	70.17±3.86	-	-	2.5244±0.0674	2.4727±0.0665	0.3080±0.0218	-
		EA	12.07±6.29	-	-	0.6154±0.0285	0.0847±0.0110	0.0190±0.0036	299.53±27.23
	Root	Water	>100	35.99±2.92	-	3.1090±0.0210	2.7980±0.0936	1.1257±0.0528	-
		MeOH	-	-	-	1.7140±0.0875	1.4720±0.0339	1.0083±0.1198	8.65±0.00
		EA	43.03±7.53	-	-	1.2424±0.0101	0.9977±0.1124	0.3130±0.0300	687.37±9.08
Moltkia coerulea	Leaf	Water	>100	80.00±3.47	15.90±3.05	1.9434±0.0248	0.7843±0.5653	0.4973±0.0138	-
		MeOH	38.79±9.22	-	-	2.9394±0.0418	2.4310±0.0752	1.2323±0.0594	-
		EA	-	-	-	0.3470±0.0172	0.1353±0.0195	0.0333±0.0087	540.62±29.76
	Flower	Water	>100	99.87±2.63	29.77±0.61	1.8684±0.0300	1.4033±0.1028	0.2910±0.0115	-
		MeOH	80.31±8.32	-	-	2.5758±0.0979	2.1333±0.1092	0.9640±0.0624	-
		EA	-	-	-	1.2720±0.0966	0.1057±0.0176	0.0157±0.0049	485.59±12.01
	Root	Water	>100	80.36±8.16	40.00±7.18	1.9684±0.0835	0.9477±0.0761	0.1500±0.0394	11.27±4.54
		MeOH	72.42±6.87	-	-	1.8060±0.0095	0.9323±0.0595	0.6330±0.0442	21.75±4.54
		EA	-	-	-	1.8114±0.0166	0.3910±0.0231	0.1100±0.0092	178.98±12.01
Reference			1 mg/mL	0.3 mg/mL	0.1 mg/mL	3 mg/mL	1 mg/mL	0.3 mg/mL	-
EDTA			98.87±0.14	96.60±3.39	95.75±0.08	NT	NT	NT	NT
Ascorbic acid			NT	NT	NT	3.5870±0.0874	3.4547±0.0852	3.0787±0.0587	NT

*Total antioxidant capacity is expressed as ascorbic acid equivalent/g extract ± SD. NT: Not tested, -: No activity, SD: Standard deviation, EDTA: Ethylenediaminetetraacetic, MeOH: Methanol, EA: Ethyl acetate

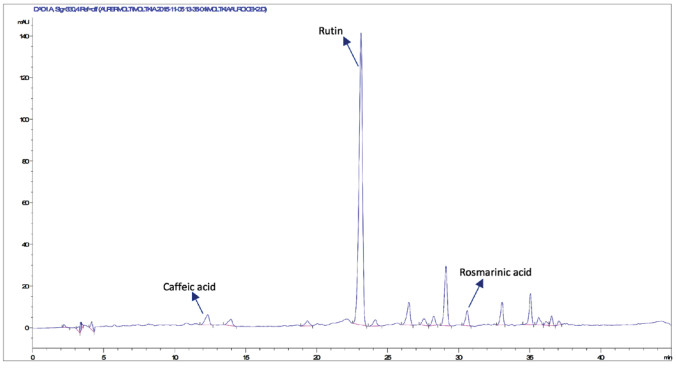

Figure 1. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia aurea* flowers

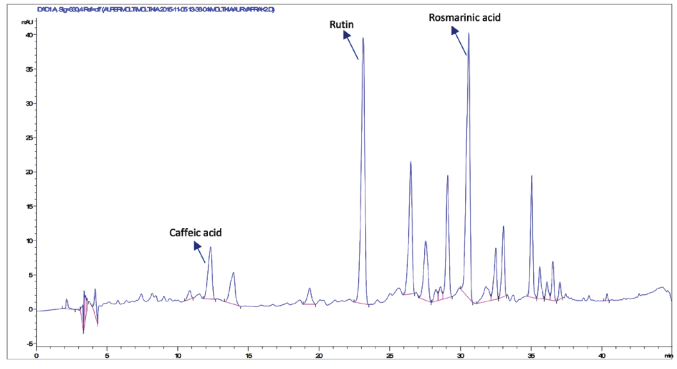

Figure 2. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia aurea* leaves

the water subextract. Erdemoglu et al.[25] studied the chemical constituents of the seed oil of *M. aurea*.

The effect of an ointment prepared from the aerial parts of *M. coerulea* (collected from Iran) on wound healing was investigated by Farahpour et al.[26] Chemical constituents of *M. coerulea* flowers collected from India included fatty acids (capric, myristic, palmitic, behenic, and undecylic acids), flavonoids (kaempferol, quercetin, nortangeretin, and rebinetin), and amino acids (ornithine, lysine, dopa, serine, glutamic acid, proline, amino-n-butyric acid, phenylalanine, and leucine).[27]

Table 4. Superoxide anion and DPPH radical scavenging activities of *Moltkia* extracts

Plant	Part	Extract	Superoxide anion scavenging activity inhibition % ± SD			DPPH radical scavenging activity inhibition % ± SD		
			3 mg/mL	1 mg/mL	0.3 mg/mL	3 mg/mL	1 mg/mL	0.3 mg/mL
Moltkia aurea	Leaf	Water	65.31±8.13	26.32±3.98	-	-	-	20.33±5.97
		MeOH	7.85±2.04	-	-	62.21±3.66	53.50±2.29	45.50±0.00
		EA	44.14±6.49	-	-	-	26.73±4.40	14.17±3.21
	Flower	Water	75.80±7.45	58.30±1.43	4.63±1.28	-	7.37±3.94	37.33±5.58
		MeOH	20.46±4.09	-	-	43.32±2.80	42.09±6.92	45.00±0.50
		EA	40.37±4.29	-	-	-	6.30±1.40	11.33±4.65
	Root	Water	96.30±6.47	81.43±9.18	45.73±5.82	-	-	-
		MeOH	91.36±0.37	58.92±1.49	30.96±5.07	10.91±7.04	24.17±3.25	35.00±9.99
		EA	80.18±1.56	34.19±6.43	-	-	-	23.50±4.33
Moltkia coerulea	Leaf	Water	61.65±3.12	24.60±4.28	-	-	-	9.00±0.00
		MeOH	34.69±5.74	5.31±1.78	-	24.88±3.94	40.17±4.04	47.00±2.50
		EA	51.09±4.82	11.56±0.74	-	-	15.21±0.00	30.67±6.53
	Flower	Water	59.63±6.16	45.03±3.15	16.97±4.47	-	-	23.17±5.80
		MeOH	14.94±4.63	-	-	44.09±2.70	41.50±0.50	44.67±0.76
		EA	51.74±1.82	14.51±1.64	-	-	4.15±0.46	-
	Root	Water	98.07±4.44	27.78±3.32	-	-	-	8.33±4.25
		MeOH	87.78±0.64	41.58±1.47	24.37±1.44	34.56±0.46	27.00±3.77	38.67±4.01
		EA	76.58±1.46	31.61±1.40	-	-	36.87±4.44	29.5±0.00
Reference			1 mg/mL	0.3 mg/mL	0.1 mg/mL	1 mg/mL	0.3 mg/mL	0.1 mg/mL
Quercetin			86.52±3.26	75.19±3.22	68.45±0.27	ND	ND	ND
BHT			>100	62.74±2.08	18.07±2.40	50.67±3.40	47.17±4.31	40.83±7.69

DPPH: 1.1-diphenyl-2-picrylhydrazy, BHT: Butylated hydroxytoluene, -: No activity, ND: Not detected, SD: Standard deviation, MeOH: Methanol, EA: Ethyl acetate

Table 5. Content of phenolic compounds in methanol extracts of *Moltkia* species

Species	Content (g/100g extract)[a]		
	Caffeic acid	Rosmarinic acid	Rutin
Moltkia aurea leaf	0.026±0.001	0.299±0.005	0.836±0.004
Moltkia aurea flower	0.032±0.003	0.111±0.005	6.198±0.271
Moltkia aurea root	0.024±0.001	3.459±0.005	0.043±0.002
Moltkia coerulea leaf	0.067±0.003	0.261±0.009	0.585±0.024
Moltkia coerulea flower	0.026±0.001	0.342±0.002	0.099±0.002
Moltkia coerulea root	0.011±0.001	2.028±0.012	ND[b]

[a]Mean ± SD (n=3); [b]ND: Not detected, SD: Standard deviation

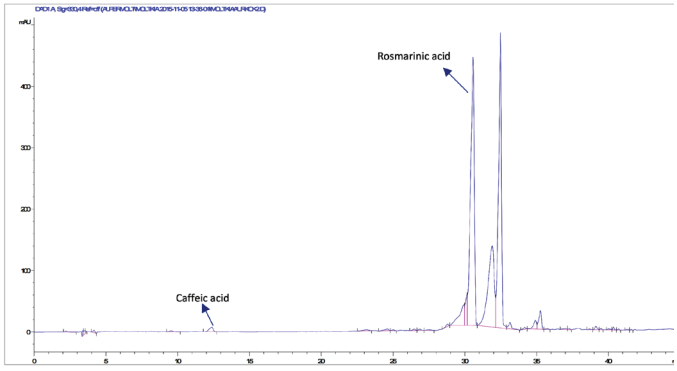

Figure 3. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia aurea* roots

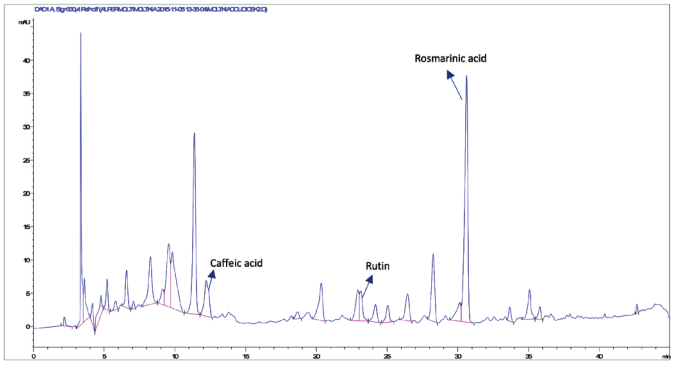

Figure 4. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia coerulea* flowers

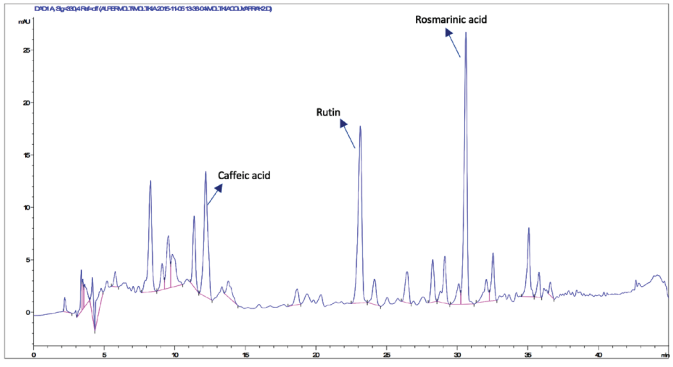

Figure 5. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia coerulea* leaves

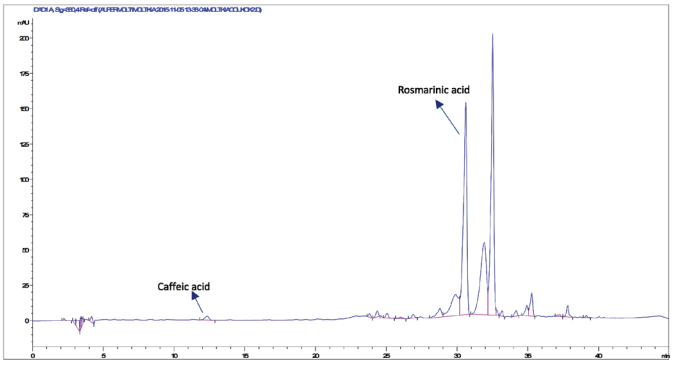

Figure 6. High-performance liquid chromatography analysis of the methanolic extract of *Moltkia coerulea* roots

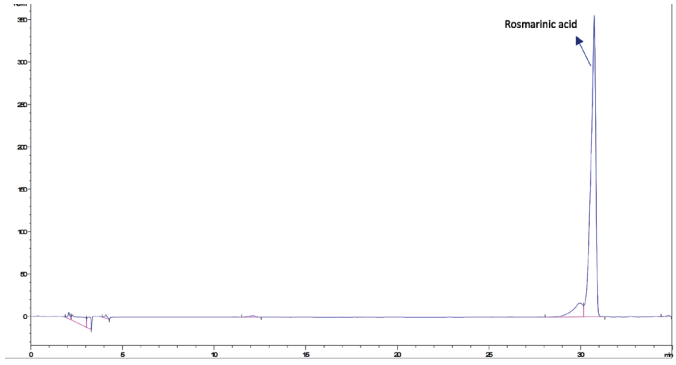

Figure 7. High-performance liquid chromatography analysis of the rosmarinic acid standard

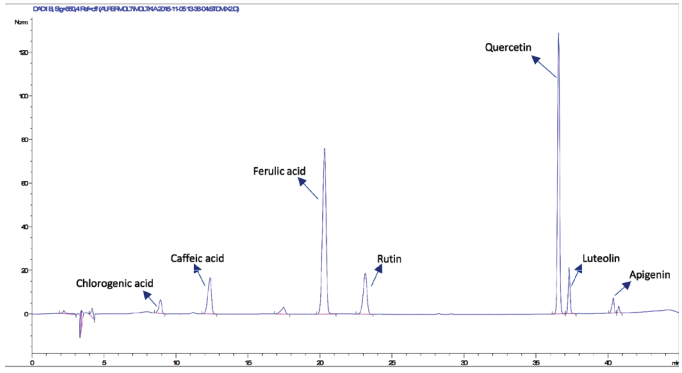

Figure 8. High-performance liquid chromatography analysis of a standard mixture containing chlorogenic acid (Rt: 8.9 min), caffeic acid (Rt: 12.3 min), ferulic acid (Rt: 20.25 min), rutin (Rt: 23.1 min), quercetin (Rt: 36.5 min), luteolin (Rt: 37.3 min), apigenin (Rt: 40.3 min)

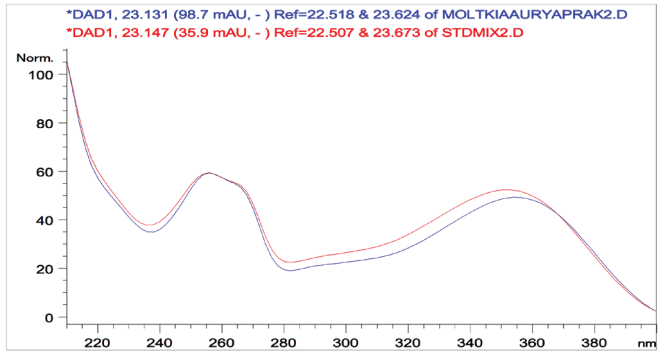

Figure 9. Overlaid ultraviolet spectra of standard rutin and rutin detected in the *Moltkia aurea* leaf extract

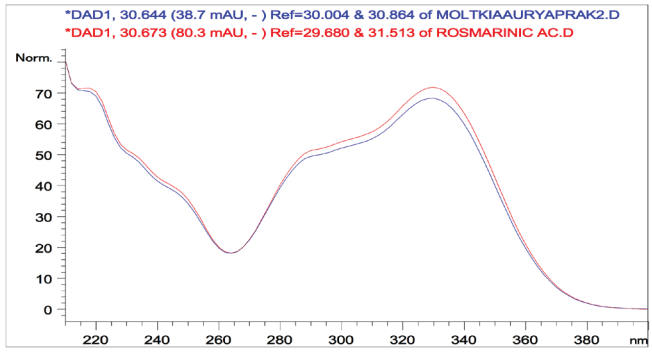

Figure 10. Overlaid ultraviolet spectra of standard rosmarinic acid and rosmarinic acid detected in the *Moltkia aurea* leaf extract

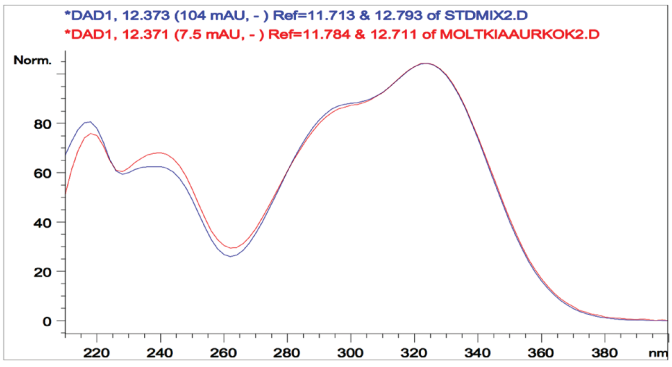

Figure 11. Overlaid ultraviolet spectra of standard caffeic and caffeic acid detected *Moltkia aurea* in the root extract

Zengin et al.[9] investigated antioxidant, antimicrobial, antityrosinase, antiacetylcholinesterase, antibutyrylcholinesterase, and antidiabetic activities of methanolic extracts of aerial parts of *M. aurea* and *M. coerulea in vitro*. Moreover, they studied the phenolic profile of the species and performed an *in vivo* assay to evaluate genotoxicity. Rutin, hesperidin, and protocatechuic acid were detected as the main components of the aerial parts of *M. aurea*, whereas rutin, protocatechuic acid, malic acid, and hesperidin were detected as the main components of the aerial parts of *M. coerulea*. In this study, we evaluated antioxidant and antidiabetic activities and phenolic profiles of three parts of the species. Consistent with the results of Zengin et al.[9], we found that a significant amount of rutin was present in the MeOH extract of *M. aurea* flowers. Moreover, rosmarinic acid was one of the most abundant compound in both species, especially in the leaf and root of *M. aurea* and all investigated parts of *M. coerulea*. The content of rosmarinic acid is the main difference between the two studies. Rutin amount was determined too high in the flower extract of *M. aurea* compared to that of *M. coerulea*. Moreover, caffeic acid should contribute to the antioxidant feature of the species.

Zengin et al.[9] reported that although both species possessed antioxidant properties, *M. aurea* was a better antioxidant. We could not categorize the species according to their antioxidant potential. Different results were obtained with different extracts, and making correlation was difficult. Both *M. aurea* and *M. coerulea* possessed antidiabetic activity as inhibitors of carbohydrate-digesting enzymes.

CONCLUSION

M. aurae and *M. coerulea* exhibited potent antioxidant and mild carbohydrate-digesting enzyme inhibitory activity in *in vitro* assays. Both species are a significant source of rosmarinic acid, rutin, and caffeic acid, which are possibly responsible for these activities. Thus, *M. aurae* and *M. coerulea* can be used as potential natural antioxidant sources. Notably, *M. aurea* extracts should be studied for the development of herbal products with antidiabetic potential.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Comparative Seed Morphology, Pharmacognostic, Phytochemical, and Antioxidant Potential of *Memecylon* L. Fruits

Memecylon L. Meyvelerinin Karşılaştırmalı Tohum Morfolojileri, Farmakognostik, Fitokimyasal ve Antioksidan Potansiyelleri

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ABSTRACT

Objectives: *Memecylon* is the genus of the family Melastomataceae and their identification is somewhat difficult as a result of similarities in their intraspecies morphologies. Thus, identification of these species is much strenuous. The present study investigated the seed morphology, pharmacognostic, phytochemical, and antioxidant potential of *Memecylon* fruits.

Materials and Methods: The species *Memecylon grande, Memecylon randerianum* and *Memecylon umbellatum* were selected for this study. The surface morphology of seeds or fruits, pharmacognostic evaluation, and phytochemical analysis are effective methods for rectifying taxonomic difficulties in authentication process and it opens a platform for pharmaceutical analysis. The surface morphology was analyzed by scanning electron microscopy (SEM) and elemental compositions were revealed by using energy dispersive X-ray spectroscopy (EDX) and inductively coupled plasma-mass spectroscopy (ICP-MS). While evaluating the pharmacognostic property of the seeds, powder microscopic analysis was used, which focusesd on the functional purity of the fruit samples. 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, nitric oxide and superoxide radical scavenging assays are carried out to determine the antioxidant potential of *Memecylon* fruits.

Results: The surface morphology of seeds had a distinct pattern of topography. SEM and EDX studies gave an insight on the elemental composition of the seeds. ICP-MS analysis showed the presence of biologically potent trace elements in the fruit samples. Quantitative estimation of secondary metabolites, such as alkaloids, terpenoids, flavonoids, and phenols, revealed that fruit extracts possess a significant amount of these phytoconstituents. The present study also reveals the antioxidant potential of *Memecylon* fruit extracts.

Conclusion: Powder microscopy, SEM, EDX and ICP-MS analyses provided knowledge about the functional purity and elemental composition of *Memecylon* fruits samples. The free radical scavenging assays showed that *Memecylon* fruits were natural antioxidant sources. Therefore, these findings can be effectively targeted toward the pharmacological utilization of *Memecylon* fruits.

