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AIMS AND SCOPE

The Turkish Journal of Pharmaceutical Sciences is the only scientific periodical publication of the Turkish Pharmacists' Association and has been published since April 2004.
Turkish Journal of Pharmaceutical Sciences journal is regularly published 6 times in a year (February, April, June, August, October, December). The issuing body of the journal is Galenos Yayinevi/Publishing House level.
The aim of Turkish Journal of Pharmaceutical Sciences is to publish original research papers of the highest scientific and clinical value at an international level. The target audience includes specialists and professionals in all fields of pharmaceutical sciences.
The editorial policies are based on the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (ICMJE Recommendations)" by the International Committee of Medical Journal Editors (2013, archived at http://www.icmje.org/) rules.

Editorial Independence

Turkish Journal of Pharmaceutical Sciences is an independent journal with independent editors and principles and has no commerical relationship with the commercial product, drug or pharmaceutical company regarding decisions and review processes upon articles.

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All correspondence should be directed to the Turkish Journal of Pharmaceutical Sciences Editorial Board

Post Address: Turkish Pharmacists' Association, Mustafa Kemal Mah 2147.Sok No:3 06510 Çankaya/Ankara, TURKEY
Phone: +90 (312) 409 81 00
Fax: +90 (312) 409 81 09
Web Page: http://turkjps.org
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ISSUING BODY CORRESPONDING ADDRESS

Issuing Body : Galenos Yayınevi
Address: Molla Gürani Mah. Kaçamak Sk. No: 21/1, 34093 İstanbul, Turkey
Phone: +90 212 621 99 25 Fax: +90 212 621 99 27
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Turkish Journal of Pharmaceutical Sciences journal is published 6 times (February, April, June, August, October, December) per year and publishes the following articles:

• Research articles

• Reviews (only upon the request or consent of the Editorial Board)

• Preliminary results/Short communications/Technical notes/Letters to the Editor in every field of pharmaceutical sciences.

The publication language of the journal is English.

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Experimental, clinical and drug studies requiring approval by an ethics committee must be submitted to the JOURNAL with an ethics committee approval report including approval number confirming that the study was conducted in accordance with international agreements and the Declaration of Helsinki (revised 2013) (http://www.wma.net/en/30publications/10policies/b3/). The approval of the ethics committee and the fact that informed consent was given by the patients should be indicated in the Materials and Methods section. In experimental animal studies, the authors should indicate that the procedures followed were in accordance with animal rights as per the Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guide.pdf) and they should obtain animal ethics committee approval.

Authors must provide disclosure/acknowledgment of financial or material support, if any was received, for the current study.

If the article includes any direct or indirect commercial links or if any institution provided material support to the study, authors must state in the cover letter that they have no relationship with the commercial product, drug, pharmaceutical company, etc. concerned; or specify the type of relationship (consultant, other agreements), if any.

Authors must provide a statement on the absence of conflicts of interest among the authors and provide authorship contributions.

All manuscripts submitted to the journal are screened for plagiarism using the 'iThenticate' software. Results indicating plagiarism may result in manuscripts being returned or rejected.

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This is an independent international journal based on double-blind peer-review principles. The manuscript is assigned to the Editor-in-Chief, who reviews the manuscript and makes an initial decision based on manuscript quality and editorial priorities. Manuscripts that pass initial evaluation are sent for external peer review, and the Editor-in-Chief assigns an Associate Editor. The Associate Editor sends the manuscript to at least two reviewers (internal and/or external reviewers). The Associate Editor recommends a decision based on the reviewers' recommendations and returns the manuscript to the Editor-in-Chief. The Editor-in-Chief makes a final decision based on editorial priorities, manuscript quality, and reviewer recommendations. If there are any conflicting recommendations from reviewers, the Editor-in-Chief can assign a new reviewer.

The scientific board guiding the selection of the papers to be published in the Journal consists of elected experts of the Journal and if necessary, selected from national and international authorities. The Editor-in-Chief, Associate Editors may make minor corrections to accepted manuscripts that do not change the main text of the paper.

In case of any suspicion or claim regarding scientific shortcomings or ethical infringement, the Journal reserves the right to submit the manuscript to the supporting institutions or other authorities for investigation. The Journal accepts the responsibility of initiating action but does not undertake any responsibility for an actual investigation or any power of decision.

The Editorial Policies and General Guidelines for manuscript preparation specified below are based on "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (ICMJE Recommendations)" by the International Committee of Medical Journal Editors (2013, archived at http://www.icmje.org/).

Preparation of research articles, systematic reviews and meta-analyses must comply with study design guidelines:

CONSORT statement for randomized controlled trials (Moher D, Schultz KF, Altman D, for the CONSORT Group. The CONSORT statement revised recommendations for improving the quality of reports of parallel group randomized trials. JAMA 2001; 285: 1987-91) (http://www.consort-statement.org/);

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).

GENERAL GUIDELINES

Manuscripts can only be submitted electronically through the Journal Agent website (http://journalagent.com/tjps/) after creating an account. This system allows online submission and review.

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Format: Manuscripts should be prepared using Microsoft Word, size A4 with 2.5 cm margins on all sides, 12 pt Arial font and 1.5 line spacing.

Abbreviations: Abbreviations should be defined at first mention and used consistently thereafter. Internationally accepted abbreviations should be used; refer to scientific writing guides as necessary.

Cover letter: The cover letter should include statements about manuscript type, single-Journal submission affirmation, conflict of interest statement, sources of outside funding, equipment (if applicable), for original research articles.

ETHICS COMMITTEE APPROVAL

The editorial board and our reviewers systematically ask for ethics committee approval from every research manuscript submitted to the Turkish Journal of Pharmaceutical Sciences. If a submitted manuscript does not have ethical approval, which is necessary for every human or animal experiment as stated in international ethical guidelines, it must be rejected on the first evaluation.

Research involving animals should be conducted with the same rigor as research in humans; the Turkish Journal of Pharmaceutical Sciences asks original approval document to show implements the 3Rs principles. If a study does not have ethics committee approval or authors claim that their study does not need approval, the study is consulted to and evaluated by the editorial board for approval.

SIMILARITY

The Turkish Journal of Pharmaceutical Sciences is routinely looking for similarity index score from every manuscript submitted before evaluation by the editorial board and reviewers. The journal uses iThenticate plagiarism checker software to verify the originality of written work. There is no acceptable similarity index; but, exceptions are made for similarities less than 15 %.

REFERENCES

Authors are solely responsible for the accuracy of all references.

In-text citations: References should be indicated as a superscript immediately after the period/full stop of the relevant sentence. If the author(s) of a reference is/are indicated at the beginning of the sentence, this reference should be written as a superscript immediately after the author's name. If relevant research has been conducted in Turkey or by Turkish investigators, these studies should be given priority while citing the literature.

Presentations presented in congresses, unpublished manuscripts, theses, Internet addresses, and personal interviews or experiences should not be indicated as references. If such references are used, they should be indicated in parentheses at the end of the relevant sentence in the text, without reference number and written in full, in order to clarify their nature.

References section: References should be numbered consecutively in the order in which they are first mentioned in the text. All authors should be listed regardless of number. The titles of Journals should be abbreviated according to the style used in the Index Medicus.

Reference Format

Journal: Last name(s) of the author(s) and initials, article title, publication title and its original abbreviation, publication date, volume, the inclusive page numbers. Example: Collin JR, Rathbun JE. Involutional entropion: a review with evaluation of a procedure. Arch Ophthalmol. 1978;96:1058-1064.

Book: Last name(s) of the author(s) and initials, book title, edition, place of publication, date of publication and inclusive page numbers of the extract cited.