Key words: *Memecylon,* SEM, EDX, powder microscopy, secondary metabolites, antioxidants

ÖZ

Amaç: *Memecylon,* Melastomataceae ailesine ait bir cinstir ve türler arası morfolojilerin belirlenmesinin zor olması nedeniyle ayırt edilmeleri zordur. Bu nedenle, bu türlerin ayırt edilmeleri güçtür. Bu çalışmada *Memecylon* meyvelerinin karşılaştırmalı tohum morfolojileri, farmakognostik, fitokimyasal ve antioksidan potansiyelleri incelenmiştir.

Gereç ve Yöntemler: *Memecylon grande, Memecylon randerianum* ve *Memecylon umbellatum* türleri bu çalışma için seçilmiştir. Tohumların veya meyvelerin yüzey morfolojileri, farmakognostik değerlendirmeleri ve fitokimyasal analizleri belgelendirme sürecinde taksonomik zorlukları rektifiye etmek için etkin yöntemlerdir. Yüzey morfolojileri taramalı elektron mikroskopisi (SEM) ile analiz edilmiştir ve elementel bileşimleri, enerji dağıtıcı X-ışını spektroskopisi (EDX) ve indüktif eşleşmiş plazma-kütle spektroskopisi (ICP-MS) ile açığa kavuşturulmuştur. Tohumların farmakognostik özelliklerini değerlendirirken, toz mikroskobik analizi kullanılmış ve meyve örneklerinin fonksiyonel saflığına odaklanılmıştır. 2,2-difenil-1-pikrilhidrazil, hidroksil, nitrik oksit ve süperoksit radikal süpürücü deneyleri *Memecylon* meyvelerinin antioksidan etkilerini belirlemek için gerçekleştirilmiştir.

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Bulgular: Tohumların yüzey morfolojileri belirgin topografik karakter göstermiştir. SEM ve EDX çalışmaları tohumların elementel bileşimi hakkında bilgi vermiştir. ICP-MS analizi meyve örneklerinde biyolojik olarak potent eser elementlerinin varlığını göstermiştir. Alkaloidler, terpenoidler, flavonoidler ve fenoller gibi sekonder metabolitlerin kantitatif oarak değerlendirilmesi meyve ekstraktlarının belirgin miktarda bu fitobileşenleri içerdiğini göstermiştir.

Sonuç: Toz mikroskopisi, SEM, EDX ve ICP-MS analizleri *Memecylon* meyve örneklerinin fonksiyonel saflık ve elementel bileşimleri hakkında bilgi vermiştir. Serbest radikal süpürücü yöntemleri *Memecylon* meyvelerinin doğal antioksidan kaynakları olduğunu göstermiştir. Bu nedenle, bu bulgular *Memecylon* meyvelerinin farmakolojik olarak kullanımlarının etkin olarak hedeflenmesini sağlayabilecektir.

Anahtar kelimeler: *Memecylon,* SEM, EDX, toz mikroskopisi, sekonder metabolitler, antioksidanlar

INTRODUCTION

Green technology and alternative eco-friendly products are brand new to several.[1] New lifestyle changes cause many perilous drawbacks, which opens a gateway for the search of new resolves. Thus, at present, the term "Green" has become much popular. The major area under "Green" consideration will be the field of traditional or herbal medicine. Herbal medicines are safe remedies for various human ailments, given their minimal side effects and low-cost treatments. Thus, there is wide acceptance of herbal medicinal system. The quality measurements of herbs are challengeable streams, where validations of herbs are more important than their usage. Adulterations are drawbacks in the field of herbal medicine, as they cause inconsistences in quality and safety of herbal preparations. This has opened a new approach to validate the quality assurance of herbs. The collection of plant materials, authentication of specimens, analysis, and formulation of drugs are the majorly value chain toward the discovery of safer natural drugs. Here in, an attempt was made to evaluate the pharmacognostic characters of the medicinally important genus *Memecylon* fruits.

Memecylon is the genus of the family Melastomataceae. There are 289 species of shrubs and trees widely distributed in tropical regions. In India, about 40 species were reported and 21 among them are endemic to the country.[2] *Memecylon* species are difficult to identify due to the fact that their morphological similarities are some what confusing. *Memecylon umbellatum* and *Memecylon randerianum* are common species found in the Western Ghats. Another selected species for the present study is *Memecylon grande*, which is present in the Western Ghats and dry deciduous forests. Previous reports have revealed that these three species of *Memecylon* have potent medicinal activity.[3,4] *M. umbellatum* possesses elliptic-lanceolate leaves with umbellate inflorescence and yellow berries. *M. randerianum* possesses ovate-oblong leaves and blueberries, while *M. grande* has ovate-lanceolate leaves with brownish blackberries.

Many systematic studies and new records are available on the genus *Memecylon*; however, evaluations of micromorphological characters are trivial. Scanning electron microscopy (SEM) analysis is the best way to analyze the surface features of the samples. In taxonomic identification, analysis of vegetative and reproductive characters through SEM is an effective method.[5] The functional purity of plant samples is essential for pharmaceutical trails. In this present study, purity of sample was analyzed by powder microscopy, SEM-energy dispersive X-ray (EDX), and inductively coupled plasma-mass spectrometry (ICP-MS) techniques. Powder microscopy acts as an effective tool for authentication plant materials.[6] SEM-EDX and ICP-MS are effective analytical methods for analyzing metal nano- and micro-particles in food products, plant, or environmental.[7] The backbone behind the performance of plants always hinge on the presence of bioactive metabolites. In majority of pharmaceutical studies, phytochemical analysis is a crucial step. Preliminary phytochemical analysis gives an insight on the phytochemical constituents present in plant extracts. It comprises qualitative and quantitative analysis. It provides valid information regarding the presence or absence of bioactive compounds in plant species. A plethora of biochemicals contribute to the specific bioactivity of plants, such as antimicrobial, antioxidant, and anticancer properties, among others.[8] The present study also emphasizes the evaluation of the antioxidant potential of *Memecylon* fruit extracts. Antioxidants are free radical scavengers that neutralize oxidative stress induced by reactive oxygen species (ROS). Furthermore, ROS can disrupt normal cellular mechanisms and lead to severe pathological conditions and diseases, such as cancer, neurological disorders, atherosclerosis, hypertension, ischemia, and diabetes.[9] The present study focused on the worth and utility of micromorphological characters and functional purity of fruit samples as an additional source of information in systematic studies of *Memecylon.*

MATERIALS AND METHODS

Plant material

Fruits of *M. grande, M. randerianum*, and *M. umbellatum* were chosen for the current study. Ripened mature fruits of the three species were collected from various parts of Kerala, India. The collected fruits were identified by Dr. A. K. Pradeep Assistant Professor, Angiosperm Taxonomy Division, Department of Botany, University of Calicut, Kerala.

SEM analysis

SEM analysis was performed using ZEISS Gemini SEM 300 machine. Samples were prepared on a carbon-coated copper grid. The technical features of Gemini SEM 300 are given below.

Gemini SEM 300 with resolutions: 0.6 nm @ 30 kV (STEM), 0.7 nm @ 15 kv, 1.2 nm @ 1 kv, and 1.1 nm @ 1kV TD. Inlens BSE resolution: 1.2 nm @ 1 kV. Resolution in variable pressure mode (30 Pa): 1.4 nm @ 3 kV and 1.0 nm @ 15 kv. Acceleration voltage: 0.02 - 30 kV. Probe current: µ3 pA-20 Na. Magnification: 12-2,000,000.

SEM-EDX analysis

SEM-EDX analysis was done using Octane plus with Gemini 300/EDS. The active area selected for the present study was 30 mm^2.

Powder microscopy

The fruit powder characteristics of *Memecylon* was analyzed under bright field microscope. The powder of the sample was treated with 4% KOH and mounted in glycerine on clean slides and the powder characters were imaged using Nikon ECLIPSE E200 trinocular microscope attached with Zeiss AxioCam Erc5s digitalcamera.

ICP-MS analysis

ICP-MS analysis was performed using Agilent 7800 ICP-MS with Integrated Sample Introduction System (ISIS3) and SPS4 autosampler. It has astandard torch of 2.5 mm-diameter injector, as well as Ni sampler and Ni skimmer cones.

Preliminary quantitative phytochemical analysis

Alkaloid content

The method of Shamsa et al.[10] was followed for the determination of total alkaloid content of the selected plant material. In brief, 2 N HCl solution was prepared and 1 mg of the plant sample was dissolved in it and filtered. Phosphate buffer (pH 4.7) was prepared and 5 mL of it and 5 mL BCG solution was added and the mixture was shivered with 1, 2, 3, and 4 mL of chloroform. The chloroform layer containing alkaloids was separated. Caffeine was used as a standard. Absorbance of the solution was read at 470 nm against a blank. Alkaloid content of the sample was calculated and expressed as mg of caffeine equivalents.

Flavonoid content

Aluminium chloride colorimetric assay is a typical method for the determination of flavonoid content.[11] 4 mL of distilled water was put in aflask and 1 mL of the extracts was added into it. 0.30 mL of 5% $NaNO_2$ and 0.3 mL of 10% $AlCl_3$ were mixed in the flask at five minutes intervals. Again, after five minutes incubation, 2 mL of 1M NaOH was taken and the final volume of 10 mL was attained using distilled water. Finally, absorbance was read at 510 nm. The total flavonoid present in the extract was calculated and expressed as mg quercetin equivalents (QE).

Phenolic content

Phenolic content of the sample was assayed by Folin Ciocalteu assay.[12] 1 mL of the extracts was put in a 25 mL flask containing 9 mL of distilled water. 1 mL of Folin-Ciocalteu phenol reagent was mixed in the flask and shaken. At 5 minutes interval, 10 mL of 7% Na_2CO_3 solution was added and the solution was kept for 90 minutes at room temperature. Absorbance was read at 550 nm against the reagent blank. Gallic acid was taken as the standard. The phenolic content was expressed as mg of Gallic acid equivalents.

Terpenoid content

Presence of terpenoid of the plant samples was estimated by the method of Ghorai et al.[13] The reaction solution contains an aliquot of the extract and few drops of chloroform and H_2SO_4. Absorbance is calculated at 538 nm against the blank. Linalool was taken as the standard. The terpenoid content of the extract was calculated and expressed as mg linalool equivalents (QE).

Antioxidant activity

The antioxidant potential of *Memecylon* fruits was documented on the basis of free radicals produced by various substrates, such as 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), Fe^{3+}-ascorbate-EDTA-H_2O_2 system, sodium nitroprusside, and potassium ferricyanide.

DPPH radical scavenging assay

The protocol of Chang et al.[14] was followed for the determination of DPPH free radical scavenging activity of *Memecylon* fruit extract. DPPH is a free radical that reacts with antioxidant agents and gets reduced to DPPH-H. The pink colored DPPH turns yellow when scavenged by antioxidants. The color change indicates the scavenging latent of the antioxidant substances. Reference was selected as ascorbic acid [10 mg/mL dimethyl sulfoxide (DMSO)]. Different volumes of the extracts [1.25-20 µL (12.5-200 µg/mL)] from a stock concentration of 10 mg/mL was taken and made up to 20 µL with DMSO and 1.48 mL of DPPH (0.1 mM) solution. The control was a test compound-free solution. A dark condition of 20 minutes was maintained for the reaction mixture at room temperature. Then, the scavenging potential was read at 517 nm. Percentage inhibition of DPPH free radical by the extracts was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{control}-\text{test}}{\text{control}} \times 100$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging potential was analyzed according to the method described by Kunchandy and Rao.[15] Different concentration of the samples (125-2000 µg/mL) from a stock solution of 10 mg/mL was mixed with 500 µL reaction mixture [2-deoxy 2-ribose (2.8 mM), $FeCl_3$ (100 µm), EDTA (100 µm), H_2O_2 (1.0 mM), ascorbic acid (100 µm) in KH_2PO_4-KOH buffer (20 mM pH 7.4)] and made up to 1 mL. The control was a solution devoid of the test material. The test sample tubes were kept at 37°C for 1 hour. 1 mL of 2.8% TCA and 1 mL of 1% aqueous TBA was mixed in the reaction tubes and allowed to stay for 15 minutes at 90°C for the color change. Absorbance was read at 532 nm against a blank. Gallic acid (10 mg/mL DMSO) was used as reference. Percentage inhibition of hydroxyl radical by the extracts was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{control}-\text{test}}{\text{control}} \times 100$$

Nitric oxide radical scavenging activity

Determination of nitric oxide radical scavenging activity of *Memecylon* fruit extracts was performed according the method described by Kumaran and Karunakaran.[16] Sodium nitroprusside (5 m/mol/L^{-1}) in phosphate buffered saline solution (pH 7.4) was added into various concentrations of extracts (125-2000 µg/mL) from a stock solution. The reaction mixtures were then

incubated at 25°C for 30 minutes. A control was also prepared and incubated accordingly. After 30 minutes incubation, 1.5 mL of the reaction mixtures were removed and 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthylethylenediamine dihydrochloride) was added. Absorbance was read at 546 nm. Gallic acid (10 mg/mL DMSO) was used as a reference compound. Percentage inhibition of nitric oxide radical by the extracts was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{control}-\text{test}}{\text{control}} \times 100$$

Superoxide radical scavenging activity

Superoxide radical scavenging assay was performed according to the method described by Valentão et al.[17] A concentration range of (125-2000 µg/mL) was taken from a stock solution (10 mg/mL). The reaction tube contained 0.05 mL of riboflavin solution (0.12 mM), 0.2 mL of EDTA solution (0.1 M), and 0.1 mL of nitro blue tetrazolium solution (1.5 mM). 2.64 mL of phosphate buffer (0.067 M) was added to the reaction mixture. Absorbance was read at 560 nm at two illumination times of 5 minutes and 30 minutes, respectively. Ascorbic acid (10 mg/mL DMSO) was used as reference. Percentage inhibition of superoxide radical by the extracts was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{control}-\text{test}}{\text{control}} \times 100$$

RESULTS AND DISCUSSION

SEM is a high-resolution surface imaging technique that uses an electron beam with superior magnification power and depth of field precision. The seed capsule micromorphology and the entire seed morphology were studied by SEM analysis. The difference in electron emission in different areas provides the surface topography of the material. In this study, all the selected species showed distinct morphological patterns. Surface morphology is an important index in taxonomic identification.[18] SEM analysis of *Memecylon* fruits is a novel report. In the case of *M. grande,* fruits show a colliculate pattern, with a seed capsule of 6.2 mm, and the seed surface possesses a tuberculate pattern, with a width of 5.8 mm (Figure 1 A1, B1, C1, and D1). SEM analysis revealed that the seed capsule of *M. randerianum* a ruminate reticulate type pattern (Figure 1 A2, B2, C2, and D2). The width of the capsule was 4.2 mm. The seed surface of *M. randerianum* was of a reticulate pattern, with a width of 3.5 mm. *M. umbellatum* seed capsule possesses a smoothened pattern, with a width of 5.6 mm, and its seed surface showed a wrinkled pattern, with a width of 3.81 mm (Figure 1 A3, B3, C3, and D3). The characteristic surface morphology is a useful tool in the identification process.

EDX is an effective way of analyzing elemental compositions at tiny level. In this study, SEM was coupled with an energy dispersive system with an electron probe for X-ray microanalysis. The surfaces of the specimens were evaluated by SEM-EDX analysis and this gave a clear picture of the elemental composition of the test sample.[19] The elemental composition of *M. grande* fruits show that nitrogen content was 91%, while that of other elements were as follows: Phosphorus (3.10%), potassium (1.53%), iron (1.41%), magnesium (0.87%), and sodium (0.55%) (Figure 2). In the case of *M. randerianum* fruit, nitrogen was the prominent element, with 93% of the weight. Copper (0.49%), cobalt (0.90%), zinc (0.09%), sodium (0.03%), magnesium (0.22%), phosphorus (4.01%), potassium (1.15%), and calcium (0.09%) were the composition of other elements (Figure 3). *M. umbellatum* fruit also possesses an elevated amount of nitrogen (93%) and the other elements were in trace amount: Phosphorus (3.4%), potassium (1%), copper (0.95%), magnesium (0.67%), cobalt (0.34%), and iron (0.22%) (Figure 4). This finding indicates that *Memecylon* fruits are reservoirs of essential elements and can be exploited in the pharmaceutical or nutritional field.

In Ayurveda, 90% of the preparations are plant-based, implying that the worthwhile usage of herbal medicines are promising remedies for diverse human diseases. In most Ayurvedic preparations, the powdered samples of plant parts are used. Therefore, the authenticity of the powdered sample is very important. Powder microscopy is a simple and easier method to analyze powdered samples and is an essential step in pharmacognostic evaluation of plant samples. Microscopic techniques examine the structural and cellular features of herbs, so as to determine their botanical origin. Microscopic

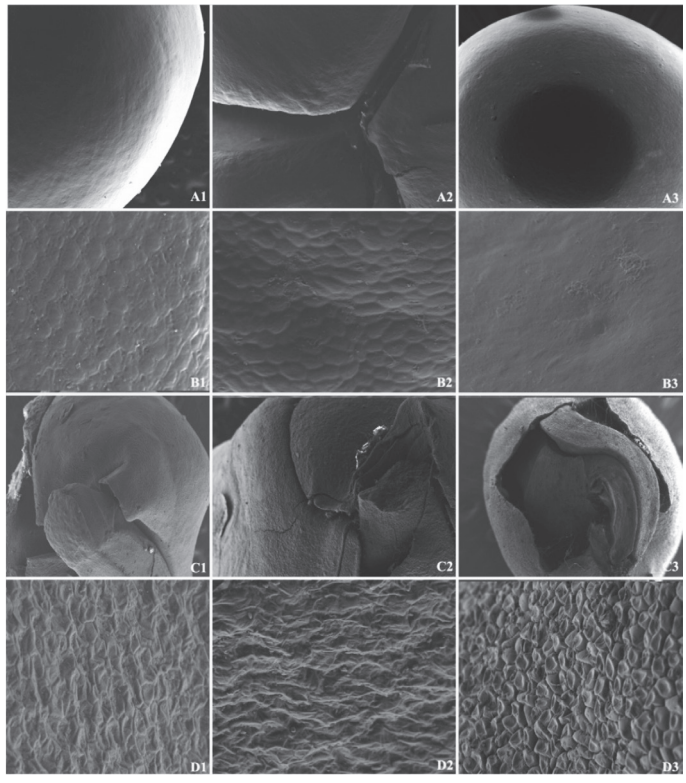
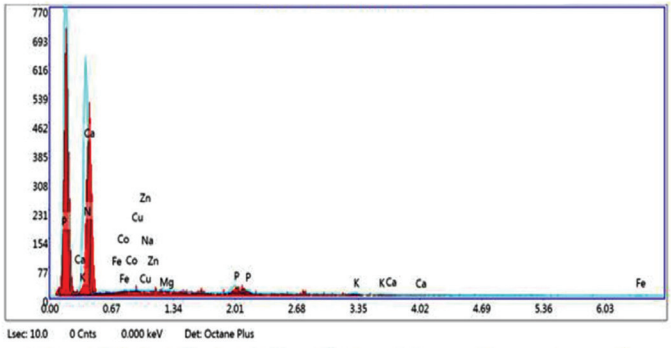


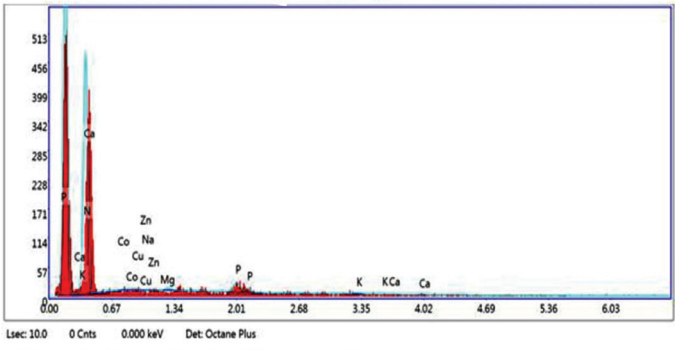
Figure 1. Scanning electron microscopic analysis of *Memecylon* fruits. A1) *Memecylon grande* seed capsule, B1) enlarged view, C1) seed surface, D1) Enlarged view. A2) *Memecylon randerianu*m seed capsule, B2) enlarged view, C2) seed surface, D2 enlarged view. A3) *Memecylon umbellatum* seed capsule, B3) enlarged view, C3) seed surface, D3) enlarged view

evaluation is now an indispensable tool for the identification of medicinal herbs and is among the important parameters in this modern time.[20] Here in, the powdered samples of *Memecylon* fruits were characterized by their microscopic characters. The powdered *M. grande* fruits was brown in color, odorless, and slightly astringent (Figure 5). The characters found in the



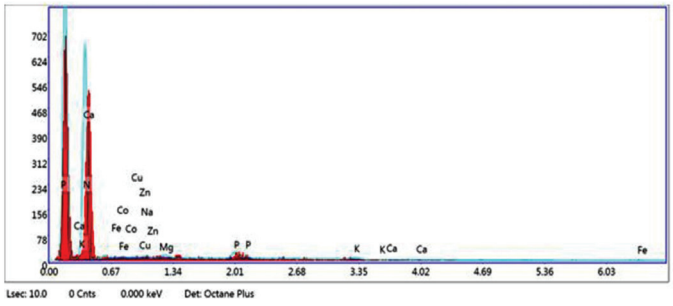
Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
N K	93.07	97.14	352.96	5.86	0.7757	1.0137	0.8222	1.0000
FeL	0.22	0.06	0.27	99.99	0.0009	0.7345	0.5352	1.0000
CoL	0.34	0.08	0.58	99.99	0.0017	0.7179	0.6777	1.0000
CuL	0.95	0.22	2.47	87.46	0.0054	0.7059	0.8034	1.0000
ZnL	0.11	0.02	0.30	99.99	0.0007	0.7054	0.8850	0.9996
NaK	0.02	0.01	0.10	99.99	0.0001	0.8829	0.6657	1.0014
MgK	0.67	0.41	4.65	75.71	0.0048	0.8943	0.7964	1.0024
P K	3.40	1.61	17.74	29.96	0.0278	0.8363	0.9699	1.0066
K K	1.00	0.37	2.78	75.64	0.0082	0.7962	1.0058	1.0263
CaK	0.22	0.08	0.47	99.99	0.0018	0.8074	1.0069	1.0336