Example: Herbert L. The Infectious Diseases (1st ed). Philadelphia; Mosby Harcourt; 1999:11;1-8.

Book Chapter: Last name(s) of the author(s) and initials, chapter title, book editors, book title, edition, place of publication, date of publication and inclusive page numbers of the cited piece.

Example: O'Brien TP, Green WR. Periocular Infections. In: Feigin RD, Cherry JD, eds. Textbook of Pediatric Infectious Diseases (4th ed). Philadelphia; W.B. Saunders Company;1998:1273-1278.

Books in which the editor and author are the same person: Last name(s) of the author(s) and initials, chapter title, book editors, book title, edition, place of publication, date of publication and inclusive page numbers of the cited piece. Example: Solcia E, Capella C, Kloppel G. Tumors of the exocrine pancreas. In: Solcia E, Capella C, Kloppel G, eds. Tumors of the Pancreas. 2nd ed. Washington: Armed Forces Institute of Pathology; 1997:145-210.

TABLES, GRAPHICS, FIGURES, AND IMAGES

All visual materials together with their legends should be located on separate pages that follow the main text.

Images: Images (pictures) should be numbered and include a brief title. Permission to reproduce pictures that were published elsewhere must be included. All pictures should be of the highest quality possible, in

JPEG format, and at a minimum resolution of 300 dpi.

Tables, Graphics, Figures: All tables, graphics or figures should be enumerated according to their sequence within the text and a brief descriptive caption should be written. Any abbreviations used should be defined in the accompanying legend. Tables in particular should be explanatory and facilitate readers' understanding of the manuscript, and should not repeat data presented in the main text.

MANUSCRIPT TYPES

Original Articles

Clinical research should comprise clinical observation, new techniques or laboratories studies. Original research articles should include title, structured abstract, key words relevant to the content of the article, introduction, materials and methods, results, discussion, study limitations, conclusion references, tables/figures/images and acknowledgement sections. Title, abstract and key words should be written in both Turkish and English. The manuscript should be formatted in accordance with the above-mentioned guidelines and should not exceed 16 A4 pages.

Title Page: This page should include the title of the manuscript, short title, name(s) of the authors and author information. The following descriptions should be stated in the given order:

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1. Title of the manuscript (Turkish and English), as concise and explanatory as possible, including no abbreviations, up to 135 characters

2. Short title (Turkish and English), up to 60 characters

3. Name(s) and surname(s) of the author(s) (without abbreviations and academic titles) and affiliations

4. Name, address, e-mail, phone and fax number of the corresponding author

5. The place and date of scientific meeting in which the manuscript was presented and its abstract published in the abstract book, if applicable

Abstract: A summary of the manuscript should be written in both Turkish and English. References should not be cited in the abstract. Use of abbreviations should be avoided as much as possible; if any abbreviations are used, they must be taken into consideration independently of the abbreviations used in the text. For original articles, the structured abstract should include the following sub-headings:

Objectives: The aim of the study should be clearly stated.

Materials and Methods: The study and standard criteria used should be defined; it should also be indicated whether the study is randomized or not, whether it is retrospective or prospective, and the statistical methods applied should be indicated, if applicable.

Results: The detailed results of the study should be given and the statistical significance level should be indicated.

Conclusion: Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

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.

Original research articles should have the following sections:

Introduction: Should consist of a brief explanation of the topic and indicate the objective of the study, supported by information from the literature.

Materials and Methods: The study plan should be clearly described, indicating whether the study is randomized or not, whether it is retrospective or prospective, the number of trials, the characteristics, and the statistical methods used.

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.

Discussion: The study results should be discussed in terms of their favorable and unfavorable aspects and they should be compared with the literature. The conclusion of the study should be highlighted.

Study Limitations: Limitations of the study should be discussed. In addition, an evaluation of the implications of the obtained findings/results for future research should be outlined.

Conclusion: The conclusion of the study should be highlighted.

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.

References: Authors are responsible for the accuracy of the references. See General Guidelines for details about the usage and formatting required.

Review Articles

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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Dear Reviewers

We would like to thank you for your contributions in "Turkish Journal of Pharmaceutical Sciences" by being as a reviewer and interest to our journal in 2021.

We wish you every success in your academic career.

Sincerely

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EDITORIAL PREFACE

Dear Colleagues,

In the last two years, the transition from E-SCI to SCI-E for Turkish Journal of Pharmaceutical Sciences' has been achieved in a rapid way with an increasing acceleration. During this transition process, I as a Chief-Editor, and the Editors Prof. Samiye Yabanoğlu Çiftçi and Prof. Pınar Erkekoğlu have joined the national and international Editorial meetings which are held by Galenos and the other publishers. I must admit that we are on the right track for SCI-E, but not completed our journey yet. In the six volumes of 2020 and 2021, we published at most one letter to the editor or short research, at least 13 research articles, and a compilation paper. To avoid the accepted review papers waiting for too long to be published, we decided to publish 12 research articles and 2 compilations in 6 volumes during the year 2022. In each volume of TJPS, we try to publish articles that contain current topics and create excitement in the field of pharmaceutical sciences for readers. The articles that will be published are planned according to acceptance order. Referee and download status of the manuscripts are posted on our journal's web site. To guide the authors, the statistics tab of our journal website, acceptance, rejection, and assessment durations of the candidate articles are shared with the authors. No articles without the Ethical permission could be shared in our journal. We appreciate Turkish Pharmacists Association that is continously supporting Turkish Journal of Pharmaceutical Science and made publishing possible.

In the sixth volume you are reading right now, I would like to thank to the academics who accepted to be reviewers and assessed the articles. The increase in the number of articles causes the need for multiple assessment by a reviewer and hence, endless thanks to all our reviewers including the Editors and the Editorial board. Lastly, I would like to congratulate all the Turkish and international authors whose articles have been published in the year 2020 and 2021.

I wish you all a happy and successful new year.

Prof. Terken Baydar
Chief-Editor

EDİTÖR ÖNSÖZÜ

Sevgili Meslektaşlarım,

Turkish Journal of Pharmaceutical Sciences isimli uluslararası dergimizin E-SCI'den SCI-E indeksleri arasında geçiş çalışmaları, son 2 yıldır ivmelenerek devam etmiştir. Bu süreçte, Galenos ve diğer yayınevleri tarafından konuyla ilgili yapılan ulusal ve uluslararası editör toplantılarına ben ve editörler Prof. Dr. Samiye Yabanoğlu Çiftçi ve Prof. Dr. Pınar Erkekoğlu ile katıldık. SCI-E için doğru yolda olduğumuzu, ancak henüz yolu kolaylamadığımızı ifade etmeliyim. 2020 ve 2021 yıllarında altışar sayı çıkardığımız dergimizde, bu sayılarda en fazla 1 editöre mektup veya kısa araştırma, en az 13 araştırma makalesi ve 1 derleme yayımladık. Ancak, kabul alan yazarları fazla bekletmemek için, 2022 yılında da planlanan 6 sayının dağılımında, 12 araştırma makalesi ve 2 derleme yayımlama kararı aldık. Her sayıda güncel konuları içeren ve eczacılık alanında merak uyandıran makaleleri yayınlamaya çalışıyoruz. Dergimizin sayılarında yayınlanacak, makaleler, kabul sırasına göre planlanmaktadır. Basılan makalelere atıfta bulunma ve indirme durumları internet sitemizde yayınlanmaktadır. Bunun dışında istatistik sekmesinde, kabul, ret ve değerlendirme süreçleri aday makale yazarları ile paylaşılmakta ve onlara yönlendirici olmayı hedeflemektedir. Dergimizde, etik izin belgesi olmayan hiçbir makale yayınlanmamaktadır. Turkish Journal of Pharmaceutical Science'ı maddi olarak destekleyen ve basılmasını sağlayan meslek örgütümüz Türk Eczacıları Birliği'ne şükranlarımızı sunarız.

Bu yazıyı okuduğunuz 6. sayımızda, yıl içerisinde hakem olmayı kabul ederek, teslim edilen makaleleri değerlendiren akademisyenlere teşekkür etmek istedim. Fazla makale teslimi, bir yılda her hakemin çok kez değerlendirmesi ihtiyacını oluşturmaktadır. Editörler ve Editörler Kurulu dahil tüm hakemlerimize sonsuz teşekkürler. 2020 ve 2021 yıllarında yazar olarak misafir olan ulusal ve uluslararası tüm yazarları da tebrik ederim.

Yeni yılınızı kutlar, sağlıklı ve başarılı bir yıl geçirmemizi dilerim.

Prof. Dr. Terken Baydar
Baş Editör

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LETTER TO EDITOR

The Potential Drug Interactions Between Multiple Sclerosis and COVID-19 Therapies

Multipl Skleroz ve COVID-19 Tedavileri Arasındaki Olası İlaç Etkileşimleri

Cansu GÖNCÜOĞLU[1]*, Aygin BAYRAKTAR EKİNCİOĞLU[1], Aslı TUNCER[2]

[1]Hacettepe University Faculty of Pharmacy, Department of Clinical Pharmacy, Ankara, Turkey
[2]Hacettepe University Faculty of Medicine, Department of Neurology, Ankara, Turkey

Key words: Multiple sclerosis, COVID-19, disease-modifying therapy, interaction
Anahtar kelimeler: Multipl skleroz, COVID-19, hastalık modifiye edici tedaviler, etkileşim

Dear Editor,

Since December 2019, the coronavirus disease-2019 (COVID-19) has spread rapidly all over the world and has severely affected the elderly population and patients with comorbidities. Drug interactions with agents used in the treatment of multiple sclerosis (MS) such as interferon beta, glatiramer acetate, ocrelizumab, natalizumab, and alemtuzumab are rare. Therefore, interactions between disease-modifying therapies (fingolimod, teriflunomide, dimethyl fumarate, and cladribine) and COVID-19 drugs[1] are outlined in this letter.

It is important to closely monitor all drugs used in COVID-19 treatment in terms of their adverse effects and drug-drug interactions. Favipiravir, remdesivir, lopinavir/ritonavir, colchicine, and tocilizumab are now used in the treatment of COVID-19 and are being studied continuously. Corticosteroids, which have been shown to be effective in COVID-19 depending on the time of use, are also utilized in the treatment of MS relapses, so no interaction is expected with their use.[2]

In patients with MS with concomitant COVID-19 infection, drug-drug interactions can partially affect the outcomes of treatment. In these patients, if disease-modifying agents and COVID-19 drugs are going to be used together, the neurologist should be aware of the potential adverse effects and drug interactions. It has been known that levels of alanin aminotranspherase (ALT) and aspartate aminotranspherase (AST) may increase with the use of teriflunomide (12-14%), fingolimod (15%), and dimethyl fumarate (4%).[3] Since increased levels of ALT and AST have also been reported with the use of COVID-19 therapies such as favipiravir (13%), lopinavir/ritonavir (1-11%), remdesivir (3-6%), tocilizumab (<22-36%), and colchicine, liver function tests should be monitored in patients with MS diagnosed with COVID-19 receiving these drugs.[3] Cladribine has a low rate of hepatic metabolism (<10%; causes hepatic injury in <1% of patients) and low risk of interaction with other drugs. Therefore, no drug interaction is expected between cladribine and drugs used in the treatment of COVID-19.[3] Moreover, colchicine may demonstrate neurotoxic effects. Hence, patients with MS who are to be given colchicine should be monitored for additional weakness and neuropathy. Considering that colchicine is a substrate of cytochrome P450 family 3 subfamily A member 4 (CYP3A4), coadministration of colchicine with lopinavir/ritonavir, which is a potent inhibitor of the CYP3A4 enzyme, is not recommended as it will increase the blood level of colchicine and its adverse effects.[4] Teriflunomide inhibits CYP2C8 and induces the CYP1A2 enzyme.[3] Thus, it should be used carefully with substrates of these enzymes such as remdesivir. Therefore, concurrent use of teriflunomide and remdesivir may increase blood concentration of remdesivir, which can potentially magnify its undesirable effects, which include rash,

*Correspondence: cansugoncuoglu@gmail.com, Phone: +90 544 679 92 70, ORCID-ID: orcid.org/0000-0003-1415-4708
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diarrhea, hypotension, nausea, abnormal liver function, and renal impairment. These were seen in 60% of patients under remdesivir treatment.[5] Fingolimod is mainly metabolized by the CYP4F2 enzyme and is a minor substrate for the CYP2D6, 2E1, and 3A4 enzymes.[3] It can be assumed that an interaction between fingolimod and lopinavir/ritonavir is possible since the latter is a potent inhibitor of CYP3A4 and CYP2D6. However, this interaction is not expected to be clinically significant because fingolimod is a minor substrate. Lastly, dimethyl fumarate is metabolized through the tricarboxylic acid cycle and is not involved in the CYP450 enzyme system.[3]

A majority of drug-drug interactions may be predicted and prevented. Therefore, it is important to be vigilant about potential drug interactions and to adjust clinical practice according to recent scientific evidence.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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ORIGINAL ARTICLE

Ethnopharmacobotanical Findings of Medicinal Plants in the Kızılcahamam District of Ankara, Turkey

Ankara'nın Kızılcahamam İlçesi (Türkiye) Tıbbi Bitkilerinin Etnofarmakobotanik Bulguları

İlhan GÜRBÜZ[1]*, Gizem ÖZATKAN[2], Galip AKAYDIN[3], Tuğba GÜNBATAN[1]

[1]Gazi University Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey
[2]Turkish Medicines and Medical Devices Agency, Department of Medicines Marketing Authorization, Ankara, Turkey
[3]Hacettepe University Faculty of Vocational Education, Department of Biology, Ankara, Turkey

ABSTRACT

Objectives: Folk medicines in Kızılcıhamam has not been investigated in detail so far. Thus, this study aimed to conduct a comprehensive investigation of folk medicine in the Kızılcahamam district.

Materials and Methods: Nine scientific field trips were organized to Kızılcahamam between April 2007 and July 2008. Data were obtained by field interviews with local people using open and semi-structured questionnaires. Results were evaluated statistically with the "use-value", "informant consensus factor" and cultural importance index.

Results: Sixty-five species (69 taxa) that belong to 58 genera of 31 families were determined to be used as folk medicines. To the best of our knowledge, this is the first study to record four of these species as folk medicines. Plants from Compositae, Lamiaceae and Rosaceae families were used most frequently as folk medicines in Kızılcahamam. Plants in the study area are mainly used for gastrointestinal system problems, respiratory disorders, and urinary tract diseases. Residents from 41% of the villages where the scientific trips were carried out, declared that they are not using or interested in folk medicines.

Conclusion: This study highlights once again the gradual reduction of folk medicinal knowledge and the urgent need for folk medicine investigations in all parts of Turkey.

Key words: Ankara, folk medicines, ethnobotany, medicinal plants, Kızılcahamam

ÖZ

Amaç: Kızılcahamam halk ilaçları şu ana kadar detaylı bir şekilde araştırılmamıştır. Bu nedenle, bu çalışmada Kızılcahamam ilçesinin halk ilaçlarının kapsamlı bir şekilde incelenmesi amaçlanmıştır.