Figure 2. SEM-EDX analysis of *Memecylon grande* fruits
SEM: Scanning electron microscopy, EDX: Energy dispersive X-ray



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
N K	93.02	97.14	265.63	6.41	0.7540	1.0139	0.7994	1.0000
CoL	0.90	0.22	1.14	92.04	0.0043	0.7180	0.6575	1.0000
CuL	0.49	0.11	0.95	99.99	0.0027	0.7060	0.7821	1.0000
ZnL	0.09	0.02	0.20	99.99	0.0006	0.7055	0.8732	0.9995
NaK	0.03	0.02	0.10	99.99	0.0002	0.8830	0.6427	1.0014
MgK	0.22	0.13	1.17	82.21	0.0015	0.8944	0.7806	1.0026
P K	4.01	1.89	16.70	30.68	0.0327	0.8365	0.9688	1.0065
K K	1.15	0.43	2.58	77.90	0.0095	0.7963	1.0061	1.0249
CaK	0.09	0.03	0.15	99.99	0.0007	0.8076	1.0071	1.0332

Figure 3. SEM-EDX analysis of *Memecylon randerianum* fruits
SEM: Scanning electron microscopy, EDX: Energy dispersive X-ray



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
N K	90.98	96.26	371.42	6.21	0.7249	1.0182	0.7826	1.0000
FeL	1.41	0.37	1.99	77.04	0.0055	0.7379	0.5306	1.0000
CoL	0.63	0.16	1.18	99.99	0.0030	0.7212	0.6641	1.0000
CuL	0.48	0.11	1.38	99.99	0.0027	0.7092	0.7867	1.0000
ZnL	0.11	0.03	0.34	90.61	0.0007	0.7087	0.8740	0.9996
NaK	0.55	0.36	3.02	95.61	0.0032	0.8870	0.6530	1.0013
MgK	0.87	0.53	6.70	69.73	0.0061	0.8985	0.7839	1.0022
P K	3.10	1.48	18.42	29.52	0.0253	0.8403	0.9655	1.0070
K K	1.53	0.58	4.86	71.10	0.0126	0.8001	1.0055	1.0260
CaK	0.35	0.13	0.86	90.39	0.0030	0.8114	1.0060	1.0326

Figure 4. SEM-EDX analysis of *Memecylon umbellatum* fruits
SEM: Scanning electron microscopy, EDX: Energy dispersive X-ray

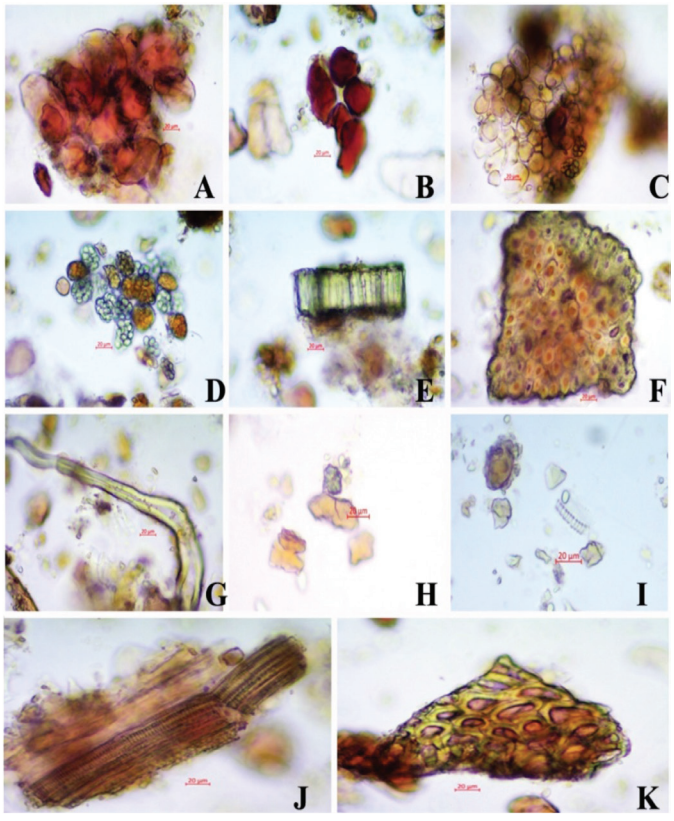


Figure 5. Powder microscopic analysis of *Memecylon grande* fruits. A, B) Epicarp cells; C, D) mesocarp parenchyma cells with starch grains; E) transversely cut testa; F) sclereidal fiber; G) rosette crystal; H) spiral vessels; I) sclereids from endocarp; J) annular vessels; K) testa in surface view

powders were epicarp cells, parenchyma cells with starch grains from mesocarp, stone cells from mesocarp, sclereids from endocarp, vessels with spiral and annular thickenings, and rosette crystals. In the case of *M. randerianum* fruits powder, it is brown-colored, odorless with a characteristic taste (Figure 6), and contains epicarp cells, mesocarp parenchyma cells, stone cells, sclereids from endocarp, tracheids, fiber bundles, and rosette crystals. The same brown colored powder was also seen for *M. umbellatum* fruits (Figure 7), which showed characteristics, such as epicarp cells, pitted parenchyma cells from mesocarp, stone cells, sclereids, spiral vessels, fibro-sclereids, and rosette crystals. These characters can be used to identify authenticated plant specimens in Ayurvedic preparations. Therefore,we can easily identify the botanical origin of the plant specimen and clearly distinguish the presence of adulterants or allied species. Microscopic evaluation of *M. umbellatum* leaves was done according to the method described by Killedar et al.[6] and the presence of lignified xylem with well-defined xylem fibers, vessels, and parenchyma was found. The presence of phloecentric vascular bundles surrounded by endodermis and crystal sheath was also reported. The powder microscopic analysis confirmed that the botanical origins of the plant samples were pure and devoid of foreign particles. Therefore, this result can be used as a future reference for identification of *Memecylon* fruits.

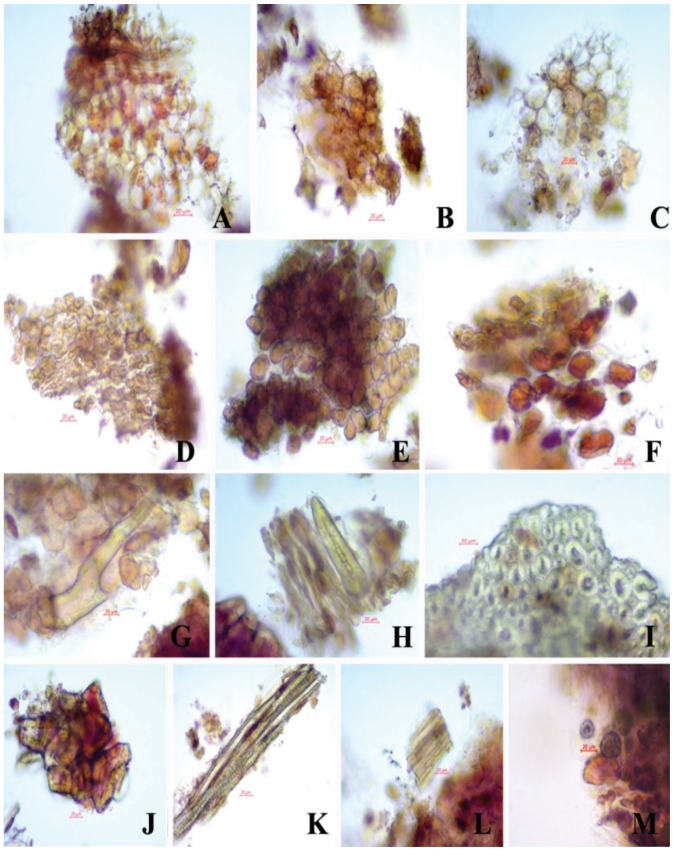


Figure 6. Powder microscopic analysis of *Memecylon randerianum* fruits. A) Mesocarp in sectional view; B) epicarp in surface view; C-F) mesocarp cells; G, H) sclereids; I, J) stone cells; K) tracheids; L) fiber bundles; M) rosette crystals

During the past decades, much attention have been focused on nutritional status. We had an insight into the profound effect of micro and macronutrients on biological processes that range from whole-organism performance to cellular function. According to the classification of trace elements, group I, which consists of carbon, hydrogen, oxygen, and nitrogen, are the key building blocks of carbohydrates, proteins, and lipids. Group II includes nutritionally important minerals, such as sodium, potassium, phosphorous, chloride, calcium, magnesium, and sulfur. Trace elements, such as like copper, iron, zinc, chromium, cobalt, iodine, molybdenum, and selenium, are found in group III.[21] Copper is important in metabolism, mainly in the proper functioning of the enzymes and its deficiency can lead to hypochromic anemia, joint pain neutropenia, hypopigmentation of hair and skin, abnormal bone formation with skeletal fragility, and osteoporosis, among others.[22] Another most important element is iron, which is a prime portion of the blood cells and its deficiency is called anemia. Anemia is the second vital cause of maternal mortality in India and 20% of the mortality is directly related to anemia, while another 50% is associated with other anemic side effects. In the case of zinc, it is crucial for normal spermatogenesis and maturation, development of thymus, epithelialization in wound healing, taste sensation, and secretion of pancreas and gastric enzymes.[23]

In addition to the SEM-EDX analysis, to substantiate the quality of the fruit samples in their elemental diversity, ICP-

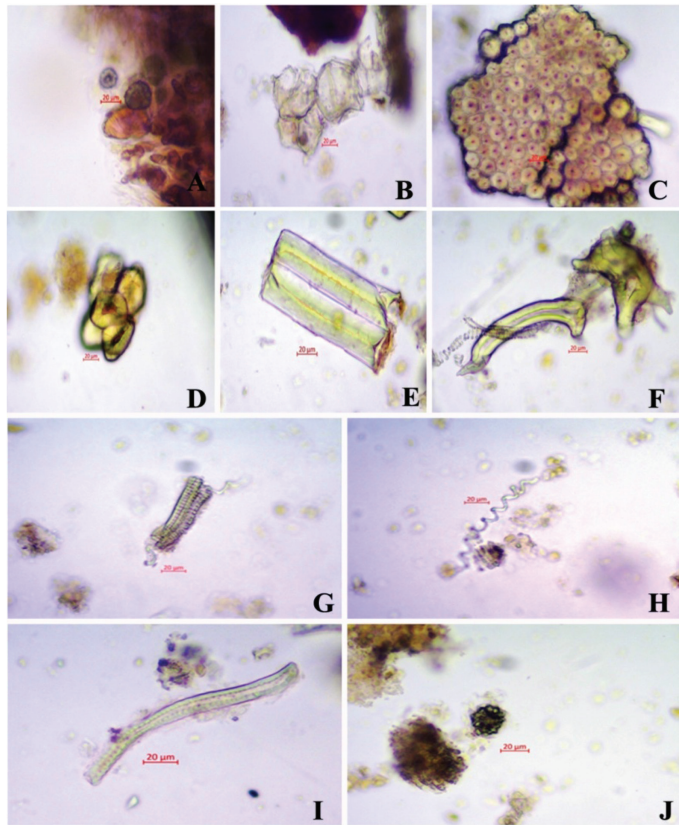


Figure 7. Powder microscopic analysis of *Memecylon umbellatum* fruits. A) Epicarp cells; B) pitted parenchyma cells of mesocarp; C) testa in surface view; D) stone cells; E, F) sclereids; G, H) spiral vessels; I) fibro-sclereid; J) rosette crystals

MS analysis was performed. This technique gave details of the elements present in the sample in parts per million and determination ofthirteen elements, including aluminum, arsenic, cadmium, cobalt, strontium, selenium, chromium, copper, molybdenum, nickel, lead, barium, and manganese, were done. *M. grande* fruits show a promising concentration of majority of the elements, except for molybdenum and lead. Lead and molybdenum concentration were found to be higher in the case of *M. randerianum* fruit (Table 1). The standard reference concentrations of trace elements present in adult human blood samples are considered, considering that it is essential for the standardization of drugs. Most of the detected elements show vital biological functions. Some elements are functional parts of vitamins, cofactors of enzymes, oxidative phosphorylation, fatty acids, and cholesterol metabolism. Lead is considered as a non-essential elemental part of the human body, since it has no biological or health roles. In the case of chromium, it acts as a modulator of enzymes and as an activator of fatty acid and cholesterol metabolism. It can also enhance sugar metabolism.[24] In the case of cobalt, it is the key factor of cobalamin (vitamin B12) and plays a vital role in the production of amino acids and neurotransmitters. Although the biological function of nickel is still somewhat unclear, nickel is found in higher concentration in RNA and is involved in protein structure.[25] Therefore, the biological role of these trace elements is significant in the maintenance homeostasis and avoidance of free radical proliferation induction and various human ailments.[26]

Preliminary quantitative analysis of various phytochemicals was done following the proposed protocols. *M. grande* fruit was found to be rich in alkaloids, flavonoids, phenolics, and terpenoids (Table 2). All these phytoconstituents have a significant biological role. Liu[27] proposes that phytochemicals are non-nutrient compounds that can reduce the risk of major non-communicable chronic diseases and that are commonly found in fruits, vegetables, grains, and other plant foods. Alkaloids have a wide spectrum of pharmacological activity,[28] which includes antifungal, antihyperglycemic, antityrosinase, antiglucosidase, antinociceptive, and antiinflammatory activities, among others. The efficiency of bioactive products in curing several diseases, such ashyperlipidemia, atherosclerosis, and hypertension, were studied by Liwa et al.[29] Polyphenols and phenolic compounds are full sources of vascular vasodilators and are used to cure hypertension and cardiovascular diseases. Flavonoids possess anti-inflammatory, anticancerous, and antimutagenic activities, which have been previously reported.[30] Tan et al.[31] confirms that the progressive use of phytochemicals through diets is an effective method to cure diseases. *Memecylon* fruit extract showed a promising antioxidant activity in four different assays. All the fruit extracts show a dose gradient scavenging activity.

Table 1. ICP-MS analysis of *Memecylon* fruits

Element	Mass	Concentration (ppm)			Reference concentration of trace elements in adult human blood (µg/L)[20]
		MGF	MRF	MUF	
Al	27	83135.864	41909.731	38739.426	2-8
Cr	52	10.223	5.923	4.016	<5
Mn	55	1790.173	786.657	272.022	8-12
Co	59	1.658	0.627	0.709	5-10
Ni	60	46.030	9.316	30.256	1-5
Cu	63	191.231	69.677	85.967	800-1100
Zn	66	320.408	119.412	118.666	6000-7000
As	75	1.620	1.048	0.958	2-20
(As)	77	19.861	5.952	5.418	2-20
(As)	78	25.097	9.117	8.453	2-20
Se	82	14.152	2.834	0.337	90-130
Sr	88	880.178	148.725	275.348	1.5-3.9
Mo	95	0.843	18.423	3.921	1-3
Cd	111	1.013	0.364	0.304	0.3-1.2
Ba	137	451.711	111.353	105.197	0.5-2.5
(Pb)	206	24.184	58.300	17.053	50-150
(Pb)	207	24.904	59.807	17.365	50-150
Pb	208	24.558	63.138	17.170	50-150

1 µg/L: 0.001 ppm. ICP-MS: Inductively coupled plasma-mass spectroscopy, MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

Among the extracts, *M. grande* fruit extract showed the highest free radical scavenging activity in all the assays, followed by *M. umbellatum* and *M. randerianum* fruit extracts (Figure 8-11). *M. grande* fruit extracts showed that highest nitric oxide scavenging activity (76.85±0.08) and the least hydroxyl radical scavenging activity (61.69±0.56). *M. randerianum* fruit showed the least hydroxyl radical scavenging activity (46.16±0.17). Based on the four assays performed, the most effective antioxidant extracts was that of *M. grande* fruit, with an IC_{50} of 83.9195±0.14. This was followed by *M. umbellatum,* with an IC_{50} of 91.1031±0.12, and *M. randerianum*, with an IC_{50} of 104.178±0.13 (Table 3). Antioxidant capacity and total phenolic content are directly related.[32] The quantitative phytochemical analysis justifies the high performance of these species in the antioxidant assays. *M. grande* fruit extract (370.28±8.36) possessed the highest phenolic content, making it a good candidate pharmaceutical agent.

Study limitations

The detailed phytochemical characterization and compound isolation can be more satisfactory in pharmaceutical applications. The present findings are useful in the pharmaceutical field, since the botanical purification of plants is the prime step in natural drug formulation. These results can be used as a future reference for the evaluation of *Memecylon* fruits.

CONCLUSION

Seed surface characteristics are used as diagnostic tools in taxonomic studies. Powder microscopy, SEM-EDX, and ICP-MS analysis have provided knowledge about the functional purity and elemental composition of *Memecylon* fruits samples. The

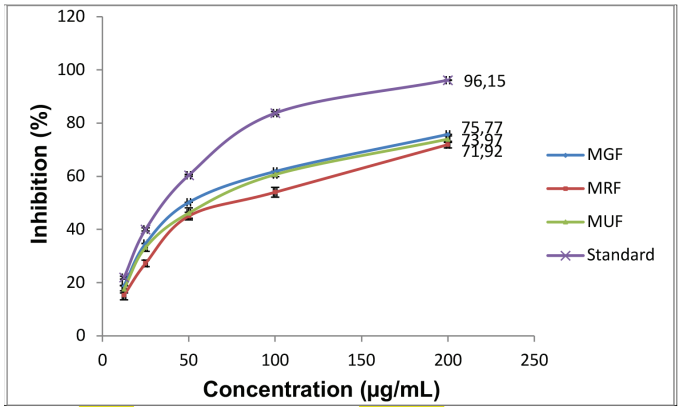


Figure 8. *In vitro* DPPH scavenging activity of *Memecylon* fruits

DPPH: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl, MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatu*m fruit

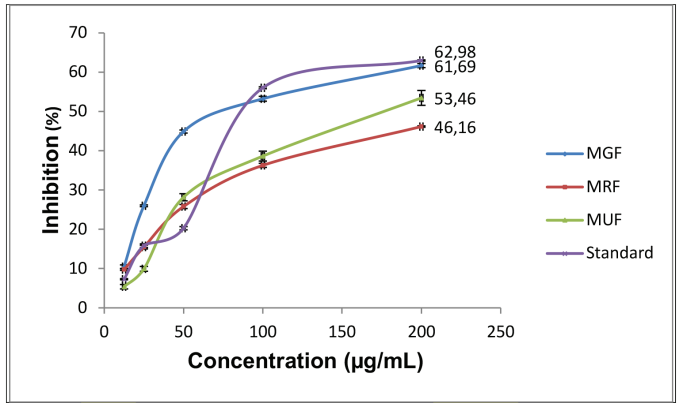


Figure 9. *In vitro* hydroxyl radical scavenging activity of *Memecylon* fruits

MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

Table 2. Preliminary quantitative phytochemical analysis of *Memecylon* fruits

Plants	Alkaloids (mg caffeine/g DW) ± SE	Flavonoids (mg quercetin/g DW) ± SE	Phenolics (mg GAE/g DW) ± SE	Terpenoids (mg linalool/g DW) ± SE
MGF	52.16±3.23^{d}	91.77±2.65^{c}	370.28±8.36^{d}	378.21±19.02^{c}
MRF	32.17±1.41^{a}	21.40±2.72^{b}	276.06±14.12^{c}	355.03±57.31^{c}
MUF	36.47±0.66^{ab}	57.57±4.40^{b}	60.83±5.70^{a}	127.5±10.50^{a}

Means within a column followed by the same letters are not significantly different at $p<0.05$, as determined by Duncan's multiple range test.
SE: Standard error, DW: Dry weight, GAE: Gallic acid equalent, MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

Table 3. Effect of methanol extracts of *Memecylon* fruits in different antioxidant assays

IC_{50} values (µg/mL)

Plants	DPPH radical scavenging assay	Hydroxyl radical scavenging assay	Nitric oxide radical scavenging assay	Super oxide radical scavenging assay
Standard	48.8412±1.5^{a}	1347.51±0.27^{b}	346.207±0.01^{a}	238.357±0.03^{a}
MGF	83.9195±0.14^{b}	1231±0.48^{a}	696.733±0.06^{b}	698.991±0.03^{b}
MRF	104.178±0.13^{d}	2029.57±0.14^{e}	1081.61±0.01^{d}	311.24±0.02^{e}
MUF	91.1031±0.12^{c}	1696.73±0.05^{c}	916.988±0.04^{c}	1129.34±0.01^{c}

Means within a column followed by the same letters are not significantly different at $p<0.05$ as determined by Duncan's multiple range test. DPPH: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl, Correct it as MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

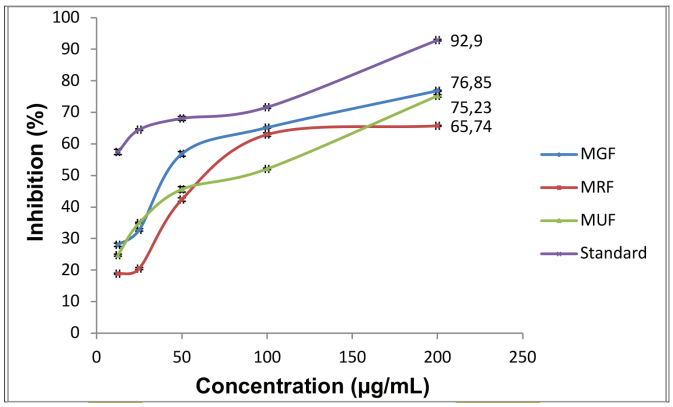


Figure 10. *In vitro* nitric oxide radical scavenging activity of *Memecylon* fruits

MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

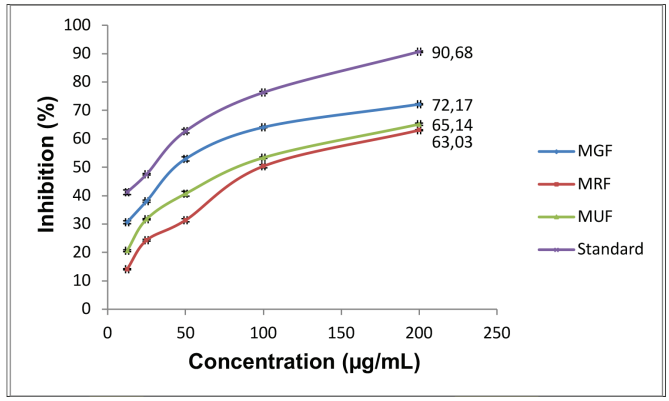


Figure 11. *In vitro* super oxide radical scavenging activity of *Memecylon* fruits

MGF: *Memecylon grande* fruit, MRF: *Memecylon randerianum* fruit, MUF: *Memecylon umbellatum* fruit

free radical scavenging assays provide an acquaintance of a natural antioxidant source. Therefore, these findings can be effectively targeted toward the pharmacological utilization of *Memecylon* fruits.