Gereç ve Yöntemler: 2007 yılı Nisan ayı ile 2008 yılı Temmuz ayları arasında Kızılcahamam'a dokuz bilimsel saha gezisi düzenlenmiştir. Veriler saha çalışmaları esnasında yerel halk ile yapılan açık ve yarı yapılandırılmış bir anket kullanılarak elde edilmiştir. Sonuçlar istatistiksel olarak "kullanım değeri", "bilgilendirici fikir birliği faktörü" ve "kültürel önem endeksi" hesaplanarak değerlendirilmiştir.

Bulgular: Otuz bir familyadan 58 cinse ait 65 türün (69 takson) halk ilacı olarak kullanıldığı belirlenmiştir. Bildiğimiz kadarıyla bu türlerden dördü ilk kez bu çalışma ile halk ilacı olarak kayıt altına alınmıştır. Kızılcahamam'da en çok Compositae, Lamiaceae ve Rosaceae familyalarından bitkilerin halk ilacı olarak kullanıldığı belirlenmiştir. Çalışma alanındaki bitkiler, ağırlıklı olarak mide-bağırsak sistemi problemleri, solunum ve idrar yolu hastalıkları için kullanılmaktadır. Bilimsel gezilerin yapıldığı köylerin %41'inde görüşülen kişiler halk ilaçları kullanmadıklarını veya halk ilaçları ile ilgilenmediklerini beyan etmişlerdir.

Sonuç: Türkiye'nin her bölgesinde halk ilacı bilgisinin giderek azaldığı ve halk ilacı araştırmalarına acil ihtiyaç duyulduğu bu çalışma ile bir kez daha vurgulanmıştır.

Anahtar kelimeler: Ankara, halk ilaçları, etnobotani, tıbbi bitkiler, Kızılcahamam

***Correspondence:** igurbuz@gazi.edu.tr or ilgurbuz@gmail.com , **Phone:** +90 312 202 31 79, **ORCID-ID:** orcid.org/0000-0002-3670-0899

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INTRODUCTION

Turkey has a quite rich flora with approximately 12,000 taxa.[1] Considering factors such as geographic position, topographical structure, climate, and richness of flora, forming a bridge between the east and west and hosting many civilizations and ethnic diversity, the Anatolian Peninsula is an extremely important region for folk medicines. This important knowledge, gained through trial and error for centuries and transferred from generation to generation, is also an important resource for herbal drug research. Nevertheless, this valuable knowledge is rapidly disappearing because of factors such as migration from villages to big cities, easy access to physicians and pharmacies, young people's indifference to folk medicines, and industrialization and destruction of nature.[2-4] For the aforementioned reasons, this rapidly disappearing treasure of Turkey should be investigated and recorded by experts comprehensively.

One of the cities that need to be studied in terms of ethnobotany in Turkey is Ankara. Despite previous investigations of the ethnobotany of some districts of Ankara, no comprehensive study was conducted in other districts. These studies, conducted in various districts of Ankara, revealed a rich ethnobotanical accumulation. For example, Şimşek et al.[5] reported that 192 usages for 85 plant species from 31 families were recorded in 25 localities of Beypazarı, Ayaş, and Güdül districts. Sarper et al.[6] found that 50 plant species from 18 families were used for treatment, food, and similar purposes in the Haymana district. Moreover, Elçi and Erik[7] revealed that 23 plant species in only six localities in Güdül and Kızılcahamam districts were used for ethnobotanical purposes. Sezik et al.[8] carried out folk medicine research in 28 localities with sampling method from six districts of Ankara (i.e., Yenimahalle, Kazan, Bala, Altındağ, Keçioren, and Çubuk) and stated that 47 species of 42 genera and 22 families were used as folk medicines. In the Çamlıdere district, the neighbor of Kızılcahamam, 79 plant taxa belonging to 66 genera and 33 families were used for the treatment of various disorders. Additionally, eight new folk medicines were included in the Turkish ethnobotanical inventory in this study.[2] However, no comprehensive folk medicine study was conducted in the Kızılcahamam district.

The Kızılcahamam district is located in the northern part of Ankara and mainly under the influence of Iran-Turan floristic area because of its location in Central Anatolia. According to taxonomic studies, the flora of Kızılcahamam is quite rich, and three floristic regions are influential in Kızılcahamam, namely, Euro-Siberian, Mediterranean, and Irano-Turanian [Eyüboğlu Ö. Kızılcahamam Soğuksu Milli Parkı'nın Florası (MSc Thesis), Ankara: Gazi University; 1991. Yıldırım A. Kocaçay Vadisi Kızılcahamam-Çeltikçi (Ankara) Arası Segetal Florası (MSc Thesis), Ankara: Gazi University; 1994]. Conversely, the district has a rich cultural heritage, as it is a transit point in Anatolia.[9] Kızılcahamam is a remarkable study area because of its geographical location, rich biota, cultural accumulation, and significant ethnobotanical findings of neighboring districts [Eyüboğlu Ö. Kızılcahamam Soğuksu Milli Parkı'nın Florası (MSc Thesis), Ankara: Gazi University; 1991. Yıldırım A. Kocaçay Vadisi Kızılcahamam-Çeltikçi (Ankara) Arası Segetal Florası (MSc Thesis), Ankara: Gazi University; 1994. http://www.kizilcahamam.gov.tr/].[9] To the best of our knowledge, no folk medicine studies have covered the entire Kızılcahamam district. Thus, this study aimed to perform a comprehensive investigation of the folk medicine in the Kızılcahamam district.

MATERIALS AND METHODS

Research area

Kızılcahamam is one of the 24 districts of Ankara (Turkey). In history, Kızılcahamam was thought to be used as a settlement place since the Hittites and had been dominated by Phrygians, Scythians, Persians, Alexander the Great, Celts, and Roman Empire. Following the occupation by Arabs in 654, it was again dominated by Roman Empire. By the Malazgirt victory (1071), the majority of the region's population began to be formed by Turks. In the Ottoman Empire period, the Kızılcahamam region was called "Yabanabad," and the region was an important accommodation place that connects Asia and Europe. The first known center of the district is Demirciören village. However, in 1915, the district center moved to Şorba village and remains the center (http://www.kizilcahamam.gov.tr/).

The Kızılcahamam district, which has 105 villages, is situated in north 40.46° latitude, east 32.65° longitude (northwest of Ankara), and A4 square according to Davis's grid system (Figure 1).[10] It is surrounded by Çubuk in the East, Çamlıdere and Güdül in the West, Ayaş and Kazan in the South, and Çerkeş and Gerede in the north (Figure 1). Its distance to Ankara is 79 km, with acreage of 1712 km^2, and altitude of 975 m. Harami Hill (2053 m) and Işık Mountain (2030 m), which are the highest places in Ankara, are within the boundaries of the district. Given its broken and mountainous physical structure, the district has plateaus, such as Yemişen, Hıdırlar, Miyala, Salın, Eldelek, Başköy, Yıldırım, and Kırık, and several streams among these plateaus (http://www.kizilcahamam.bel.tr/2103/Cografi-Konum). Aluç, Beykaya, Yıldırım, and Kavaklı Mountains are the important mountains of the district. Kızılcahamam is quite rich in water resources, in addition to three damns (namely, Kurtboğazı, Eğrekkaya, and Akyar) that satisfy the water requirement of Ankara, and it attracts attention with an abundance of underground water resources. Kocaçay, Kirmir, and Kurtboğazı are important streams of Kızılcahamam.

The region is under the influence of continental and Black Sea climate. It is cold and snowy in winters and hot and droughty in summers. As it has many forests, it is rainy in every season. The average temperature is +11°C, and the average humidity is 66%. The highest temperature is observed in August as +34°C, while the lowest temperature is observed in February at -20°C (http://www.kizilcahamam.gov.tr/). It has a population of 32,647, and the main sources of livelihood are agriculture, livestock, apiculture, and spa tourism (http://www.tuik.gov.tr/Start.do).[9]

The Soğuksu National Park, one of the most important national parks of Turkey, is located within the borders of Kızılcahamam. As it constitutes a transition between steppe and forest

zones, the Soğuksu National Park, covering 1195 hectares, has an extremely rich biota [Turan M. Fayda-Maliyet Analizi Kapsaminda Kızılcahamam Soğuksu Milli Parkı İncelemesi (MSc), Ankara: Ankara University; 2007). In a study investigating the flora of the Soğuksu National Park, a total of 474 species from 74 families were identified, and 49 of these species were endemic. Researchers also reported that the Soğuksu National Park has the floristic elements of the Euro-Siberian and Mediterranean geographical regions. Compositae, Leguminosae, Poaceae, Lamiaceae, and Brassicaceae were the most common families. Forest vegetation mainly consists of *Pinus sylvestris* L., *Pinus nigra* J. F. Arnold, *Abies nordmanniana* (Steven) Spach subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen, and *Quercus pubescens* Willd. [Eyüboğlu Ö. Kızılcahamam Soğuksu Milli Parkı'nın Florası (MSc Thesis), Ankara: Gazi University; 1991].

Field trips

Nine scientific field trips were organized to Kızılcahamam between April 2007 and July 2008. Generally, brief information about the aim and scope of the study was given to the local authority (called *mukhtar*) at each settlement area. Afterward, people that are knowledgeable about the folk remedies and elders of villages were reached through mukhtars. Face-to-face interviews were conducted in a suitable setting with 57 people by using open and semi-structured questionnaires. Data obtained during the interviews were recorded. General questions (local name of medicinal plants, used parts, purpose of usage, preparation and application method, source of information, etc.) were asked. After each interview, the plants that used as folk medicines were found for in the field under the guidance of the informants. Subsequently, plant samples were taken and appropriately prepared as herbarium materials. To increase the accuracy in identification, flowering or fruiting plants were collected. Therefore, scientific trips were organized to the region between April and July to coincide with the flowering or fruiting period. Plant specimens were identified by Prof. Dr. Galip Akaydın, through Davis's "Flora of Turkey and the East Aegean Islands,"[1,10,11] and the Latin names of identified plant species were updated according to The Plant List (http://www.theplantlist.org/, revision date: 23.02.2021), while the endemism status were checked from "Türkiye Bitkileri Listesi"[12] After botanical identification, plant specimens were preserved in Gazi University Faculty of Pharmacy Herbarium (GUEF). Visited locations are shown in Figure 1.

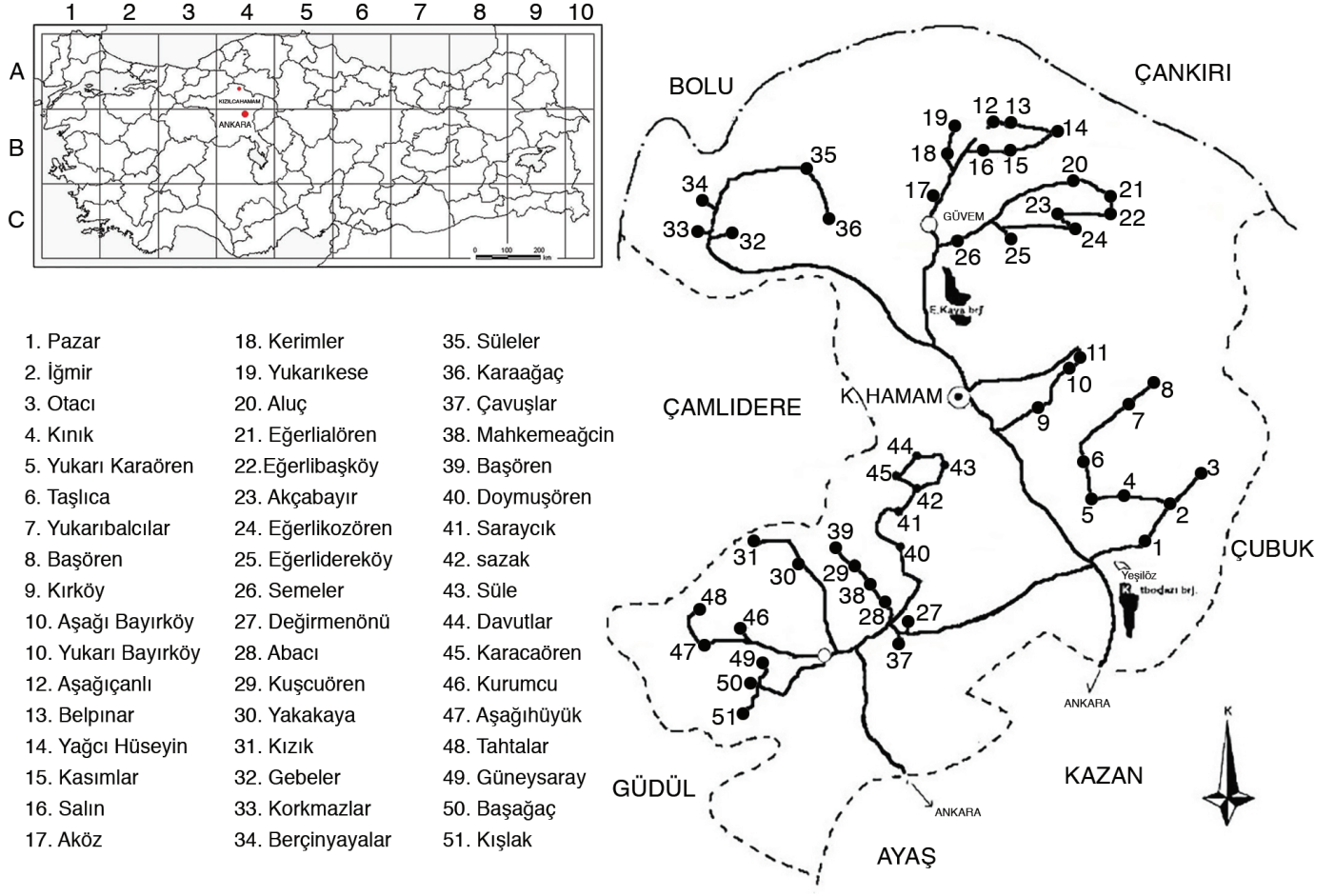

Figure 1. Position of Ankara and Kızılcahamam in Davis's grid system and visited locations

Statistical analysis

Initially, the distribution of folk medicines among 11 pharmacological categories was determined. Subsequently, the informant consensus factor (FIC), use value (UV), and cultural importance index (CI) were calculated using previously described formulas.[2] High FIC degrees (close to 1) correlate with the consent for the use of folk medicine in specific situations by the informant, while the high UV and CI signifies the importance of a plant and high frequency of declaration.[13-16]

RESULTS

Kızılcahamam folk remedies have not been investigated in detail previously. Thus, the ones that are still not forgotten in the folk medicine accumulation of the district were determined and recorded by field studies. During our research, nine field trips were organized to the district, and 51 localities were visited (Figure 1). After the study, people knowledgeable about folk medicine could be reached in 30 of these locations, while there were no knowledgeable people in the other 21 locations. After the identification of plant specimens, 65 species (69 taxa) from 58 genera and 31 families were determined to be used as folk medicines. While seven of the species used as folk medicines in the district were cultivated plants, the rest were wild. The plants used as folk medicine in Kızılcahamam are compiled together and presented in Table 1. In this table, the Latin names of the medicinal plants are given alphabetically (by family and species name) with other relevant data (GUEF numbers, local names, purpose of usages, preparation, and administration methods).