The present investigation focuses on the seed morphology, pharmacognostic, phytochemical, and antioxidant potential of *Memecylon* fruits. *M. grande, M. randerianum and M. umbellatum* are the selected experimental plant materials. Powder microscopy, SEM-EDX and ICP-MS analysis were carried out to analyze the surface features and elemental composition of the *Memecylon* fruits. The antioxidant potential of fruit samples are analyzed by DPPH, hydroxyl, super oxide and nitric oxide radical scavenging assays. The analysis of morphological features are key diagnostic tools in taxonomic studies. In the present study SEM analysis of fruit samples gave valuable results in species identification of *Memecylon.* The each sample shows distinct surface morohology. In ICP-MS analysis, elemental composition of fruit samples reveals the diverse elemental presence in the sample. It may helpful in pharmaceutical field for valuable medicine preparations. In the four antioxidant assays, all the selected extracts shows promising free radical scavenging activity. So all these results pointing that selected *Memecylon* fruit samples become a potential contributor in the pharmaceutical field.

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ORIGINAL ARTICLE

In Vitro Caco-2 Cell Permeability Studies of Ziprasidone Hydrochloride Monohydrate Nanocrystals

Ziprasidon Hidroklorür Monohidrat Nanokristallerinin Caco-2 Hücre *İn Vitro* Geçirgenlik Çalışmaları

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ABSTRACT

Objectives: The current study focused on the evaluation of the cytotoxic effect and permeability of ziprasidone hydrochloride monohydrate (ZHM) nanocrystals on Caco-2 cells.

Materials and Methods: ZHM nanocrystals were prepared by the microfluidization method in the presence of polyvinylpyrrolidone as a stabilizer. Particle size (PS), particle size distribution (PDI), and zeta potential (ZP) values were measured in characterization studies. *In vitro* cytotoxic effects of ZHM nanocrystals were investigated using the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Caco-2 transport studies were conducted with formulations of ZHM coarse powder and nanocrystals.

Results: Nanocrystals were obtained with 400-600 nm PS, 0.1-0.4 PDI, and >20 mV ZP values. The cell viability remained 100% for all sample groups. The permeability value of ZHM nanocrystals through Caco-2 cells increased 2.3-fold in comparison with ZHM coarse powder. Cumulative drug transport also increased at the end of the sampling period.

Conclusion: Nanocrystal technology helps to increase the permeability of drug particles by increasing the saturation solubility.

Key words: Caco-2 cells, permeability, ziprasidone, nanocrystal

ÖZ

Amaç: Bu çalışma, ziprasidone hidroklorür monohidrat (ZHM) nanokristallerinin Caco-2 hücreleri üzerindeki sitotoksik etkisini ve geçirgenliğini değerlendirmeye odaklanmıştır.

Gereç ve Yöntemler: ZHM nanokristalleri, stabilizan olan polivinilpirolidon varlığında mikrofluidizasyon yöntemi ile hazırlanmıştır. Partikül büyüklüğü (PB), partikül büyüklüğü dağılımı (PBD) ve zeta potansiyel (ZP) değerleri karakterizasyon çalışmalarıyla ölçülmüştür. ZHM nanokristallerinin *in vitro* sitotoksik etkisi 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide testi ile belirlenmiştir. Caco-2 hücreleri taşınma çalışmaları, ZHM kaba tozu ve nanokristal formülasyonları ile gerçekleştirilmiştir.

Bulgular: ZHM nanokristalleri 400-600 nm PB, 0,1-0,4 PBD ve >20 mV ZP değerleri ile elde edilmiştir. Tüm çalışma gruplarında hücre canlılığı %100 kalmıştır. Caco-2 hücrelerinden geçiş çalışmalarında, ZHM nanokristalleri, permeabilite değerini ZHM kaba tozuna kıyasla 2,3 kat artırmıştır. Aynı zamanda kümülatif ilaç taşınımı örnek alma süresinin sonunda yükselmiştir.

Sonuç: Nanokristal teknolojisi doygunluk çözünürlüğünü artırmasına bağlı olarak permeabiliteyi artırmada yarar sağlayabilir.

Anahtar kelimeler: Caco-2 hücreleri, geçirgenlik, ziprasidon, nanokristal

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INTRODUCTION

New drug candidates have poor water solubility, which limits absorption and results in low bioavailability.[1] Saturation solubility depends on the radius of the particles according to the Ostwald-Freundlich equation (equation 1).[2,3]

$$\mathrm{Ln}\left(\frac{S}{So}\right) = 2\,M\,\gamma\,/\,\rho\,r\,R\,T \qquad \text{equation 1}$$

Where S is the solubility, r is the radius of the particles, S_o is the normal solubility value (plane surface), M is the molecular weight, γ is the interfacial tension, ρ is the density, R is the gas constant, and T is the temperature (Kelvin).

Reducing particle size (PS) to the nanometer range can increase the surface area, which leads to an increase in kinetic saturation solubility and dissolution velocity according to the Kelvin and Noyes-Whitney equations. Membrane penetration and, finally, enhanced bioavailability can be achieved by PS reduction.[4,5]

Nanocrystals are 100% drug molecules with a PS <1000 nm (typically 200-600 nm).[6] They consist of a minimum amount of stabilizer, such as a polymer and/or surfactants.[7] Because of the advantage with regard to PS, they increase the saturation solubility and hence the permeability and dissolution rate of the drug component, which increases the bioavailability.

Ziprasidone hydrochloride monohydrate (ZHM) is a Biopharmaceutical Classification system (BCS) class II antipsychotic drug with low water solubility and high permeability.[8] The absorption of ZHM is affected by the presence of food.[9] The dissolution-rate-limited performance causes a highly variable bioavailability and absorption profile, which is affected by the fed/fasted state of the patient.[10] Preparing nanocrystals of ZHM can increase the saturation solubility and hence the dissolution rate, which results in elimination of the food effect due to drug absorption and enhances the permeability and bioavailability of ZHM.[11-13]

Intestinal drug absorption is affected by the permeability of drugs, and there are several methods for investigating permeability during the drug development process.[14] One of the methods uses cultured monolayers of suitable cells, which is recommended by the food and drug administration for determining drug substance permeability. The Caco-2 human colon epithelial cancer cell line is used as an *in vitro* model to predict drug permeability. Caco-2 cells are differentiated to mimic the small intestinal epithelium when cultured as monolayers under conventional culture conditions.[15] An excellent correlation has been shown between *in vivo* absorption and *in vitro* apparent permeability obtained from a Caco-2 cell model.[15,16] Besides compound screening in high throughput format during the discovery phase, a Caco-2 model can be used to investigate the formulation effect on permeability. Some excipients can compromise tight junction integrity or cause changes in the efflux system and increase permeability;[17] therefore, it is important to investigate the permeability of formulations during drug development.

Previous studies showed that saturation solubility was dramatically enhanced with nanocrystal formulations of ZHM,[18] and an orally disintegrating tablet form was developed successfully.[19] This study focused on the *in vitro* cytotoxicity as well as the Caco-2 cell permeability of ZHM nanocrystals, and the effect of PS on permeability was investigated.

MATERIALS AND METHODS

Materials

ZHM was a kind gift from Abdi İbrahim Pharmaceuticals (İstanbul, Turkey). Polyvinylpyrrolidone K30 (PVP K30) was purchased from Sigma Aldrich (USA). Human epithelial colorectal adenocarcinoma cells (Caco-2, ATCC HTB 37) were obtained from the ATCC. Hank's balanced salt solution (HBSS), 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma Aldrich (USA). Other chemicals for high-performance liquid chromatography (HPLC) were of analytical grade.

HPLC analysis of ZHM

The ZHM concentration was determined using HPLC. An Agilent 1200 HPLC (Agilent Technologies, California, USA) system equipped with an autosampler and a ultraviolet (UV)-visible detector was used for this purpose. The concentration range was 12-20 µg/mL. The analytical column used for sample separation was an ODS C18 (RP) column (150 mmx4.6 mm, 5 µm) (TSKgel). The mobile phase consisted of potassium dihydrogen phosphate (6.8 g/L and with pH 3.0 o-phosphoric acid) 10:90 (v/v). The flow rate was 1 mL/min, the injection volume was 20 µL, and the column temperature was 25°C. The detection wavelength was 229 nm, and the retention time was 2.4 minutes.

Preparation of ZHM nanocrystals

ZHM nanocrystals were prepared according to a previous study.[18] The microfluidization technique (Microfluidics LV1, Microfluidizer® Processors, USA) was used to obtain nanocrystals. For this purpose, 0.5% (w/w) ZHM was dispensed into the 0.5% (w/w) PVP K30 solution. Macro suspensions were stirred with a homogenizer (Ultraturrax, Heidolph, Germany) at 15,000 rpm for 10 minutes. The microfluidization method was performed at 30 000 psi pressure for 20 cycles. PS, PS distribution, and zeta potential (ZP) values were measured using Malvern ZetaSizer (Malvern Instruments, UK).

The ZHM amount in ZHM nanocrystals was analyzed with a validated UV spectrophotometric method. Lyophilized nanocrystals were dissolved completely in methanol, filtered through 0.45 µm membrane filter, and then measured at wavelength of 314 nm.

Cell culture studies

Cell culture studies were conducted on Caco-2 cells to determine the permeability values of the ZHM coarse powder and ZHM nanocrystals. The effect of the sample groups on Caco-2 cell viability was investigated with an MTT test.

Preparation of Caco-2 cells

Firstly, Caco-2 cells were removed from a -180°C nitrogen tank and thawed in a water bath at 37°C. The Caco-2 cells in the vial were transferred into 15 mL of culture media under laminar flow. The media was centrifuged for 3 minutes at 2,000 rpm, and the supernatant was removed. The precipitated cells were re-suspended and transferred to 25 cm^2 flasks. The cells were incubated at 37°C, in air containing 5% CO_2, in DMEM with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 IU/mL penicillin and 50 μg/mL streptomycin. The media was changed every other day. After 6-7 days, when the cell frequency reached 80%-90%, the cells were removed by treatment with trypsin-EDTA (0.25%) and transferred to new flasks. Cells were used for permeability studies at passage 24.

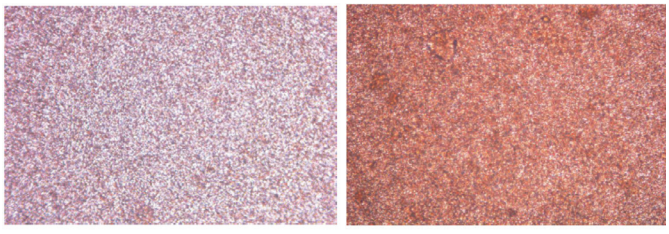
Transfer of Caco-2 cells into inserts

After 24 passage cycles, cells were counted using microscopy with a hemocytometer using trypan blue. The suspended cells were seeded into 6-well plate inserts (Snapswell™), 6 well, 0.4 μm pore diameter) at a density of 70,000 cells per well. Media (1.5 mL and 0.5 mL) were placed into the basolateral and apical sides, respectively. The culture medium was changed every other day for 21 days. Images of the cells in the culture media were acquired with an inverted microscope (Figure 1).

In vitro cytotoxicity

The MTT cell viability test was executed to investigate the effect of the coarse powder of ZHM and nanocrystals on Caco-2 cells and to determine the concentrations to be applied in the permeability study.[20] Caco-2 cells were seeded into 96-well plates at a density of 5,000 cell/well. The ZHM coarse powder and ZHM nanocrystals were dissolved in dimethyl sulfoxide (DMSO) or dispersed in culture media (DMEM) and added into the 96-well plates. DMSO (0.4%) was added as a control group. The plates were incubated at 37°C, in air containing 5% CO_2 for 4 hours, which was the duration for permeability studies. After the incubation period, 25 μL of MTT (5 mg/mL) solution was added to the wells, and the plates were incubated for a further 4 hours. Then, the media in the wells was removed, 200 μL of DMSO was added to each well, and the absorbance was read using a microplate reader at 570 nm to measure optical density. Cell viability was calculated according to equation 2.

$$\text{Cell viability (\%)} = (Abs_{test}/Abs_{control}) \times 100 \qquad \text{equation 2}$$



(a) (b)

Figure 1. Photo of the inserts taken with an inverted microscope. (a) Empty inserts; (b) Caco-2 cells

Evaluation of the monolayer integrity of the cells

The transepithelial electric resistance (TEER) of the Caco-2 cell monolayer was measured with a Millicell-ERS voltohmmeter. Monolayers with TEER >300 Ω.cm^2 were used for permeability studies.[21]

TEER values were calculated according to equation 3, where R_{sample} is the resistance of inserts, which contain cells; R_{empty} is the resistance of inserts, which are empty; a is the surface area of the cell culture inserts (cm^2).

$$TEER_{cell\ layer} = (R_{sample} - R_{empty}) \times A \qquad \text{equation 3}$$

In vitro permeability

HBSS buffer with 10 mM Hepes (pH 7.4) was used for in vitro permeability studies. The samples were prepared in 0.4% DMSO containing HBSS. Stock ZHM concentration of the formulations was 100 μg/mL. The other concentrations were diluted from stock solution. The stock concentration was selected according to the oral ZHM dose.

Firstly, the culture media in the basolateral and apical sides was removed, and these compartments were treated with HBSS. After 30 minutes of incubation, all of the medium was removed. A 0.5 mL aliquot of the sample was placed into the apical compartment, and 1.5 mL of the HBSS buffer was placed into the basolateral compartment (n=6). The plates were incubated at 37°C, at 60 rpm, for 4 hours, and samples were taken at intervals of 30, 60, 90, 120, and 240 minutes. At each sampling time point, 0.5 mL of sample was withdrawn, and fresh HBSS buffer was added to the basolateral side. The samples were analyzed using a validated HPLC method. The apparent permeability coefficient (P_{app}, cm/s) was calculated according to equation 4.

$$P_{app} = dC/dt \times 1 / (A \times C_0) \qquad \text{equation 4}$$

Where dC/dt is the drug permeation rate (μg/s); a is the surface area of the inserts (cell monolayer) (cm^2); and C_0 is the initial concentration at the apical side (μg/mL).

Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance, followed by Tukey's post-hoc test using SPSS statistics, Version 20 (Armonk, NY: IBM Corp.). The significance level was selected as $p<0.05$. The results were express as means ± standard deviation.

RESULTS AND DISCUSSION

Preparation of nanosuspensions

Microfluidization is one of the top-down methods of producing nanocrystals. The main advantages of this method are the repeatability of experiments, ease of scale up, and relatively fewer process validation parameters.[22]

In this study, ZHM nanocrystals characterized by a 532.4±13.7 nm PS, 0.304±0.01 PS distribution, and 20.5±0.3 mV ZP values were used for cell culture studies. The ZHM amount was found to be 95% ± 5% for the nanocrystals according to the validated UV spectrophotometric method.[18]

Cell culture studies

Caco-2 cell viability

An MTT assay was conducted to understand the *in vitro* toxicity of the coarse ZHM and ZHM nanocrystals. Cell viability (%) was determined after the interaction between Caco-2 cells and ZHM formulations for 4 hours. ZHM coarse powder or ZHM nanocrystals were not cytotoxic, even at the highest dose, which was 200 µg/mL. DMSO 0.4% was used to dissolve ZHM particles and applied to the cells as a control group. No cytotoxic effect was observed for DMSO at this concentration (Figure 2). As the ZHM treatment dose is 20 mg and considering that the drug is taken with 200 mL of water, permeability studies were continued with a concentration of 100 µg/mL. In the literature, it was reported previously that the PVP K30 polymer was not cytotoxic at the concentration we used.[23]

Caco-2 cell permeability

Saturation solubility, dissolution rate, and drug permeability are crucial factors for improving oral bioavailability. It is well known that the dissolution rate can be increased by reducing the PS of a drug substance to the sub-micron level, due to increased surface area. Nanocrystal technology, by taking advantage of this phenomenon, is a useful method for improving the bioavailability of poorly soluble compounds.[24] Besides *in vitro* solubility and dissolution studies, nanocrystal formulations should be tested regarding their permeability to investigate the influence of PS on drug permeability and to complement solubility and dissolution studies.[25] In this regard, the permeability of the nanocrystal formulation was investigated across the Caco-2 cell monolayer.

The monolayer integrity of Caco-2 cells was shown with TEER values of 300-1000 ohm.cm^2, which is in accordance with the literature.[26] The permeability value of coarse ZHM was found to be 8.887x10^{-6} cm/s at the end of 4 hours. The permeability value increased 1.34-fold with the nanocrystal formulation, to a value of 11.931x10^{-6} cm/s (Figure 3). In a previous study, it was shown that ZHM nanocrystals increased the saturation solubility 2.3-fold in comparison with the coarse powder of ZHM.[18] The increase in the permeability value can be explained by the increase in saturation solubility caused by the nanocrystal formulation.[27] The reduced PS of the nanocrystal formulation increases the saturation solubility of ZHM; therefore, the amount of dissolved ZHM on the apical side is greater for the nanocrystal formulation compared with coarse powder.[25] Hence, this results in an increased permeation rate for ZHM nanocrystals.

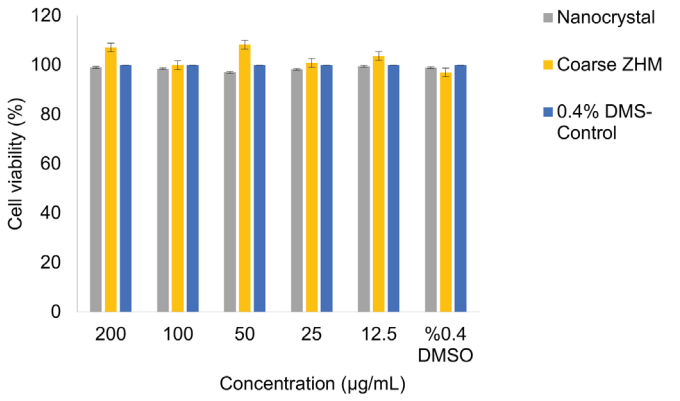


Figure 2. Cell viabilities (%) of the sample groups (n=6)
ZHM: Ziprasidone hydrochloride monohydrate, DMSO: Dimethyl sulfoxide

ZHM is known as a BCS class II drug, which has low water solubility as well as high permeability.[8,28] The log P value of ZHM is 3.6, and the drugs that have a log P value between 2.9 and 5.2 have an expected permeability value of 10^{-5}-10^{-4}.[29] The drugs, which are completely absorbed at Caco-2 monolayers, have a high permeation coefficient. Permeability values can be classified as Papp <1x10^{-6} cm/s, >10x10^{-6} cm/s and between 1 and 10x10^{-6} cm/s for the drugs which have low, high, and moderate permeability properties, respectively.[16] Considering this information, nanocrystal formulation increased the permeability value of ZHM from moderate to high.

The cumulative drug transport was also found to be higher with nanocrystal formulation in comparison with coarse powder, which is considered to be related to the improved solubility of ZHM in nanocrystal form (Figure 4).