Table 1 shows that Compositae (13 genera, 12 species, and 13 taxa), Lamiaceae (6 genera, 9 species, and 10 taxa), and Rosaceae (7 genera, 9 species, and 9 taxa) are the most commonly referred families in Kızılcahamam as folk medicines (Figure 2). In addition, the most cited plant species are *Malva neglecta*, *Urtica dioica*, *Pinus nigra* subsp. *pallasiana*, *Pinus sylvestris,* and *Rosa canina* (with UV values of 0.29, 0.28, 0.21, 0.17, and 0.17, respectively). Some differences were noted in this order according to the CI, *Urtica dioica* at the first place (CI: 0.98), *Malva neglecta* and *Rosa canina* at the second place (CI: 0.43), and *Cota tinctoria* and *Viscum album* at the third rank (CI: 0.35) (Table 1).

Folk medicines are generally used after certain preparation methods in the area, while only 32% of them are used directly. For example, half of the folk medicines used internally are prepared as tea (33.9% decoction and 16.7% infusion) in Kızılcahamam. Some of the internally used folk remedies were prepared as meals (e.g., *Beta lomatogona* and *Malva neglecta*), jam (e.g., *Rosa canina*), or poultice (e.g., *Malva neglecta* and *Quercus robur*) before use. Interesting uses such as intrauterine administration of *Malva neglecta* for women's infertility were also noted (Table 1). Regarding forms of administration, folk medicines were generally used orally (83.4%) (Table 2).

Folk remedies identified in Kızılcahamam are generally monocomponent and process in a simple preparation. The most preferred plant parts in Kızılcahamam folk medicine are aerial parts, leaf, and fruit. However, inflorescence (e.g., *Tilia rubra* subsp. *caucasica*), fresh shoot (e.g., *Pinus nigra* subsp. *pallasiana*), phloem (the tissue that appears after peeling the peridem tissue of *Pinus sylvestris* stem), and whole plant (e.g., *Viscum album*) are the least used parts (Figure 3).

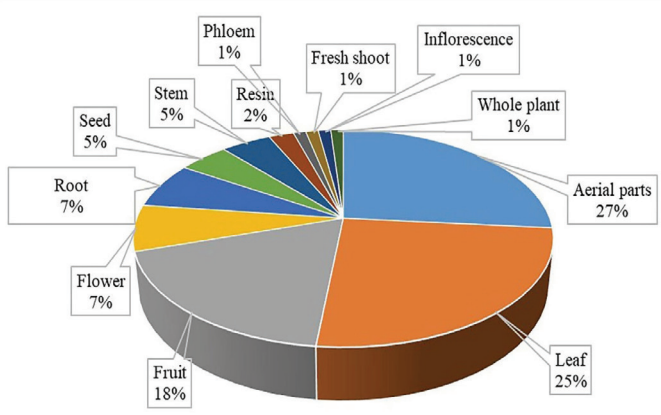

Figure 3. Distribution chart of plants according to used parts

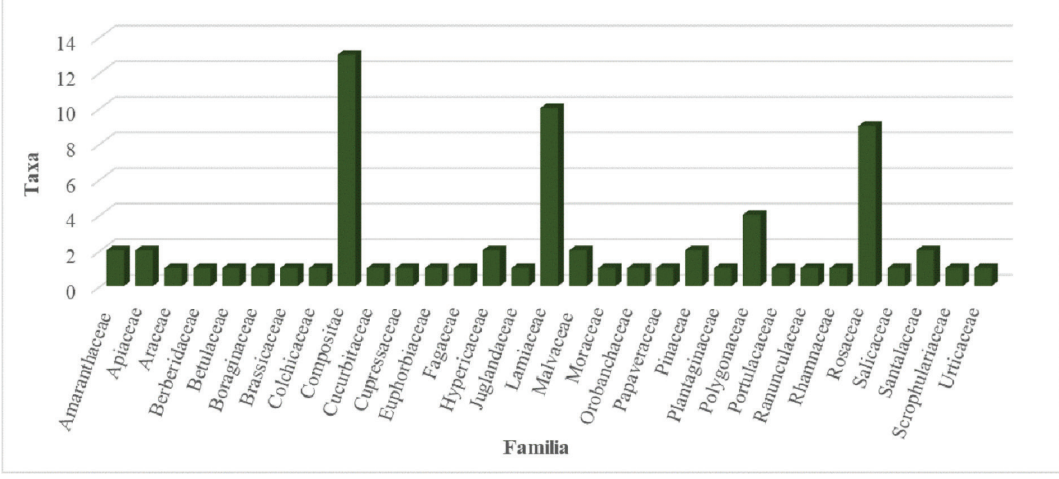

Figure 2. Distribution chart of plants according to family

Table 1. Ethnobotanical usages of plants in Kızılcahamam district

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Amaranthaceae						
Beta lomatogona Fisch. & C. A. Mey. (2690)	27	Göverek	A.p.	For constipation; (I), e.d. or cooked as meal.	0.03	0.01
Chenopodium album L. (2693)	27	Sirken	A.p.	For constipation; (I), eaten fresh.	0.01	0.01
Apiaceae						
Foeniculum vulgare Mill. (2752)*	51	Rezene	L.	For stomach disorders; (I), dec.	0.01	0.01
Petroselinum crispum (Mill.) Fuss*	-	Maydanoz	A.p.	For inflammation; (I), e.d.	0.01	0.01
Araceae						
Arum euxinum R. R. Mill (2631, 2645)	5, 12	Gavur mancarı	Ro.	For hemorrhoid; (I), mixed with rye flour and honey to obtain a pill and eaten on empty stomach for a few days. Or smashed Ro. are mixed with honey and eaten. For leukemia; (I), e.d. or cooked. For hypercholesterolemia; (I), e.d.	0.07	0.12
			Ro./ L.	As foodstuff; (I), e.d. (just L.) or roasted, mixed with honey and eaten.		-
Berberidaceae						
Berberis crataegina DC. (2732)	46	Ak çalı	Fr.	For pain; (I), dec.	0.01	0.01
Betulaceae						
Corylus avellana L.	-	Fındık	L.	For snake bite; (E), crushed leaves are wrapped on affected area.	0.01	0.01
Boraginaceae						
Echium italicum L. (2653)	19	Kızılcık otu, kızılbacak	Ro.	For wound healing and abscess; (E), Ro. is roasted with butter to obtain an ointment, then app. aff. and covered with cloth. Or Ro. collected in the autumn are chopped, mixed with soap, sugar and egg yolk (this mixture is named as "sweet ointment"), app. aff., covered with cloth.	0.07	0.01
Brassicaceae						
Sinapis arvensis L. (2728)	46	Hardal	Se.	For kidney stone; (I), eaten.	0.01	0.01
Colchicaceae						
Colchicum szovitsii Fisch. & C. A. Mey. (2644)	12	Siğilotu, boru otu	L.	For hyperglycemia; (I), as dec.	0.01	0.01
Compositae						
Achillea sp. (2627)	3	Kokağan otu	A.p.	As perfume; (E), applied to hands.	0.01	0.01
Arctium minus (Hill) Bernh. (2651, 2704, 2717)	16, 42, 46	Kabalak	L.	For rheumatismal pain; (E), wrapped on affected area until recovery with intervals.	0.05	0.05
Cota tinctoria (L.) J. Gay (2666, 2680, 2694, 2698, 2711)	32, 36, 27, 38, 45	Beyaz papatya, papatya, akbaba	F.	For stomach disorders; (I), dec. with *Rosa canina* L. Fr. (daily 3x1-2 tea glass full dec. for 1-2 month). For cough and stomachache; (I), dec. with apple Fr. rind. For urinary tract inflammation; (I), inf. is drunk until recovery. To lose weight, as diuretic; (I), dec.	0.10	0.35

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Centaurea solstitialis L. (2656)	25	Çayır dikeni	Flowering A.p.	For hemorrhoid and constipation; (I), a glass of dec. is drunk on empty stomach.	0.03	0.01
Chondrilla juncea L. (2686)	36	Ciklet	Ro. Lt.	As stomachic; (I), Lt. obtained from Ro. is air dried and chewed.	0.01	0.01
Cichorium intybus L. (2731, 2750)	46, 51	Sütleğen	Ro. Lt.	For dental disorders; (I), Lt. obtained from drilled Ro. is dried and chewed.	0.03	0.03
Cirsium arvense (L.) Scop. (2709)	45	Isırgan	A.p.	For urinary tract and prostate disorders; (I), as dec.	0.03	0.01
Cyanus depressus (M. Bieb.) Soják (2707)	42	-	A.p.	For heart health and vascular occlusion; (I), dec. is prepared with a sprinkle of A.p. and drunk after gets warm.	0.03	0.01
Inula oculus-christi L. (2743)	50	Mayasılotu	L.	For hemorrhoid; (I), dried and crushed leaves are drunk with a glass of water.	0.01	0.01
Onopordum turcicum Danin (2695)	27	Galgan	F.	For hyperglycemia; (I), dec.	0.01	0.01
Taraxacum scaturiginosum G. E. Haglund (2633)	6	Ağacakavağı, hindiba, eşek karakavuğu	L.	For hyperglycemia, hypertension and hypercholesterolemia; (I), e.d.	0.05	0.03
Tragopogon dubius Scop. (2730)	46	Yemlik	L.	For wound healing; (I), e.d.	0.01	0.01
Tripleurospermum elongatum (DC.) Bornm. (2641, 2714)	11, 46	Papatya otu	L.	For bronchitis; (I), inf. (daily 1 glass on empty stomach in the mornings or 3-4x1 glass) or inf. with A.p. of *Urtica dioica* L. For stomach disorders; (I), dec. For prostate disorders; (I), inf. with A.p. of *Urtica dioica* L.	0.05	0.10
Cucurbitaceae						
Citrullus lanatus (Thunb.) Matsum. & Nakai*		Karpuz	Fr.	For stomachache and prostate disorders; (I), Fr. juice is drunk (daily 2x1 glass) on empty stomach for at least one week.	0.03	0.03
Cupressaceae						
Juniperus communis L. var. *saxatilis* Pall. (2652, 2712)	16, 46	Ardıç	Ro., S. Fr.	For eczema; (I), tar obtained from Ro. and S. by dry distillation is drunk with water on empty stomach. For shortness of breath; (I), as dec.	0.03	0.07
Euphorbiaceae						
Euphorbia condylocarpa M. Bieb. (2648)	12	Sütleğen	Lt.	For inflamed wounds; Lt. that obtained from torn plant were app. aff.	0.01	0.01
Fagaceae						
Quercus robur L. (2653, 2721)	21, 46	Meşe, karaağaç	G. L. Ro.	For heart disorders; (I), e.d. For stomachache; (I), dec. For knee pain; (E), prepared po. from fresh Ro., app. aff. for one night. This application is repeated for 3-4 day.	0.05	0.10
Hypericaceae						
Hypericum heterophyllum Vent. (2677)	33	Yağşan otu	A.p.	For wounds on animals: (E), app. aff.	0.01	0.01

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Hypericum perforatum L. (2663, 2670, 2691)	32, 33, 27	-	A.p., F.	For stomachache or gastric ulcer; (I), as dec., is drunk until the pain is over.	0.05	0.05
Juglandaceae						
Juglans regia L. (2652, 2697, 2705, 2720)	19, 38, 42, 46	Ceviz	Se.	For diabetes; (I), 15-20 Se. crushed and macerated in a glass of water for one night. In the morning, a tea cup of maceration water is drunk and 1-2 Se. are eaten. Or juice of half lemon and 3 Se. are added to a glass of water and waited for one night. In the morning, maceration water is drunk and Se. is eaten. This application is repeated for 15-20 days. For, high cholesterol; (I), fresh cotyledons macerated with water for one night. In the morning, maceration water is drunk and cotyledons are eaten. For goiter; (I), marble sized immature Se. is swallowed with water.	0.07	0.14
Lamiaceae						
Ajuga chamaepitys (L.) Schreb. subsp. *chia* (Schreb.) Arcang. (2647)	12	Ömür çiçeği	F.	For hemorrhoid; (E), directly app. aff.	0.01	0.01
Ballota larendana Boiss. & Heldr. (2724)	46	-	A.p.	For women's infertility; (E), boiled with milk and used as sitz bath for one week. Application starts in the morning and continues until the evening. It causes excessive sweating. At this time, patient should be protected from cold.	0.01	0.01
Mentha aquatica L. (2628, 2748)	3, 51	Su nanesi	L.	For stomachache; (I), dec.	0.03	0.03
Mentha longifolia (L.) L. (2667)	32	Yarpuz	A.p.	For inflammation; (I), inf.	0.01	0.01
Mentha x *piperita* L.* (2737)	46	Nane	A.p.	For abdominal pain and common cold; (I), e.d. or as dec.	0.07	0.03
Phlomis sp. (2640)	11	Kedi kulağı	A.p.	For gastric ulcer; (I), inf. (daily 2-3x1 glass).	0.01	0.01
Sideritis germanicopolitana Bornm. (2672)	33	Adaçayı	A.p.	For cough; (I), inf. with lemon and thyme. For kidney inflammation and other inflammations; (I), inf. with one slice of lemon (people who have liver diseases should not use this preparation).	0.05	0.05
Thymus leucostomus Hausskn. & Velen. (2681, 2699, 2702, 2729)	36, 38, 42, 46	Kekik	A.p.	For stomachache, asthma, bronchitis and as panacea; (I), inf. For cough; (I), inf. with F. of *Cota tinctoria*.	0.08	0.21
Thymus leucotrichus Halácsy (2669)	34	Kekik	A.p.	For cough; (I), inf. is prepared by a sprinkle of *Thymus leucotrichus*, A.p. of sage and lemon for 5-10 minutes, drunk after adding sugar.	0.01	0.01
Thymus longicaulis C. Presl subsp. *chaubardii* (Rchb.f.) Jalas (2646, 2664)	12, 32	Kekik	A.p.	For asthma and bronchitis; (I), inf. (daily 3x1-2 tea cup, for 1-3 months). For stomach disorders; (I), inf. As spice.	0.07	0.08