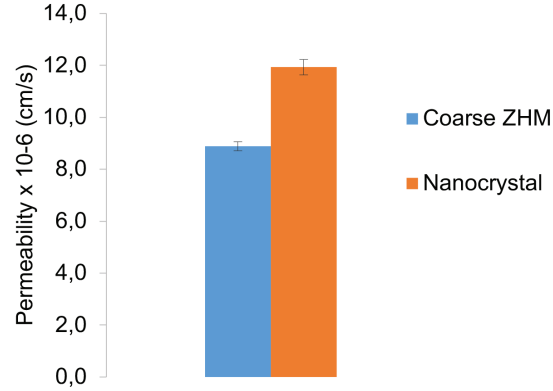


Figure 3. Permeability values of coarse ziprasidone hydrochloride monohydrate and nanocrystals
ZHM: Ziprasidone hydrochloride monohydrate

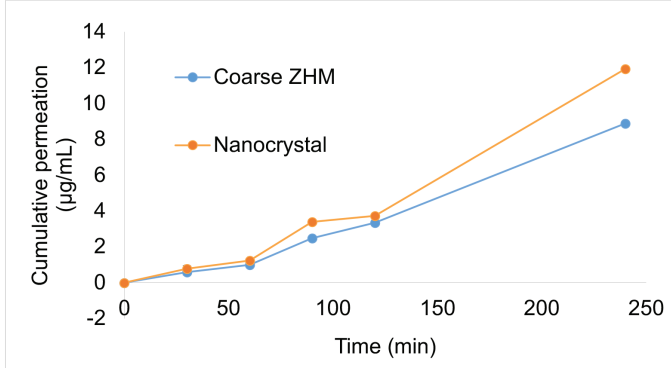


Figure 4. Cumulative amount of ziprasidone hydrochloride monohydrate that penetrated the basolateral membrane
ZHM: Ziprasidone hydrochloride monohydrate

CONCLUSION

The microfluidization method was found to be an effective and easy technique to prepare nanocrystal formulations. ZHM

nanocrystals were successfully obtained, lyophilized, and applied to Caco-2 cells. Coarse powder of ZHM or nanocrystals had no toxic effects on Caco-2 cells regarding the applied dose. ZHM nanocrystals showed an enhanced permeability value in comparison with coarse powder. In addition, the cumulative penetrated drug amount reached a higher concentration by using nanocrystals. In light of these results, it can be concluded that nanocrystal formulations can enhance the permeability of drug substances.

ACKNOWLEDGMENTS

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Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

A Prospective Study on Medication Errors in an Intensive Care Unit

Yoğun Bakım Ünitesinde İlaç Hataları Üzerine Prospektif Bir Çalışma

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ABSTRACT

Objectives: Any preventable event related to drugs that may cause harm to a patient is known as "medication error". Errors occur due to a lack of knowledge, poor performance, and psychological lapses. The pharmacists has a major role along with physicians, nurses, and administrators to examine and improve the healthcare system in order to ensure patient safety. The objective of this study was to determine the frequency, causes, and types of medication errors in the secondary-care intensive care unit.

Materials and Methods: All medical records of intensive care unit patients, above 14 years of age, listing their co-morbid/non-co-morbid conditions, occupation, caste, and gender, were checked for medication errors for a period of 6 months at Government Head Quarters Hospital, Udhagamandalam.

Results: According to the results of this study, 116 medication errors were found in 103 patients in the intensive care unit. The number of medication errors was higher in men than in women. The most common medication errors were prescription errors, which were due to illegible handwriting; the use of lookalike drugs; and incomplete dose, dosage, and frequency.

Conclusion: Considering the results of this study, it is important to increase awareness among healthcare professionals of varying stature about the significance of medication errors. It is also necessary to change the existing prescribing techniques and clearly differentiate lookalike drugs to avoid medication errors.

Key words: Error, drug, prescription

ÖZ

Amaç: Hastada ilaca bağlı zarar oluşturabilecek her önlenebilir hata "ilaç hatası" olarak bilinir. Hastalar bilgi eksikliği, düşük performans ve psikolojik sorunlar nedeniyle ortaya çıkabilir. Eczacıların doktor, hemşire ve yöneticilerle birlikte hasta güvenliğini sağlamak üzere sağlık sistemini geliştirmede büyük rolleri vardır. Bu çalışmanın amacı yoğun ikincil bakım ünitelerinde ilaç hatalarının sıklığı, nedenleri ve tiplerini belirlemektir.

Gereç ve Yöntemler: On dört yaş üzerindeki tüm yoğun bakım hastalarının komorbid/non-komorbid durumları, işleri, kastı ve cinsiyeti liste edilerek 6 ay içinde Udhagamandalam Devlet Merkez Hastanesi'ndeki ilaç hataları kontrol edilmiştir.

Bulgular: Çalışmanın sonuçlarına göre, yoğun bakımdaki 103 hastada 116 ilaç hatası bulunmuştur. Kadınlarda ilaç hatalarının sayısı erkeklere göre yüksektir. En sıklıkla rastlanan ilaç hataları okunamayan el yazısı ve benzer isme sahip ilaçlar nedeniyle ortaya çıkan yanlış ilaç verme, tam olmayan dozlama, dozajlama ve uygulama sıklığı hatalarıdır.

Sonuç: Çalışmanın sonuçları dikkate alındığında ilaç hataları hakkında farklı konumlardaki sağlık profesyonelleri arasında duyarlılığı artırmanın önemli olduğu görülmektedir. Ayrıca, var olan reçeteleme tekniklerini değiştirme ve benzer özelliklerdeki ilaçları belirgin bir şekilde ayrıştırma ilaç hatalarını önlemek için gereklidir.

Anahtar kelimeler: Hata, ilaç, reçete

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INTRODUCTION

The term medication error can be described as improper/incorrect administration of a medication that occurs due to an incorrect dosage or route of administration, failure to prescribe or administer the appropriate medication or formulation for a specific disease or condition, use of obsolete medicines, failure to follow the correct time to administer the medication or lack of knowledge of adverse effects. Causes of prescription errors include difficulty reading handwritten instructions, misunderstanding about different medications with similar names, and lack of knowledge about allergies or sensitivities to a patient's prescription. Drug errors occur when a patient is selected by a health care provider for an inadequate form of treatment. This is one of the main issues, and the correction of drug errors is the first step towards improving patient health. In this, the clinical pharmacist, along with health care practitioners, will play a significant role in patient safety. Errors in drug administration can be considered as lethal as any disease. Harvard University reported in a 2013 report that each year India reports a whopping 52 lakh accidents (out of the 430 lakhs worldwide) due to medical errors and adverse events (May 5, 2016). The key explanation for this is the lack of interaction amongst health care professionals.

A drug error can be categorized as (1) a prescription error, (2) an interpretation/transcription error, (3) an administration error, or (4) a documentation error.

1. Prescription error: An error made by the doctor while writing a prescription, such as poor handwriting, incorrect spelling of medications, incorrect dosage type, and no drug strength specified on the prescription. For example, the drug name is specified correctly, but the dosage type or frequency is not. This type of error can be fatal.

2. Transcription error: A form of error created by nurses and physicians when entering the specifics of the medication in the system or in the patient's profile. Errors in this category include double drug entry, absence of drug entry, or entry of a drug that was not administered.

3. Administration error: The most common error made by nursing staff during patient administration of medications. Such errors include prescribing the medications to the wrong patient, incorrect prescription, directions, dosage, indications, cause, time, duration, etc.

4. Documentation error: Patient care nurses play a significant role. Records of the patient's prescription history are kept by nurses. Mistakes in reporting details of the drug, such as no recording of the drug, double entry of the medications, or losing critical information when reporting, are known as documentation errors.

The main causes of medication error are missing patient information, missing drug information, miscommunication of a drug order, lack of education of nurses, lack of drug details from the patient's side, improper drug storage, illegible handwriting, incorrect drug selection, drug interactions, improper training of nurse's, etc.[1]

The following control measures can reduce the severity of medication error:

a. Ensuring proper drug administration to the correct patient, such as the correct medication, dose, path, and duration.

b. Following proper reconciliation of the medication when moving a patient from one unit to another, verifying the appropriate drug, dose, path, and time for the appropriate patient against the order of transfer.

c. Having nurses who work different shifts check prescription orders two or three times.

d. Ensuring proper preservation of records and documents.

e. Maintaining proper medicinal.

f. Avoiding the use of abbreviations.

Types of prescription error:

Computerized pharmacy order entry (CPOE) is a very helpful way for doctors to enter medication directly into a hospital's computer system that avoids poor handwriting to complete all vital details and can reduce the rate of medicine error. The use of this system will strengthen contact efforts during care transition. Doctors should be interested in tailoring CPOE to ensure that it is both user friendly and unique to their medical ordering needs.[2,3] In addition to providing essential reminders and warnings, the computerized decision support systems can improve the clinical performance related to prescribing practices. It includes a summary of the orders as they are issued, comparing new and current orders, scanning for potential drug reactions, correct dosing schedules, and alerting the physician to appropriate laboratory results, all of which affect the physician's decisions and the patient care plan.[4]

Pharmacist-aided rounds ensure that medications are used rationally and cost effectively, facilitate safe living, and enhance clinical outcomes by regularly participating in direct patient care and partnering with other healthcare practitioners, thus reducing prescription errors. The use of CPOE can help to remove handwriting errors that can result in adverse effects on the patient. By using CPOE, we can prevent errors introduced by handwritten abbreviations, drug names, dosages, and frequencies. Abbreviations are one of the main causes of prescription drug errors when doctors write prescriptions by hand. Therefore, to prevent prescription errors, we must stop using abbreviations for product names, doses etc. The decimal point should be used with a leading zero so the patient or caretaker does not miss the decimal point on the prescription. For example; write 0.1 mg drug instead of writing 1 mg.

Electronic order transcription with CPOE improves transcription speed and accuracy. Therefore, we can eliminate a few prescription mistakes using this.[5] Mishearing often leads to the use of incorrect words or terms due to poor listening. To prevent this form of mistake, the pharmacist should be attentive to the doctors' orders. Security is the primary concept upon which verbal commands are recognized. Verbal directives have a greater potential for errors because such instructions may be misunderstood, misinterpreted, and mistranscribed.[6] The patient's written documentation records are essential to

the preparation and assessment of procedures and patient care. The written reports include the patient's medical history and drug history, the patient's course of treatment, care, and reactions while under the health care provider's supervision.[7] Many of the good documentations can be reliable, complete, timely, truthful, and structured. Patient data, such as name, title, and other information, must be cross-checked with the medical record registry by the pharmacist or nurses before administration of the drug to the patient.[8] A pharmacist must verify the appropriate dosage, route, and time written on the prescription before drug administration.[9] Drug reconciliation is a procedure to strengthen communication so that mistakes can be avoided by obtaining the patient's medication history and reconciling the medication as soon as possible when the patient is admitted to hospital. The drug records will be reconciled with the hospital's discharge medicines. This is one of the best ways to prevent mistakes in recovery, such as when a patient is given their list of medications on hospital admission, thus decreasing likelihood of errors and harm. A medication reconciliation strategy that includes healthcare providers and patients can minimize medication morbidity and mortality and is a vital factor in patient safety.[10-12]

MATERIALS AND METHODS

The study was performed at government headquarters hospital, Ooty over a span of six months as a prospective analysis involving purposeful sampling. The criteria for inclusion in the research included all patients admitted to the intensive care unit, patients over 14 years of age, patients with co-morbid or non-comorbid conditions who belonged to any occupation, caste, or gender. The exclusion criteria covered pregnant or lactating mothers and patients under 14 years of age. The resources used for the research were patients' medical histories and types of drug errors (method of data collection). The drug error form was developed with the assistance of a healthcare professional. Forms were printed and kept in the ICU for the reference of healthcare professionals. Patients clinical histories were checked, and regular patient interviews were performed. Once the incidence was confirmed, the medication error was filed and documented. Institutional Review Board Ethical Approval was obtained for the study (JSSCP/IEC/ 01/2018-19).

Statistical analysis

No statistical analysis was carried out in this study.

RESULTS

A total of 116 drug errors were discovered among 103 patients according to the findings of this report. Considering the type of error, prescription errors were found to be more frequent than other errors, such as administration errors, paperwork errors, and transcription errors shown in Figure 1. Illegible handwriting was the main cause of medication error. The use of lookalike drugs and incomplete dose, dosage, and frequency information were other features, as outlined in Figure 2. The number of drug errors was higher in men than in women, as seen in Figure 3. The cardiovascular system was the most affected system (Table 1). For this analysis, the age groups 55-64 years and 45-54 years were the most affected (Table 2).

DISCUSSION

Medication errors are a major concern in many healthcare settings. This can have serious implications for patients and healthcare providers alike. It contributes to extended hospital stays with detrimental impacts. The main objective of this

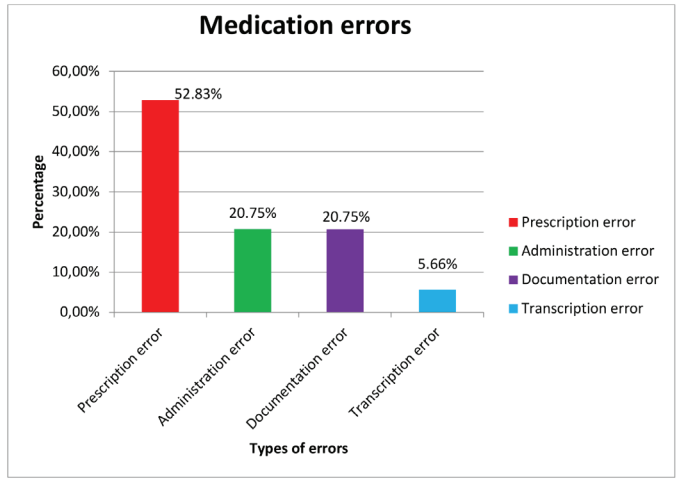

Figure 1. Type of medication errors

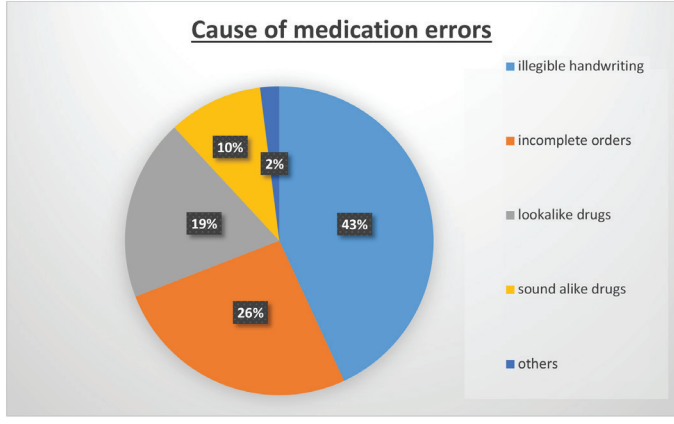

Figure 2. Causes of medication errors

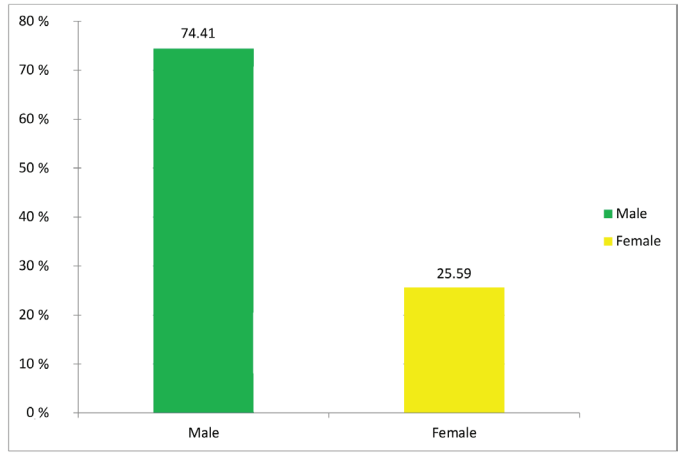

Figure 3. Male predominance of the effect of medication error

research was to determine the prevalence of medication errors in an intensive care unit with patient safety as the goal by defining and reducing the major cause of medication errors to recognize various forms of medication errors and to raise awareness among health care professionals about medication errors.

The goal of drug treatment is to optimize the clinical outcome and enhance the patient's overall quality of life. Drug mistakes, however, have major consequences for safety. These errors may occur at various stages of drug usage, such as prescribing, dispensing, and administration. Early identification of these errors is of great importance for patient health. The most common source of medication error in the current study was prescription error at 52.83%, followed by administration and reporting error at 20.75%, and lastly, transcription error at 5.66%. Published research papers show that not every drug error causes harm. Hospitals need to build and create mechanisms to avoid medication errors by identifying and recognizing the cause of such errors. Pharmacists must collaborate actively with doctors, nurses, and other personnel to ensure that medication prescriptions are safe and correct. The patient's medication files were consulted in the current study, and reports of errors were produced. The study emphasizes the importance of recording errors in developing prevention approaches aimed at minimizing medication errors.

CONCLUSION

Defining the characteristics and patterns of medication error can direct error prevention. Patients with life-threatening diseases are treated in intensive care units. The intensive care unit atmosphere produces potentially high-risk iatrogenic events. Critically ill patients, due to their underlying comorbidities and acute organ dysfunction, are particularly vulnerable to drug errors. Identifying medication errors is thus critical for reporting and preventing this risk. Perhaps more than one would expect, the underlying causes of medication error are in need of fundamental changes in healthcare systems. Additionally, the current prescription methods need to be updated and lookalike drugs specifically distinguished to prevent medication errors. One of the required improvements is to include pharmacists in the healthcare team and use their experience to mitigate medication error. Knowledge campaigns for healthcare professionals should be implemented and, likewise, awareness of the serious side effects of drugs and how to manage them should be provided to lay people. There is an urgent need for a systemic approach to reducing organizational vulnerability to errors by providing the tools needed to track, assess, and execute successful interventions.

Table 1. Systems affected by medication error

	System	No of cases
Affected systems	Digestive	17
	Cardiovascular	43
	Muscular	13
	Nervous	6
	Respiratory	19
	Excretory	5

Table 2. Effect of medication error on the cardiovascular system by age group

	Years	Percentage
Age group	15-24	2.0
	25-34	6.97
	35-44	12.0
	45-54	23.25
	55-64	27.88
	65-74	18.60
	75-84	4.65
	85-94	4.65

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ORIGINAL ARTICLE

Development of an *In Vitro-In Vivo* Correlation for Sitagliptin and Metformin Prolonged-release Tablet Formulations

Sitaglipin ve Metformin Uzatılmış Salınımlı Tablet Formülasyonları İçin *İn Vitro-İn Vivo* Korelasyon Geliştirme

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ABSTRACT

Objectives: The objective of this study was to establish and validate an *in vitro-in vivo* correlation (IVIVC). To investigate the safety of a fixed-dose combination (FDC) versus the reference formulations (Januvia® 100 mg Filmtabletten co-administered with Glucophage® SR 1000 mg prolonged-release tablets), a bioequivalence study was conducted in the fasted and fed states, and the data generated were used to establish the correlation.

Materials and Methods: The formulations used in the bioequivalence study were a FDC (sitagliptin hydrochloride equivalent to 100 mg of sitagliptin and metformin hydrochloride 1000 mg prolonged release) and Januvia® 100 mg co-administered with Glucophage® SR 1000 mg. The plasma profiles from the bioequivalence study and respective dissolution data were then utilized to establish "level A" IVIVC. The procedure comprises pharmacokinetic modeling to derive the empirical constants for further use in deconvolution and convolution procedures. Levy plots were constructed to understand the relationship between *in vitro* and *in vivo* properties. The internal and external predictabilities were evaluated by comparing the predicted pharmacokinetics with the observed values from the bioequivalence study.

Results: The formulations showed approximately 91%-95% and 89%-91% dissolution, respectively in fasted and fed-state dissolution media for sitagliptin. The dissolution of metformin was 96%-98% and 89%-95%, respectively, in fasted and fed-state media. The regression coefficients of all the Levy plots were more than 0.900, indicating a linear correlation between *in vitro* release and *in vivo* parameters. The prediction error value of internal and external predictabilities was below 10 and met the US Food and Drug Administration criteria. Therefore, it can be stated that the correlation models are validated and can be used for predictions and to setting the dissolution specifications. The safety and tolerability of the FDC was found to be superior to those of the reference formulations, as fewer adverse events occurred following administration of the FDC.

Conclusion: Correlation models can be useful for the prediction of FDCs during the management life cycle of the product. The models can also serve as a surrogate for *in vivo* studies. The FDC was tolerable, and the adverse events were mild and similar to those observed with the reference products. Therefore, the FDC is safe for use in human subjects.

Key words: IVIVC, level A correlation, Levy plot, sitagliptin hydrochloride, metformin hydrochloride

ÖZ

Amaç: Bu çalışmanın amacı sabit doz kombinasyonunun (FDC) güvenliliğini araştırmak için referans formülasyonlara (Januvia® 100 mg Filmtabletten ie birlikte uygulanan Glucophage® SR 1000 mg uzatılmış salınımlı tablet) karşı *in vitro-in vivo* korelasyonu (IVIVC) kurmak ve valide etmektir. Bu nedenle, aç ve tok durumlarda bir biyoeşdeğerlik çalışması yapılmış ve elde edilen veriler korelasyon kurmak için kullanılmıştır.

Gereç ve Yöntemler: Biyoeşdeğerlik çalışmalarında kullanılan formülasyonlar bir FDC (100 mg sitaglipine eş staglipin hidroklorür ve uzun salınımlı metformin hidroklorür 1000 mg) idi. Biyoeşdeğerlik çalışmasının plazma profilleri ve takip eden dissolüsyon verileri "düzey A" IVIVC kurmak için kullanılmıştır. Bu prosedür, dekonvülüsyon ve konvülüsyon prosedürlerinde kullanılmak üzere ampirik sabiteleri derive etmek için kullanılan bir farmakokinetik modellemeden oluşmaktadır. *İn vitro* ve *in vivo* özellikler arasındaki ilişkiyi anlamak için için levy grafikleri düzenlenmiştir. İç ve dış tahmin edilebilirlikler biyoeşdeğerlik çalışmasından elde edilen tahmini gözlemlenen değerler ile farmakokinetikleri karşılaştırarak değerlendirilmiştir.

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Bulgular: Sitaglipin formülasyonları aç ve tokluk durumları için dissolüsyon medyasında sırasıyla yaklaşık %91-%95 ve %89-%91 dissolüsyon göstermiştir. Metforminin dissolüsyonu aç ve tokluk durumları için dissolüsyon medyasında sırasıyla yaklaşık %96-%98 ve %89-%95 idi. Tüm levy grafiklerinin regresyon katsayıları 0,900'ün üzerindeydi ki bu *in vitro* salım ile *in vivo* parametreler arasında doğrusal bir korelasyon varlığını göstermekteydi. İç ve dış tahmin edilebilirlikler için tahmini hata değeri 10'un altında idi ve Amerikan Gıda ve İlaç Dairesi kriterlerine uymaktaydı. Bu nedenle, korelasyon modellerinin valide olduğu ifade edilebilir ve tahmin edilebilirlikler ve dissolüsyon spesifikasyonlarını belirlemek için kullanılabileceği söylenebilir. FDC'nin güvenliliği ve tolere edilebilirliği referans formülasyonlardan daha üstün bulunmuştur; zira FDC'nin uygulanmasını takiben daha az sayıda advers etkiler görülmüştür.

Sonuç: FDC'lerin tahmininin korelasyon modelleri için ürünün yaşam döngüsünün idare edilmesinde faydalı olabileceği söylenebilir. Bu modeller *in vivo* çalışmalar için yedek modeller olarak da işlev görebilir. FDC tolere edebilir özelliktedir ve advers etkileri hafiftir ve referans ürünlerle gözlenen etkilere benzerdir. Bu nedenle, FDC'nin insanlarda kullanımı güvenilirdir.

Anahtar kelimeler: IVIVC, A düzeyi korelasyon, levy plotu, sitagliptin hidroklorür, metformin hidroklorür

INTRODUCTION

Sitagliptin, a dipeptidyl peptidase 4 inhibitor, is indicated for hyperglycemia.[1] Sitagliptin exerts its action by prolonging the action of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide but facilitates insulin production and reduces the secretion of glucagon, which in turn decreases hepatic glucose overproduction.[2] Food does not show a significant influence on the pharmacokinetics of sitagliptin; hence, sitagliptin can be taken without regards to food. Metformin is prescribed as a first-line therapy in type-2 diabetes.[3] Metformin exerts a glucose-lowering effect (i) via inhibition of gluconeogenesis in the liver, (ii) by delaying the action of glucagon, (iii) by facilitating the action of insulin, and (iv) by delaying glucose absorption from the intestine.[4,5] As per the biopharmaceutical classification system (BCS), both sitagliptin and metformin possess high solubility and poor permeability; therefore, both drugs belong to BCS class III.[6,7]

A fixed-dose combination (FDC) comprising sitagliptin hydrochloride equivalent to sitagliptin 100 mg as an immediate-release form and metformin hydrochloride1000 mg as a prolonged-release form was developed. FDCs offer numerous merits,[8] in comparison with individual drug products, including the simplicity of dosage forms in terms of the dosing schedule. This leads to improved patient compliance and results in an overall better treatment outcome. This aspect is especially important in elderly patients or those suffering from multiple disorders. The *in vivo* behavior of Januvia®, Glucophage® 1000 mg SR, and the FDC were evaluated in a bioequivalence study including healthy subjects.[9] The FDC was developed in order to avoid administration of two individual products for the routine treatment of type-2 diabetes mellitus. The formulations (FDC vs individual innovator products) as a part of development have been evaluated and their safety, bioequivalence, and tolerability proved in human volunteers in the fasted and fed states.[9]

In the management life cycle of formulations, the need always arises for changes in parameters, such as the composition, process, equipment, scale-up or scale-down, or the manufacturing site. In certain instances, post-approval changes may trigger the conduct and reporting of further bioequivalence testing for the modified and marketed formulations.[10] Furthermore, the availability of an *in vitro-in vivo* correlation (IVIVC) can simulate and predict plasma profiles and can serve as a surrogate for *in vivo* studies. Therefore, an IVIVC was developed and validated for predictability using bioequivalence data collected in the fasted and fed states. The present paper describes the developmental aspects of the IVIVC and those of internal and external validation. Correlation models can be useful for the prediction of FDCs during the management life cycle of the product.

MATERIALS AND METHODS

Formulations tested

The formulations evaluated for bioequivalence are shown in Table 1. A FDC (composition is not disclosed) containing sitagliptin hydrochloride equivalent to sitagliptin 100 mg and metformin hydrochloride 1000 mg prolonged-release tablets (AET Laboratories Pvt. Ltd, India) was used as the test formulation. The *in vivo* behavior of the test formulation as a FDC was compared with those of individual reference formulations, namely Januvia® 100 mg Filmtabletten (lot no. 362117, marketed by Merck Sharp & Dohme Ltd., UK) and Glucophage® SR 1000

Table 1. Scheme of the clinical study

Period	Cohort 1 (n=12)		Cohort 2 (n=12)	
	Group 1 (n=6)	Group 2 (n=6)	Group 1 (n=6)	Group 2 (n=6)
1	*FDC	#Januvia® 100 mg and Glucophage® SR 1000 mg	*FDC	#Januvia® 100 mg and Glucophage® SR 1000 mg
2	#Januvia® 100 mg and Glucophage® SR 1000 mg	*FDC	#Januvia® 100 mg and Glucophage® SR 1000 mg	*FDC

*: FDC comprising sitagliptin hydrochloride, equivalent to 100 mg sitagliptin and metformin hydrochloride 1000 mg and is manufactured by AET Laboratories Pvt Ltd., India. #: Originators comprises of sitagliptin phosphate monohydrate, equivalent to 100 mg sitagliptin (Januvia® 100 mg) and metformin hydrochloride 1000 mg as prolonged release (Glucophage® SR 1000 mg). FDC: Fixed-dose combination

mg prolonged-release tablets (lot no. GXC15222, marketed by Merck Serono Ltd., UK). In cohort 1, the study was conducted under fasting conditions and in cohort 2, in the fed state.[9]

Dissolution method

The *in vitro* release of sitagliptin and metformin from FDC, Januvia® 100 mg Filmtabletten and Glucophage® SR 1000 mg prolonged-release tablets was performed using a USP I (basket) apparatus (Electrolab, Mumbai, India). Individual tablets were placed in dissolution vessels containing 900 mL of dissolution media. Phosphate buffer, pH 6.8 and acetate buffer, pH 5.5 were selected as the dissolution media for fasted- and fed-state conditions, respectively. The pH of the dissolution media was based on the pH of the gastrointestinal (GI) tract in the fasted and fed states. The preprandial GI pH was 1 to 7.5, and the postprandial pH was 2.7-6.4 (stomach) and 4-8 (intestine).[11-13] Due to the high solubility and pH-independent soluble nature of the molecules, aqueous buffers at one pH for each condition was selected. The study was conducted at a rotational speed of 100 rpm. The samples were collected for up to 60 min for sitagliptin and 12 h for metformin and replenished with the respective fresh media. The drug released from the samples was analyzed using a high-performance liquid chromatography (HPLC) system (Waters, Singapore) equipped with a quaternary pump, ultraviolet-visible spectrophotometric detector (Perkin Elmer, Lambda 25, Massachusetts, USA), and C_8 column (100x2.1 mm, particle size of 1.7 µm). The mobile phase consisted of acetonitrile, 10 mM potassium dihydrogen phosphate buffer, and 2 mM sodium hexane-1-sulfonate. The pH of the mobile phase was adjusted to 5.5 using phosphoric acid. The flow rate was 1 mL min^{-1}, and the detection wavelength was 210 nm.[14] The precision and accuracy of the chromatographic method were checked and were found to be within the required limits (coefficient of variation <15%). The dissolution profiles were subjected to similarity assessment in accordance with the guidelines.[10]

In vivo characterization

Subjects and study approval

A mixed population comprising 24 healthy subjects (including 9 male and 15 female subjects) after screening were enrolled in the bioequivalence study (Table 1). The subjects were grouped in two cohorts, each consisting of 12 subjects. Subjects were included in accordance with the guidelines,[15] and the inclusion criteria were age ≥18 years and body mass index 18.5-30 kg/m^2. The study protocol was approved (refer to letter number: 429) by the National Ethics Committee for Drugs Clinical Trials and to the Medicines and Medical Devices Agency Chisinau, The Moldavian Republic (date: 27.12.2017, no: 429). The study was conducted[9] in agreement with the Declaration of Helsinki (1964 and subsequent amendments), ICH-good clinical practice (GCP) R2,[16] EEC rules and in accordance with GCP for the conduct of clinical studies. The subjects' medical histories were recorded by the clinical investigator. A medical examination was conducted to record systolic arterial pressure (SAP), diastolic arterial pressure (DAP), heart rate, electrocardiogram (ECG), body temperature, and respiratory frequency. Biological samples (urine and blood) were collected for analysis from a clinical chemistry perspective.

Study design

The study was performed as an open label, two-period, two-way crossover, randomized controlled, single-dose comparative bioequivalence study between the FDC and reference formulations in healthy subjects with a wash-out period of 14 days between periods. Blood samples were collected before the study drug administration and at 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.33, 4.67, 5.00, 5.33, 5.67, 6.00, 7.00, 8.00, 9.00, 10.00, 12.00, 16.00, 24.00, 48.00, 72.00, 96.00, and 120.00 hours post dose. The blood samples were centrifuged for 10 minutes at 4°C nominal with a force of 1500 (±5) g. After centrifugation, the plasma was separated into two aliquots and stored at -20°C until sample analysis.

Bioanalytical procedure: estimation of analytes from plasma

Sample analysis and processing were performed by the Analytical Laboratory of 3S-Pharmacological Consultation & Research GmbH (Bucharest, Romania). The concentrations of sitagliptin and metformin were measured by reversed-phase HPLC coupled to a tandem mass spectrometry detector (LC/MS/MS). The separations were performed isocratically on a reversed-phase column (Agilent Zorbax 300-SCX, 2.1x50 mm, 5 µm) with a flow rate of 1.00 mL/min. The mobile phase consisted of methanol and 75 mM ammonium acetate buffer (80:20). The internal standards used were sitagliptin-D4 and metformin-D6. Detection was carried out by triple quadrupole MS/MS with an AB-Sciex model, API 5500 QTRAP, equipped with an atmospheric pressure ionization interface (Model, Turbo Spray). The precursor and product ions used for detection were 408,123/235,100 for sitagliptin, 412,088/239,100 for sitagliptin-D4, 129,975/71,200 for metformin, and 136,026/60,000 for metformin-D6.The analytical method was validated in accordance with GLP principles, US Food and Drug Administration (FDA) rules,[17] European Medicines Agency (EMA) guidelines,[18] and the current Romanian GLP guidance. The method was developed and validated in the concentration range of 1.0-800 ng/mL and 5.0-4000 ng/mL, respectively, for sitagliptin and metformin. During validation, the stability of the internal standard working solution (up to 16 hours at room temperature), system suitability test solution stability (up to 1 week when stored below -20°C), spiked plasma sample stability (up to 6 hours at room temperature, up to 1 week at -5°C, up to 11.5 months below -20°C, up to 1 week below -70°C), freeze-thaw stability (up to 5 cycles), and stability of spiked plasma sample extract (up to 48 hours at 10°C) were evaluated.

Sample preparation

An aliquot of 0.150 mL of plasma sample was transferred to 2 mL multi-well plates. Fifty microliters of working internal standard solution was added to the plasma sample and mixed for 3 minutes followed by addition of 0.800 mL of acetonitrile. The contents were mixed for 5 minutes and centrifuged for 5 minutes at 4000 rpm (20°C, nominal). The supernatant was separated and diluted, and 20 µL was injected in to HPLC.

Pharmacokinetic variables

The pharmacokinetic parameters, peak drug concentration (C_{max}) and area under the curve from time zero to time t (AUC_{0-t}) as primary parameters and area under the curve from time zero to infinity (AUC_{0-Inf}) and time of the peak drug concentration (T_{max})as secondary parameters were calculated.

$$c\delta\,(t) \;=\; \sum_{j\,=\,1}^{n} \mathrm{Aj}\; e^{-\alpha j\, t}$$

Safety and tolerability

The clinical safety of the formulations was assessed via medical history, clinical examination (physical and systemic examination), 12-lead ECG, and vital signs (blood pressure, heart rate, respiratory rate, and temperature), and biochemical parameters. The parameters were measured at the time of check-in to the study center and before treatment in each study period. Before dosing in each period at at follow-up, the subjects were asked about their health status and medication consumption. The SAP, DAP, heart rate, and body temperatures were measured before dosing and during the study for each period.

Development of in vitro-in vivo correlation

Phoenix® Version 8.1 software was used for the pharmacokinetic modeling, deconvolution, and convolution procedures. Pharmacokinetic modeling was performed in order to fit the best model by varying the parameters, e.g., with and without lag time and choosing one- or two-compartment models. Among the attempts, the one that yielded a high correlation coefficient was chosen for further consideration. Accordingly, the empirical constants (A and alpha) were chosen for the deconvolution and convolution procedures. "A" and "alpha" refer to the parameters of a poly exponential unit impulse response function of the form

Where, "N" is the number of exponential terms, "Cδ" represents to concentration time course, and "t" stands for time.

The *in vivo* plasma concentrations versus time profiles were deconvolved to derive the fraction absorbed (F_a). Then, the fraction of drug absorbed was correlated with the drug dissolved in order to construct a "levy" plot.

Validation of the IVIVC

The empirical constants were chosen for the convolution of dissolution profiles in order to derive the plasma-concentration-time-profile. The simulated plasma profile was further subjected to the calculation of pharmacokinetics to compute C_{max}, AUC_{0-t}, and AUC_{0-Inf}. The prediction error (PE) was calculated for C_{max}, AUC_{0-t}, and AUC_{0-Inf} for each formulation and for each drug substance using the equation below:

PE=[(observed-predicted)/observed] x100

The predictability of the correlation model was evaluated using the internal and external predictabilities as per FDA guidance.[19] For both internal and external validation, the mean PE was required and should be not more than 10% for C_{max}, AUC_{0-t}, and AUC_{0-Inf}.

No statistical methods were used for the data treatment.

RESULTS

In vitro release

Sitagliptin

The *in vitro* drug-release profiles of FDC and Januvia® using the selected method (USP 1, 100 rpm at pH 6.8 and pH 5.5) are presented in Figure 1. The immediate-release form of sitagliptin showed approximately 91%-95% of dissolution in phosphate buffer pH 6.8 and 89%-91% dissolution in acetate buffer at pH 5.5. Despite the differences in salt from Januvia® and FDC, the dissolution appeared to be complete and gradual. However, differences existed in the dissolution pattern. To check the impact of the difference in dissolution between the reference and test formulations, the similarity factor (f_2) was assessed for the dissolution profiles. The f_2 values were found to be 54 for both fasted and fed-state dissolutions. An f_2 value greater than 50 is an indication of similarity.[10]

Metformin

The *in vitro* drug-release profiles of FDC and Glucophage® SR 1000 mg using the selected method (USP 1, 100 rpm at pH 6.8 and pH 5.5) are presented in Figure 2. The prolonged-release form of metformin HCl showed approximately 96%-98% dissolution at pH 6.8 and 89%-95% dissolution at pH 5.5. The dissolution profiles appear to be gradual and complete. The f_2 values were found to be 65 (at pH 6.8) and 55 (at pH 5.5), respectively, also an indication of the similarity of the dissolution profiles.[10]

IVIVC model

The plasma profiles of sitagliptin (Figure 3A) and metformin (Figure 3B) from the bioequivalence study were deconvoluted to derive the respective *in vivo* absorption profiles (Figure 4A, B). Levy plots were constructed to understand the relation

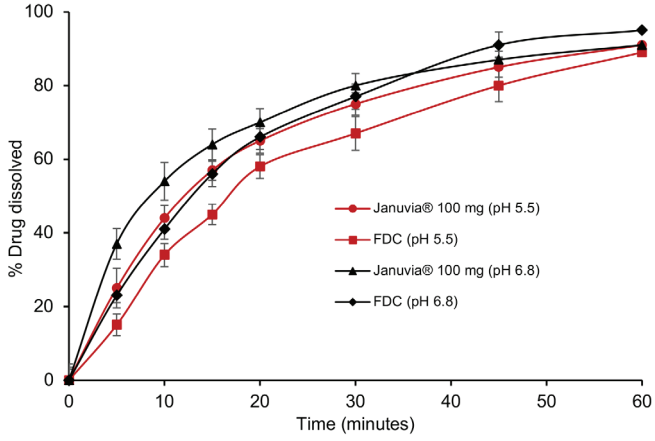

Figure 1. *In vitro* release of sitagliptin from FDC and Januvia® 100 mg at pH 6.8 and pH 5.5. FDC contains sitagliptin HCl, and Januvia® 100 mg contains sitagliptin phosphate monohydrate equivalent to 100 mg sitagliptin, respectively

FDC: Fixed-dose combination

between the *in vitro* sitagliptin dissolved and *in vivo* sitagliptin absorbed (Figure 5A, B). Regression coefficients of 0.952 and 0.976 for Januvia® and FDC indicate that a good linear correlation existed between *in vitro* and *in vivo* parameters under fasting conditions. Similarly, the correlation coefficients were 0.996 and 0.963, respectively, for Januvia® and FDC tested under fed conditions (Figure 5C, D). Despite the differences in the salts used in the formulations, the *in vitro* tool showed good discrimination. Hence, the employed dissolution method can be used for the characterization of formulations containing either of the salts.

The levy plots of metformin (Figure 6A, B) using the data obtained in the fasted state study showed a regression coefficient of 0.971 and 0.937, respectively, for Glucophage® SR 1000 mg and FDC. The regression coefficients of fed-state data were 0.965 and 0.959, respectively, for Glucophage® SR 1000 mg and the FDC (Figure 6C, D). The results indicate that the chosen *in vitro* conditions are appropriate and are mimicking the *in vivo* environment.

Internal and external validation

The internal and external predictability of sitagliptin for the C_{max}, AUC_{0-t}, and AUC_{0-Inf} are presented in Table 2. The mean PE

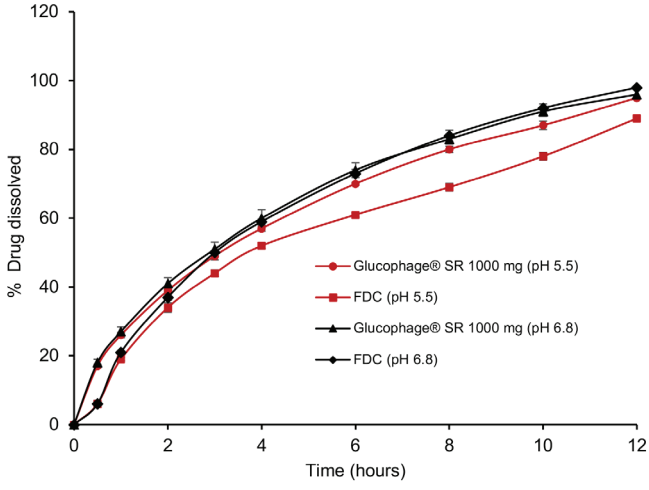

Figure 2. *In vitro* release of metformin HCl from FDC and Glucophage® SR 1000 mg at pH 6.8 and pH 5.5. Both the formulations contain 1000 mg metformin HCl in the prolonged-release form

FDC: Fixed-dose combination

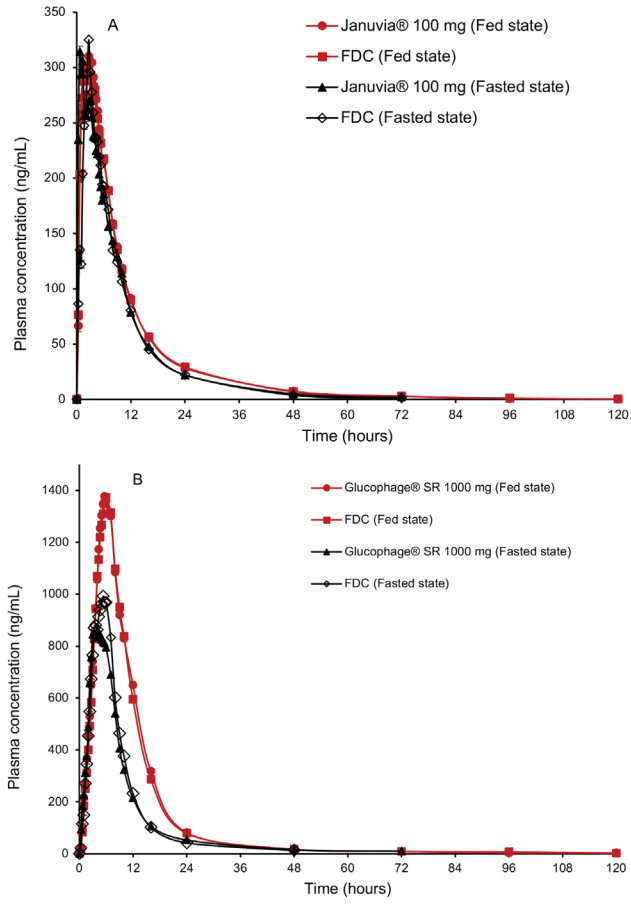

Figure 3. Mean plasma profile of sitagliptin in the fasted and fed states (A) and metformin in the fasted and fed states (B) for the formulations used in the study

FDC: Fixed-dose combination

Table 2. Validation of the correlation model for sitagliptin

Parameter	Januvia® 100 mg internal validation			FDC external validation		
	Observed	Predicted	PE	Observed	Predicted	PE
Fasted state						
C_{max} (ng/mL)	314.0	302.6	**3.64**	324.7	309.4	**4.69**
AUC_{0-t} (ng-h/mL)	3141.7	3024.8	**3.72**	2964.9	2931.0	**1.14**
AUC_{0-Inf} (ng-h/mL)	3178.9	3044.1	**4.24**	2983.2	2951.2	**1.07**
Fed state						
C_{max} (ng/mL)	383.2	370.2	**3.40**	347.7	338.6	**2.62**
AUC_{0-t} (ng-h/mL)	3595.0	3395.0	**5.56**	3531.5	3355.0	**4.99**
AUC_{0-Inf} (ng-h/mL)	3630.5	3400.6	**6.33**	3565.6	3362.0	**5.72**

FDC: Fixed-dose combination, PE: Prediction error, C_{max}: Peak drug concentration, AUC_{0-t}: Area under the curve from time zero to time t, AUC_{0-Inf}: Area under the curve from time zero to infinity

values were below 10 for all the parameters and for both the formulations under fasted and fed conditions. The internal and external PE values were below 10% for metformin for all the principle pharmacokinetics (Table 3).

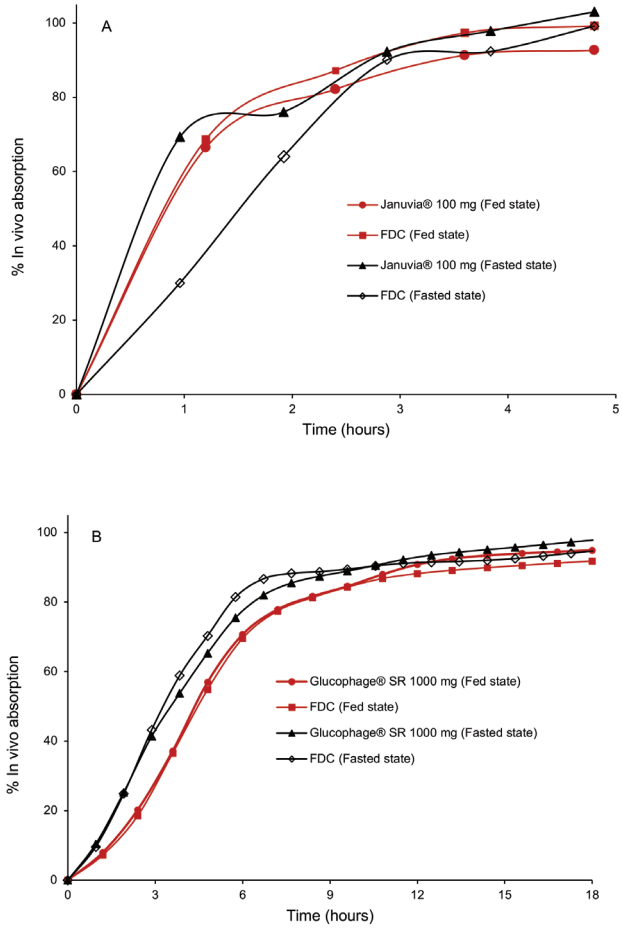

Figure 4. Mean *in vivo* absorption profiles for sitagliptin in the fasted state and the fed state (A) and metformin in the fasted state and the fed state (B) for the reference and test formulations

FDC: Fixed-dose combination

Safety and tolerability

No serious adverse events (AEs) were reported in the bioequivalence study, and the intensity of reported AEs were deemed by the principal investigator as mild in severity. A total of five subjects showed eight AEs; among them, two subjects showed AEs for FDC and five showed AEs for the reference formulation.[9] Despite the differences between salts of sitagliptin from reference (sitagliptin phosphate monohydrate) and those of the test formulation (FDC comprised of sitagliptin hydrochloride), the number of AEs with test formulation was "low" compared with the reference formulation under both fasted and fed conditions. The test and reference products were well tolerated, considering the AEs observed in the study. Therefore, sitagliptin hydrochloride can be deemed safe and is behaving in line with sitagliptin phosphate monohydrate from a safety perspective.

DISCUSSION

As per EMA guidelines,[20] a validated IVIVC serves as a surrogate for *in vivo* performance. Internal and external validation of the IVIVC was performed in the current study. Therefore, the changes in the manufacturing process and some formulation modifications, including the product strength using the same formulation, can be justified using IVIVC, without the need for additional bioavailability/bioequivalence studies. Therefore, in present study, a correlation was established between *in vitro* drug release at pH 6.8 (pre-prandial state) and pH 5.5 (post-prandial state) versus the fraction of respective drug absorption in the fasted and fed states.

Sitagliptin

At pH 6.8, sitagliptin release was initially slow from the FDC compared with that of Januvia® and similar from 20 min onwards. The slow pattern of dissolution from the FDC could be due to the combination of immediate and prolonged-release formulations, where sitagliptin hydrochloride was present as an immediate-release form along with the prolonged-release form, metformin HCl, whereas Januvia® is an immediate-release tablet, and dissolution progresses as the tablets disintegrate. A similar

Table 3. Validation of the correlation model for metformin

Parameter	Glucophage® SR 1000 mg internal validation			FDC external validation		
	Observed	Predicted	PE	Observed	Predicted	PE
Fasted state						
C_{max} (ng/mL)	946.7	869.9	**8.11**	991.8	924.9	**6.75**
AUC_{0-t} (ng-h/mL)	8785.2	8720.9	**0.73**	8802.8	9093.4	**-3.30**
AUC_{0-Inf} (ng-h/mL)	9015.1	9057.6	**-0.47**	9003.8	9438.9	**-4.83**
Fed state						
C_{max} (ng/mL)	1255.9	1250.6	**0.42**	1375.68	1273.3	**7.44**
AUC_{0-t} (ng-h/mL)	15196.5	15549.6	**-2.32**	15215.12	16380.5	**-7.66**
AUC_{0-Inf} (ng-h/mL)	16263.7	15815.8	**2.75**	15640.22	16653.2	**-6.48**

FDC: Fixed-dose combination, PE: Prediction error, C_{max}: Peak drug concentration, AUC_{0-t}: Area under the curve from time zero to time t, AUC_{0-Inf}: Area under the curve from time zero to infinity

trend was also evident at pH 5.5. Despite the differences in dissolution, sitagliptin HCl from the FDC showed bioequivalence with Januvia®,[9] indicating that both salts demonstrated similar *in vivo* behavior, which could be due to the high solubility and high bioavailability of sitagliptin.[9,21] The physiological conditions of the GI tract, such as peristaltic motility,[22] appear to resemble the *in vitro* conditions, as evidenced from the similar *in vivo* performance. The *in vitro* difference during the initial phase of dissolution does not impact on the *in vivo* performance.

Metformin

The dissolution of FDC was slow at 0.5 h and followed by a similar release pattern in comparison with that of Glucophage® 1000 mg SR at pH 6.8. Similarly, the dissolution of FDC at pH 5.5 was slower by 5%-11% up to 10 hours. A similar trend was reflected *in vivo*,[9] indicating the discrimination ability of the dissolution method to mimic the *in vivo* environment.

Furthermore, levy plots were constructed to establish a mathematical relationship linking F_a to fraction of dissolved (F_d) for both sitagliptin and metformin. To establish said relationship, the respective *in vivo* plasma concentration profiles for sitagliptin and metformin were deconvoluted into the F_a and the F_d, which were derived from the respective *in vitro* dissolution profiles. The so-derived least-square regressions from the levy plots yielded essentially linear patterns (correlation coefficient >0.900) under both preprandial and postprandial conditions, demonstrating that the proposed *in vitro* biorelevant dissolutions can indeed explain the absorption of both sitagliptin and metformin satisfactorily. The constructed level "A" levy plots demonstrated that the chosen *in vitro* conditions are appropriate and are mimicking the *in vivo* environment.

Further, the predictability of the developed IVIVC models were estimated in terms of PE values for the validation of the correlation models. An evaluation of internal and external

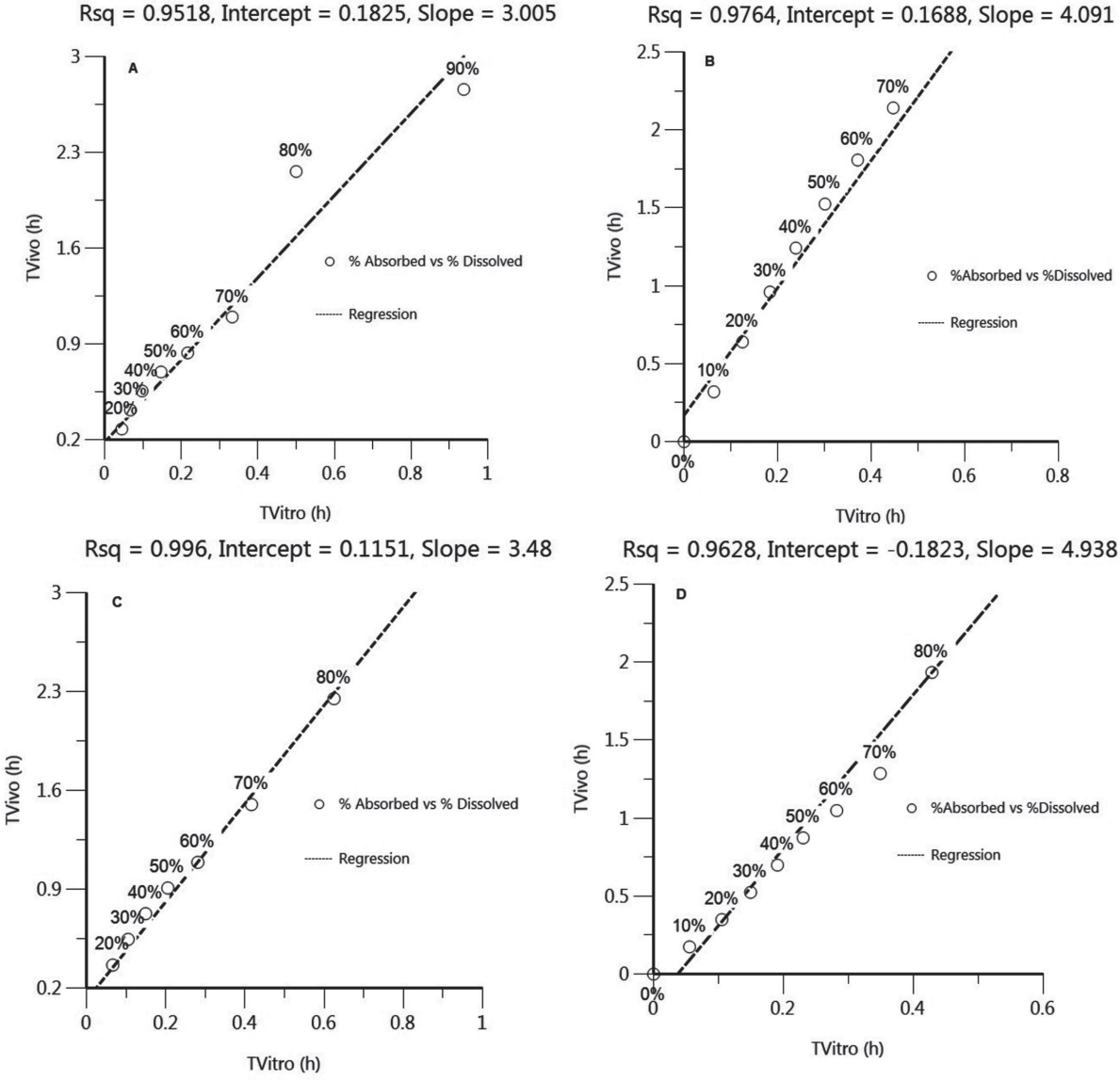

Figure 5. Levy plots for sitagliptin constructed using the fasted state data (A, B) and fed-state data (C, D). "A and C" denotes Januvia®, and "B and D" denote the fixed-dose combination

predictability for the pharmacokinetic parameters (C_{max}, AUC_{0-t}, and AUC_{0-Inf}) was below 10% and demonstrated the predefined internal and external validation criteria.[19] Overall, the attained IVIVC models yielded predicted C_{max} and AUC parameters below 10% of the observed values for both internal and external validations in both the fasted and the fed state. A proven IVIVC and a discriminatory *in vitro* method can serve as a surrogate for *in vivo* characterization and be used to select suitable formulations for *in vivo* studies.[23] The bioequivalence results also substantiated the validity of the developed IVIVC models for both sitagliptin and metformin; hence, the correlation models can be deemed to be robust and can therefore be considered for predicting *in vivo* performance.

The bioequivalence results reveal that FDC is well tolerated and safe for use in humans. From an *in vivo* behavior and safety perspective, the FDC product consisting of sitagliptin HCl and prolonged-release metformin HCl showed more or less similar *in vivo* behavior in comparison with the individual reference formulations. Therefore, FDC can enhance patient compliance, as it minimizes the consumption of individual products.

CONCLUSION

A robust IVIVC (level A) that meets the validation criteria for both internal and external predictability was established for sitagliptin and metformin prolonged-release formulations. Although the sitagliptin salt form is different in the test and

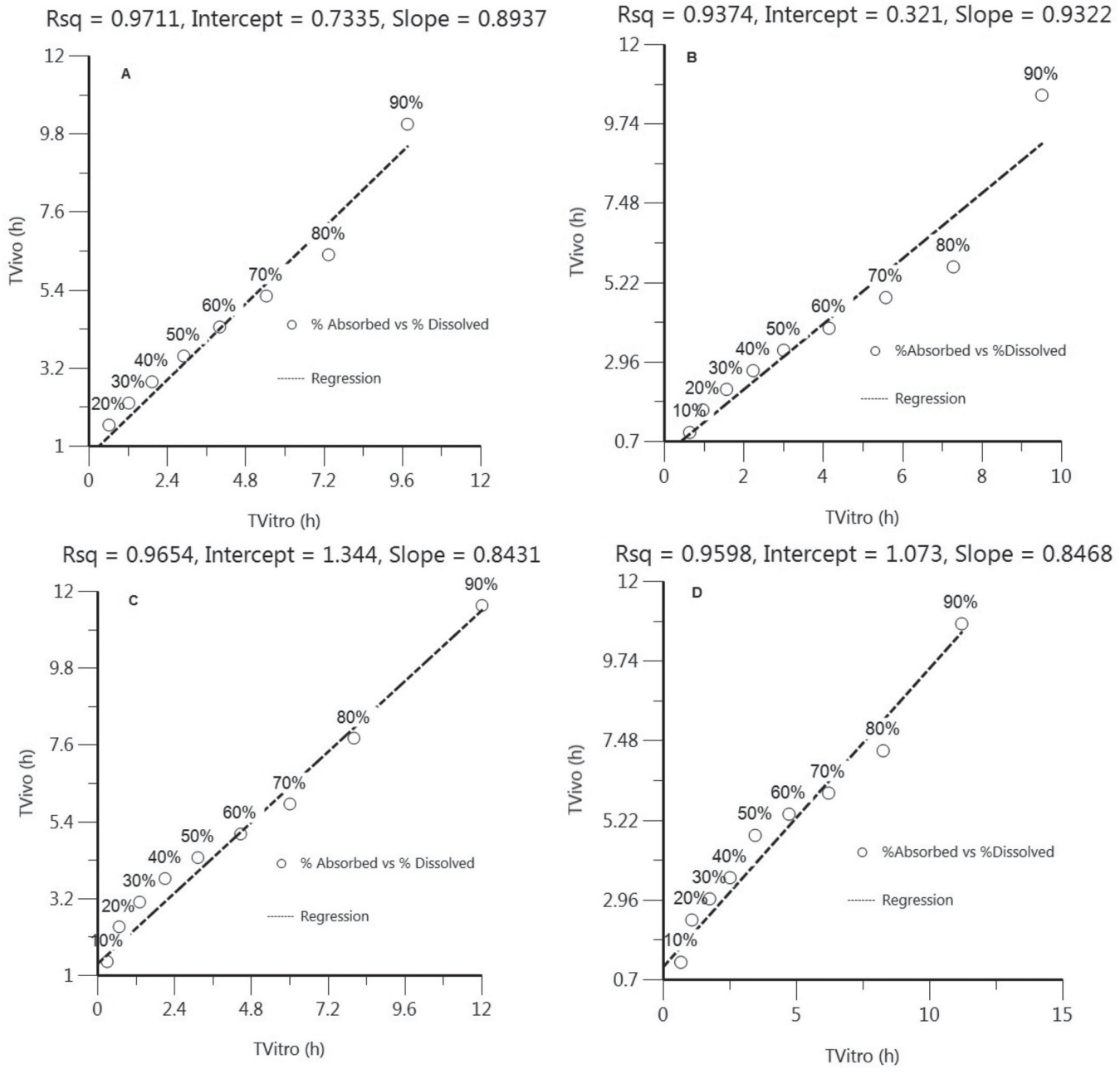

Figure 6. Levy plots for metformin constructed using the fasted state data (A, B) and fed-state data (C, D). "A and C" denotes Glucophage® SR 1000 mg, and "B and D" denote the fixed-dose combination

reference products (FDC contains sitagliptin hydrochloride, and Januvia® contains sitagliptin phosphate monohydrate), the developed IVIVC model exhibited good predictability. The correlation models can be used to predict formulations containing immediate-release sitagliptin salts (as HCl or phosphate monohydrate as the salts) and prolonged-release metformin HCl. The IVIVC can also be used as a surrogate for bioequivalence studies in the case of future formulation changes that are covered by the IVIVC release rates. The FDC comprising sitagliptin HCl and Metformin HCl was well tolerated in human volunteers, and the rate of AEs was similar to that of reference products; hence, FDC can be deemed safe for use in human subjects.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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REVIEW

Factors Associated with Antidepressant Medication Non-adherence

Antidepresan İlaç Uyunçsuzluğu ile İlişkili Faktörler

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ABSTRACT

Medication non-adherence is one of the major problems in treating patients with depression. Non-adherence results in an increased risk of relapse and reduced quality of life. The objective of this review was to review and summarize studies that focused on the factors associated with antidepressant medication non-adherence in patients with depression. Literature searches were performed using PubMed/Medline and Google Scholar. The search was limited to articles published in the English language in peer-reviewed journals between January 2000 and December 2019. Studies that analyzed factors of non-compliance in patients with depressive disorders were included in the review.

Patient-related factors such as forgetfulness, comorbidities, and misconceptions about the disease and medication, medication-related factors, polypharmacy, side effects, pill burden and cost, healthcare system-related factors, including physician-patient interactions, sociocultural factors such religious and cultural beliefs and stigma, and logistic factors were found to be the major factors associated with antidepressant non-adherence.

Efforts should be made to increase patient adherence to antidepressants by strengthening physician-patient relationships, simplifying medication regimens, and rectifying myths and beliefs held by patients with scientific information and explanations.

Key words: Adherence, antidepressants, depression, associated factors

ÖZ

İlaç uyumsuzluğu depresyon için hastaları tedavi ederken karşılaşılan önemli sorunlardan biridir. Uyumsuzluk relaps riskini artırır ve hayat kalitesini düşürür. Bu derlemenin amacı depresyonu olan hastalarda antidepresan ilaçlara uyumsuzluk ile ilgili çalışmaları değerlendirmek ve özetlemektir. Literatür araştırması PubMed/Medline ve Google Scholar kullanılarak yapılmıştır. Araştırma Ocak 2000 ile Aralık 2019 arasında İngilizce olarak bağımsız değerlendirme yapan dergilerde yayınlanan makalelerle sınırlandırılmıştır. Depresif bozukluğu olan hastalardaki uyumsuzlukla ilgili faktörler analiz eden çalışmalar derleme kapsamına alınmıştır. Antidepresan uyumsuzluğundaki önemli hasta ile ilgili faktörlerin unutma, komorbidite, hastalık ve ilaç hakkında yanlış bilgisi olma; ilaçla ilgili faktörlerin polifarmasi, yan etkiler, ilaç yükü ve tutarı; sağlık sistemiyle ilgili faktörlerin hekim-hasta ilişkileri ve sosyo-kültürel faktörlerin dini ve kültürel inançlar, ayıplama ve mantıksal faktörler olduğu bulunmuştur.

Antidepresanlara uyumu artırmak için hekim-hasta ilişkilerini güçlendirmek, dozlama rejimlerini basitleştirmek ve bilimsel enformasyon ve açıklamalarla hastalardaki mit ve inançları düzeltmek gibi konularda gayret gösterilmelidir.

Anahtar kelimeler: Uyum, antidepresanlar, depresyon, ilgili faktörler

INTRODUCTION

Depression has become a major public health concern with an increased prevalence and global disease burden due to the associated mental, social, and interpersonal dysfunction.[1] According to the World Health Organization, by 2020, depression will be the second-highest known cause of disability worldwide.[2] It is characterized by a sad mood, pessimistic thoughts, lowered interest in day-to-day activities, poor concentration, insomnia or increased sleep, significant weight loss or gain, decreased energy, continuous feelings of guilt and worthlessness, decreased libido, and suicidal thoughts occurring at least once every two weeks.[1,3] Antidepressant drugs are the most effective and widely used forms of treatment for depression.[4] Despite the availability of many effective antidepressants, 50% patients do

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not achieve a complete cure of symptoms and even experience recurrence.[5,6] Therefore, in many patients, depression becomes a chronic disorder and may require lifelong antidepressant treatment. For the desired treatment outcome, adherence to antidepressant medication plays a crucial role, and non-adherence is the key problem associated with antidepressant treatment. Adherence has been defined as "the extent to which a person's behavior regarding taking medication, following a diet, or executing a lifestyle (change) corresponds with recommendations from a healthcare provider".[7] The failure of patients to follow medical advice results in a risk of relapse and reduced quality of life. Many factors, be they patient, medicine, health system, or social and cultural factors, all are associated with patient non-adherence to prescribed antidepressants. Hence, this study was conducted to review and summarize studies focused on the factors associated with antidepressant medication non-adherence in patients with depression.

METHODS

Data sources, literature search, and selection

A comprehensive literature review was conducted using PubMed/Medline and Google Scholar. The search was limited to articles published in the English language in peer-reviewed journals between 2000 and 2019. The keywords used for the article search were depression, depressive patients, antidepressant, antidepressant adherence, patient compliance, and discontinuation of antidepressants. We also included articles listed in the author's reference lists and those listed in other systematic reviews. Studies were selected on the basis of relevance. Full articles on those studies that were deemed relevant to our study title were fully reviewed. This study is a narrative review and does not include any statistical analysis (Figure 1).

Study selection

For inclusion in our review, studies must have included adult or elderly patients, irrespective of gender, diagnosed with depression, and prescribed antidepressants by physicians. Literature could be of varying methodologies i.e. observational, prospective, cross-sectional, retrospective, or survey.

Our study outcomes were the factors that caused non-adherence to antidepressants among patients with depressive disorder. Studies that did not meet our criteria were excluded during the review. Studies were discarded if they were clinical trials or reviews.

RESULTS

One hundred fifty-five articles were selected by title/abstract; finally, 21 articles were included. Table 1 shows the main findings of these studies. Two studies were performed in Ethiopia,[8-11] one in Nepal,[9] one in Malaysia,[10] one in Island,[12]

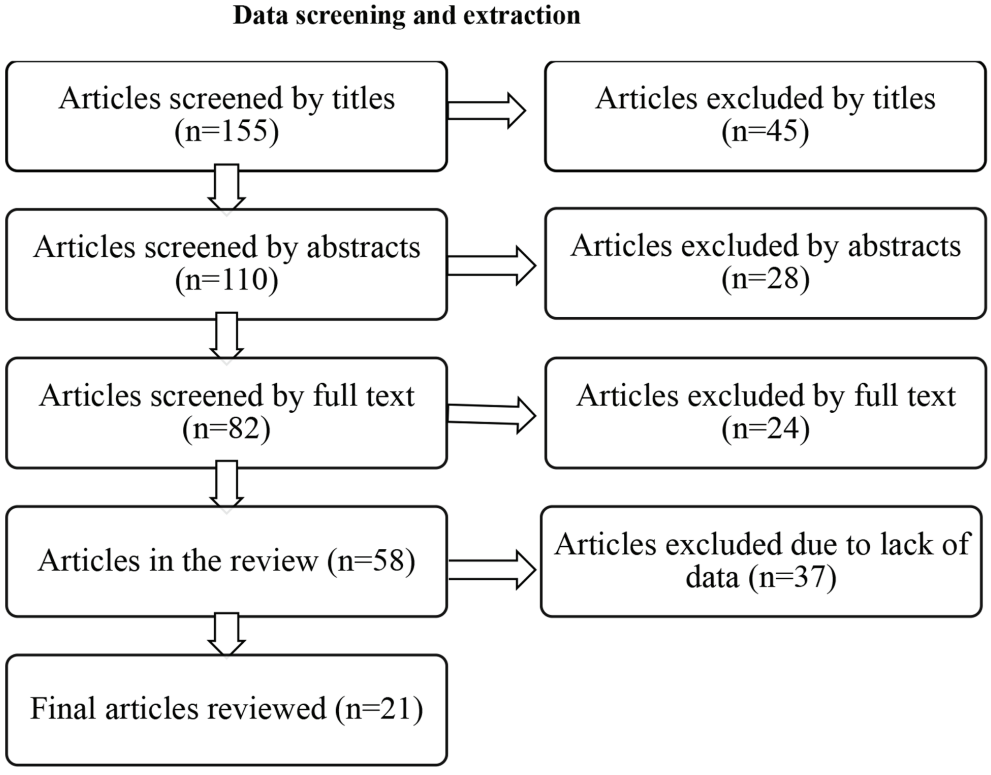

Figure 1. Data screening and extraction

Table 1. Objectives, methods, and major findings of the included studies

Study	Objective	Methodological review	Major findings
Woldekidan et al.[8]	To determine the patient satisfaction level and factors associated with psychiatric outpatient care	Institution-based cross-sectional study n=250 Age: ≥18 years Structured questionnaires used to collect data	Unsatisfied patients: 69.2% factor associated: Insufficient information on disease condition and drugs from healthcare professionals
Shrestha Manandhar et al.[9]	To determine the medication adherence pattern and factors associated with non-adherence	Hospital-based prospective study n=60 Age: >18 years Structured questionnaire for demographic details and medication adherence pattern	Only 37% were adherent. Factors in non-adherence: Forgetfulness, occupation of patients, cost of medicine, treatment duration, occurrence of adverse effects, patients' perception toward disease and drug, physician-patient relationship, and availability of medication
Ho et al.[10]	To determine the facilitators and barriers to antidepressant adherence among outpatients with MDD	Qualitative study n=30 Age: ≥18 years Semi-structured and individual in-depth interviews were conducted	Facilitators: perceived health benefits, regular activities, effective patient-provider relationships, and social support networks. Barriers: Incorrect beliefs about disease or medications, forgetfulness, negative attitudes, insufficient knowledge, and comorbidities
Abegaz et al.[11]	To determine the level of antidepressant adherence, clinical outcome, and magnitude of ADRs	The hospital-based prospective cross-sectional study n=217 Age: ≥18 years MMAS-8 was used	Low medication adherence was present in 57.1% of patients. Factors affecting adherence: Long-standing depression, distance from the follow-up clinic, and comorbid psychiatric illness
Telinoiu[12]	To measure adherence to antidepressant medication	Retrospective cohort study n=22.977 Age: 18-75 years Health Plan Employer Data and Information Set, and the proportion of days covered, to measure adherence	Adherent group: Older patients (aged ≥50 years), high economic status, females, patients with more follow-up visits, and patients with comorbid conditions such as diabetes, cardiovascular, respiratory, and mental illness
Lu et al.[13]	To determine the beliefs associated with antidepressants and associated adherence among older Chinese patients with major depression	Cross-sectional survey n=135 Age: >60 years MMAS, to measure adherence	Factors in non-adherence: Forgetfulness, discontinuing medications when feeling well, concern for long-term effects and addiction, and high-income respondents
Alekhya et al.[14]	To determine the sociodemographic factors that influence compliance with the treatment of depression	Cross-sectional study n=103 Age: >18 years Questionnaire format (for sociodemographic factors)	Non-adherent group (majority): 21-50 years of age, males, unmarried, low socioeconomic status, and a high level of education
Al Jumah et al.[15]	To determine antidepressant adherence and the factors associated with it	Non-experimental cross-sectional study n=403 Age: 18-60 years Used the MMAS	Factors in low adherence: Specific belief concerns, general harm, overuse beliefs about antidepressant medications and younger age (<40 years). More psychiatrist follow-up visits, effective physician-patient contact, and patient satisfaction increased adherence in patients
Serrano et al.[16]	To determine the adherence level and to analyze the sociodemographic factors and clinical profiles involved in adherence	Longitudinal observational study n=29 Age: ≥18 years Sociodemographic and clinical variables questionnaire, Simplified Medication Adherence Questionnaire, were used	Factors addressing compliance: Awareness of illness, positive attitude toward drugs, and tolerability (in particular regarding the side effects of medication)
Sultana et al.[17]	To determine the rate and predictors of antidepressant treatment discontinuation in depressed older patients	Population-based study n=39.557 Age: ≥65 years A nationwide Italian general practice "Health Search" database was used	Predictors of discontinuation of antidepressants: Polypharmacy, use of other classes of antidepressants other than SSRIs due to intolerance

Table 1. Continued

Study	Objective	Methodological review	Major findings
De las Cuevas et al.[18]	To determine the risk factors for antidepressant non-adherence	Population-based cohort study n=145 Age: ≥18 years The Morisky self-report scale was used to assess adherence	Factors associated with non-adherence: Negative attitude of patients toward their treatment, increased severity of depression, and the presence and severity of side effects
Banerjee and Varma[19]	To determine treatment non-adherence among patients with unipolar depression	Cross-sectional study n=239 Age: 18-60 years The MMAS was used	Non-adherent patients: 66.9% non-adherent group: Women (three times more than men), low socioeconomic status, lack of awareness about diagnosis, and inappropriate intake of medication
Jeon-Slaughter[20]	To determine the effect of patients' income on selective SSRI non-adherence	Population-based study n=280 Age: 18-64 years The National Comorbidity Survey-Replication was used	Factors leading to non-adherence: Low income level combined with lack of health insurance, African Americans vs Whites, and major depressive episode comorbidity
Fawzi et al.[21]	To determine antidepressant adherence in older patients in relation to their beliefs and Knowledge about these medications	Population-based study n=108 Age: >55 years The MARS and a Global Adherence Measure was used	Older patients are more likely to adhere to medication. Potential predictors of adherence: Sociodemographic, medication, and illness variables. Reasons for non-compliance: Insufficient knowledge about the prescribed medication
Park et al.[22]	To determine the factors associated with treatment-seeking in respondents with MDD	Population census n=362 Age: 18-64 years Data used from the Korean Epidemiologic Catchment Area study	Factors affecting treatment-seeking by individuals: Sociocultural factors such as misconception and stigma, severity of depression and comorbid conditions like anxiety, and obsessive-compulsive disorder
Tamburrino et al.[23]	To survey antidepressant adherence among primary care patients so as to better understand the factors associated with non-adherence	Survey n=148 Age: ≥18 years The Medication Adherence scale and the Medical Outcome study were used to measure adherence	The majority were female. Factors on non-adherence: Young age (<40 years), carelessness about taking medications, consciousness of side effects, dissatisfaction with physicians, and patients' demand for a specific antidepressant
Sawada et al.[24]	To determine the persistence and compliance with antidepressant drugs	Retrospective study n=367 Age: 16-82 years Medication Possession Rate was used	Persistent and compliant group: Older people, males, and sertraline users
Taj et al.[25]	To determine the predictors of non-adherence among psychiatric patients	Questionnaire-based cross-sectional study n=128 Age: ≥18 years A 19-item questionnaire was used	Mean age of patients: 39.49 years. Reasons for non-adherence: Comorbid condition, sedation, high medication cost, forgetfulness, and insufficient patient information
Russell and Kazantzis[26]	To determine belief and antidepressant adherence in primary care	Prospective study n=85 Age: 18-65 years The Beliefs about Medication Questionnaire and MARS were used	Reasons for antidepressant adherence: Patients were aware of the need for medication and had fewer symptoms of depression
Yeh et al.[27]	To determine the predictors of antidepressant adherence among depressive patients	Cross-sectional study n=181 Age: 23-61 years A self-report questionnaire was used to collect data	Predictors of adherence: Treatment efficacy, severity of depression, mental health professional-patient interaction, awareness of the need to continue medication, social support, and patient income. Negative beliefs and perceptions decrease medication adherence
Ashton et al.[28]	To identify the reasons for non-compliance with antidepressant medications	Survey n=344 Age: 18-65 years A 42-question survey was used	Treatment discontinuation: 60% of patients. Reason for non-compliance: Trouble remembering to take the drug, weight gain, inability to have an orgasm, and loss of interest in sex

MDD: Major depressive disorder, ADRs: Adverse drug reactions, SSRI: Selective serotonin reuptake inhibitor, MMAS: Morisky Medication Adherence scale, MARS: Medication Adherence Rating scale

two in China,[13,27] two in India,[14,17] one in Saudi Arabia,[15] two in Spain[16,19], one in Italy,[18] three in the United States,[20,23,28] one in the UK,[21] one in Korea,[22] one in Japan,[24] one in Pakistan,[25] and one in New Zealand.[26]

DISCUSSION

a. Sociodemographic factors

From this review, it was observed that the majority of patients who were non-adherent to their prescribed antidepressants were younger patients aged <40 years.[15,21,23] Non-adherence among younger patients could be due to having less experience with depression and associated medications. In contrast, older patients may have more experience with depressive episodes and antidepressants, which makes them more willing to complete their prescribed doses.[15,23] Additionally, antidepressants are associated with common side effects such as weight gain and impaired sexual function, which may make it troublesome for younger patients to adhere to antidepressant drugs.[15,21,23,29-31]

Female patients were found to be less adherent to prescribed antidepressants than males.[19,23,24,32] Women play multiple roles in the family and society, for example, as homemakers, spouses, mothers, professionals, and caregivers, which might cause them difficulty visiting the hospital and making them unable to adhere to their prescribed medications. However, findings contrary to these studies were reported by other works,[12,14] where males were less adherent to their regimens. This could be because they were not permitted leave from the office, or they may have been concerned about a pay deduction on the particular day they took leave to visit the hospital. Their inability to attend hospital visits on work days might have kept them from adhering to their treatment.[12,14,19,29,33]

One of the study[14] showed that non-adherence and level of education are inversely proportional. This means that when the level of education increases, adherence decreases, and vice versa. Highly educated people fear side effects and long-term effects of the drugs, which might be due to lack of appropriate information about disease and the drugs prescribed to them and their unwillingness to communicate with their healthcare personnel.[14,34]

Many studies predicted that people of low socioeconomic status would be less adherent to antidepressants.[12,14,19,20,27] This could be due to unemployment or an unstable income, inability to afford medications in the long term, and frequent appointments with their physician becoming expensive for them, which leads to premature discontinuation and non-adherence to drug regimens.[27,35] In contrast, another work[13] found that patients with a high socioeconomic status were less adherent to antidepressants. This could be because higher-income people generally have a high social status and education level. They might be more concerned about the side effects or potential for dependence on antidepressants, which finally leads to decreased adherence.

b. Patient-related factors

From the literature review, patients often forgetting to take their medications was found to be a common patient-related factor in antidepressant non-adherence.[9,10,13,25,28,36,37] Inappropriate intake of medication or patient carelessness were other reasons for non-adherence.[9,19,23]

Patients have incorrect beliefs about the disease itself or prescribed antidepressants.[10,13,15,22,27] From the findings of our review, a poor understanding of mental illness and medication was found to be the main barrier to depression treatment and hence, to adherence. Even after being diagnosed with depression, many did not consider themselves as having a mental illness. They believed they could easily overcome their mental illness on their own.[22,38] They also believed that it would be resolved by positive thinking or having a complete rest without taking antidepressants.[3] Some people accept depression as a normal part of their aging process, and some even consider it as a result of bad fortune in their lives or a weak personality, rather than a mental illness.[39,40]

The majority of patients taking antidepressants have misconceptions about them. They believe there is no need to take medication in the absence of any signs or symptoms. They believe they can take less medication or simply discontinue the medication themselves when they feel better.[10,13,41,42]

The patient's decision to adhere to antidepressants mainly depends on the balance between necessity and concern about the safety and efficacy of the prescribed medication.[43,44] Therefore, many people believe that long-term use of antidepressants is toxic and may lead to kidney damage. They were also concerned with the potential for addiction and psychological dependence on antidepressants, all of which affect their adherence to treatment.[10,13,15]

Patients with a positive attitude towards their disease and medication and an awareness of their illness adhere more to their regimens.[9,10,16,18,19,26,27] They believe they will return to normal functioning if they continue to take their medications. They will communicate regularly with their physicians to enhance their knowledge about their own mental illnesses and medications.[10] All this will help them cope with the response and lead to better health outcomes.[45]

Comorbidity, polypharmacy, and non-adherence are inter-related with each other. Comorbidity increases the number of medications be taken by the patients.[10,17,25] Patients will feel pill burdens and even an economic burden because they must take different types of medications. The complex dosing schedule and the intention to save money may force patients to discontinue their medication, leading to non-compliance.[10,20] Comorbidity also leads to logistic problems, as patients must seek medical advice from more than one physician, obtaining appointments and timely medication refills time and again.[25]

c. Medication-related factors

It is evident from many studies that the majority of patients refuse to continue antidepressants due to the prevalent side effects of the drug prescribed.[9,11,13,18,25,26,28] Patients mostly prefer

medications that have a lower risk of weight gain, sexual dysfunction, and fatigue.[28] Antidepressants are reported to cause many adverse effects such as sedation, restlessness, tremor, dry mouth, decreased libido, weight gain, irregular menstrual cycles, and impotence. These unpleasant effects may impair patient quality of life and self-esteem, resulting in non-adherence to prescribed antidepressants.[46-48] In two of the studies,[17,24] patients prescribed with antidepressants other than selective serotonin reuptake inhibitors (SSRIs) were more likely to be non-adherent. This might be due to the lower side effect profile, low overdose-related cardiotoxicity, better tolerability, and overall favorable risk-benefit ratio attributed to SSRIs.[11,49-52]

Adherence to treatment is greatly influenced by physician-patient interactions. One of the common reason for non-adherence was the failure of physicians to provide adequate information on patients' illness and medications prescribed.[9,15,21,25,27] The studies found that the most common patient complaint was the failure of the physician to explain the timing and dosing of the medication completely, along with the benefits of therapy, and the consequence was non-adherence.[21,25,46,53] Similarly, not trusting the physician and dissatisfaction with the physician and their prescribed medication also led to patients being non-adherent to their treatment.[10,15,23,54,55]

Due to multiple prescribers, problems communicating with physicians, frequent follow-up, long waiting times in hospitals, repeated medication refills, and unavailability of prescribed medications,[19] many patients choose to discontinue their medications.[9,10] Patients lose confidence in their physician when there are multiple prescribers, which ultimately affects their medication-taking behavior. Some patients alter/stop their medication without informing their physician, as they find it difficult to communicate with them. To avoid long wait times, patients skip their appointments, leading to an insufficient supply of medication at home.[10]

d. Sociocultural factors

Lower adherence to medication depends on the patient's perception of their illness, which may differ according to the religion and culture to which they belong.[13,56] A study[10] collected the beliefs of people from different religions and cultures. It stated that people from Malaysia believe that mental illness is a social punishment for particular people, or an illness of the soul caused by weakness of the spirit. Similarly, Chinese people believe that mental illness is a symbol of lack of self-worth, which is measured in terms of education and monetary gain that brings honor to the family. Likewise, Indians believe that one gets a mental illness from evildoers.[57] All of these beliefs create barriers to pursuing and sticking to antidepressant regimens. Some patients want to determine experimentally whether being prayerful will cure their depression, which forces them to stop taking the medication.[10,13,38]

Depression is still considered a social stigma. Many regard it as a sign of a personal weakness.[26] Regular social support and motivation from family members and co-workers help depressive patients get going their antidepressant treatment.[22,27,58] Due to fear of being stigmatized by society, many patients do not reveal their mental illness,[22] which influences their adherence to medication. Unsupportive family members and co-workers also discourage patients from continuing their medication,[10] which further worsens their mental illness.

e. Logistical factors

Many patients who live far from city areas or hospitals have poor access to healthcare facilities and hence hinder patients from adhering to antidepressants.[9-11]

CONCLUSION

From our review, we conclude that patient-related factors such as forgetfulness, comorbidity, and misconception about the disease and medication; medication-related factors such as polypharmacy, side effects, pill burden, and cost; healthcare-system-related factors including the physician-patient interaction; sociocultural factors such as religious and cultural beliefs and stigma; and logistic factors are the major barriers to antidepressant adherence. Hence, efforts should be made to increase patient adherence by strengthening physician-patient relationships. Physicians should emphasize patient education that includes an explanation of the drug, dosage, duration, and timing of administration, possible side effects, adverse effects, lag time before the onset of treatment and relief of symptoms, and consequences of non-adherence. In the case of comorbid conditions, physicians should simplify the medication regimen. Additionally, they should focus on rectifying the myths and beliefs held by patients with scientific information and explanations.

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