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Malvaceae						
Malva neglecta Wallr. (2635, 2665 2682, 2689, 2723, 2747)	10, 32, 36, 27, 46, 51	Ebemgömeci, ebemkömeci	A.p.	For hyperglycemia; (I), inf. or e.d. or cooked as meal. For stomach disorders; (I), inf. or e.d. or cooked as meal. (E), po. m., applied on abdomen. For constipation; (E), po. m., applied on abdomen. (I), e.d. or dec. For hemorrhoid; (E), fresh or dried A.p. boiled with milk and app. aff. This application drains the inflammation. Or (I), cooked as meal. For pain in waist and knee, rheumatismal pain; (E), boiled to obtain a po. and app. aff. or (I), dec. Women's infertility; crushed and boiled in water and milk added to obtain a po., after the milk has absorbed, placed into uterus. Or boiled in milk, as sitz bath. Or crushed and boiled in milk and water to obtain a po., then placed into uterus. Or boiled in milk, as sitz bath.	0.29	0.43
			L.	For prostate disorders; (I), dec.		
Tilia rubra DC. subsp. *caucasica* (Rupr.) V. Engl. (2675, 2678)	33, 36	Yabani ıhlamur, dağ ıhlamuru	Inflorescence with bracts	For common cold; (I), inf. For asthma, cough, tonsillitis, abdominal pain and as sedative for children; (I), as dec. Residue could be used to prepare dec. for 2-3 times.	0.10	0.10
Moraceae						
Morus nigra L.* (2735)	46	Dut	Fr.	As blood-former; (I), e.d.	0.01	0.01
Orobanchaceae						
Rhinanthus serotinus (Schönh.) Oborny subsp. *aestivalis* (N.W.Zinger) Dostál (2715)	46	-	Se.	For urinary tract disorders; (I), dec.	0.01	0.01
Papaveraceae						
Chelidonium majus L. (2685)	36	Temreotu	Lt.	For "temre" (a skin disorder); (E), the Lt. that running from plucked leaves is app. aff.	0.01	0.01
Pinaceae						
Pinus nigra J. F. Arnold subsp. *pallasiana* (Lamb.) Holmboe (2639)	11	Çam	Fresh shoot	For shortness of breath and asthma; (I), inf. is drunk at two times a day after waiting in cold for one night. Or e.d.	0.21	0.03
			S.	For shortness of breath and asthma; (I), dec., for 10 days. For inflamed wounds; (I), dec.		
			R.	For shortness of breath and asthma; (I), mixed with honey. For abscess and wound healing; (E), directly, or mixed with honey or mixed with soap, app. aff. It heals the wound and drains the inflammation. For removing foreign objects from skin (e.g. splinter); (E), app. aff.		

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Pinus sylvestris L. (2625, 2638, 2654, 2746)	2, 11, 21, 51	Çam	Ph.	For lung disorders, tuberculosis, asthma, stomachache; (I), mixed with honey and eaten.	0.17	0.28
			Se.	Stomach disorders; (I), crushed, mixed with honey and eaten. For shortness of breath; (I), crushed Se. is mixed with honey and eaten.		
			R.	For removing foreign objects from skin (e.g. splinter); (E), app. aff.		
			C.	For shortness of breath: (I), dec. prepared with a handful of C. that collected in the November. Or small immature C. is mixed with honey and eaten. Women's urinary tract disorders; (I), dec. with C., equal amount of leaves of *Cydonia oblonga* and A.p. of nettle (3 times in a day, for 1-3 months).		
Plantaginaceae						
Plantago major L. (2703, 2706, 2710, 2716)	42, 45, 46	Siğil otu	L.	For abscess; (E), app. aff. For gastric ulcer; (I), mixed with honey, butter and eaten.	0.05	0.10
Polygonaceae						
Polygonum cognatum Meissn. (2629, 2634, 2688)	3, 9, 27	Madımalak, kadımalak, dağ mancarı, üfelek	A.p.	For stomach and intestinal disorders; (I), boiled and eaten. As foodstuff: (I), e.d.	0.05	0.10
Rumex crispus L. (2649, 2749)	12, 51	Kenger, kuzukulağı	L.	For hyperglycemia; (I), e.d. As appetite suppressant; (I), eaten.	0.03	0.07
Rumex scutatus L. (2626)	3	Acıkıcı	A.p.	As foodstuff; (I), e.d.	0.01	0.01
Rumex sp. (2739)	46	Roka	L.	For hyperglycemia; (I), e.d.	0.01	0.01
Portulacaceae						
Portulaca oleracea L. (2733)	46	Sirkenotu, semizotu	A.p.	For hyperglycemia, inappetency and cancer; (I), e.d.	0.05	0.01
Ranunculaceae						
Nigella sativa L.*	-	Çörekotu	Se.	For shortness of breath; (I), mixed with honey.	0.01	0.01
Rhamnaceae						
Paliurus spina-christi Mill. (2708, 2751)	42, 51	Karaçalı	Fr.	For stomach disorders and kidney stone; (I), dec.	0.03	0.07
Rosaceae						
Crataegus orientalis Pall. ex M. Bieb. (2740)	46	Kürdili	Fr.	For diarrhea and stomach disorders; (I), mature Fr. are eaten.	0.07	0.05
			F.	For hypertension and hyperglycemia; (I), inf. is drunk in the mornings on empty stomach.		
Cydonia oblonga Mill. (2660, 2692)	28, 27	Ayva	L.	For shortness of breath, bronchitis and cough; (I), dec. is drunk with lemon juice. For women's urinary tract disorders; (I), as dec. with equal amount of leaves of *Cydonia oblonga*, C. of *Pinus sylvestris* L. and A.p. of *Urtica dioica* L. (daily 3x1, for 1-3 month).	0.07	0.03

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Malus sylvestris (L.) Mill. (2683)	36	Acı elma, yabani elma	Fr.	For hyperglycemia; (I), sugar free compote is prepared with dried Fr. and drunk. Or dried layers of Fr. pulp (this preparation is called as "pestil") are eaten. Or pestil is macerated in water and drunk.	0.05	0.01
Prunus cerasus L.*(2734)	46	Vişne	Fr.	For hyperglycemia; (I), sugar free compote that prepared from Fr. is drunk, marc is eaten as well.	0.01	0.01
Prunus spinosa L. (2636, 2713, 2725)	10, 46	Karamuk	Fr.	For hyperglycemia; (I), dec. prepared from mature black Fr.	0.03	0.03
Rosa canina L. (2632, 2662, 2673, 2687, 2718)	6, 32, 33, 36, 46	Kuşburnu	Fr.	For bronchitis; (I), dec. Or Fr. juice is drunk. Or jam prepared from Fr. is consumed twice a day. For cough and shortness of breath; (I), inf. For hemorrhoid; (I), dec. prepared by boiling for 15 minutes is drunk after get warm. For hyperglycemia; (I), Fr. juice is boiled and drunk on empty stomach (daily 2x1 tea cup). For hypertension; (I), viscous dec. is mixed with water, drunk in the evenings (daily 2-3 glass). For stomach disorders; (I), dec. of Fr. with F. of *Cota tinctoria* (daily 3x1-2 tea cup, for 1-2 month). For prevent diseases; (I), mature Fr. are boiled for 1-2 hour, squashed, sieved, remaining pulps is drunk with water or eaten.	0.17	0.43
Sanguisorba minor Scop. (2700)	41	Tere	L.	For wound healing; (E), crushed, mixed with butter, app. aff. For lung disorders; (I), mixed with honey and eaten.	0.03	0.03
Sorbus domestica L. (2736, 2742, 2744)	46, 50, 51	Övez	Fr. Fr. and L. L.	For children's diarrhea; (I), e.d. For urinary tract disorders; (I), dec. For kidney stone; (I), prepared inf. is drunk on empty stomach after waiting in cold for one night.	0.05	0.10
Sorbus umbellata (Desf.) Fritsch (2741)	46	Alıç	Fr.	For hyperglycemia and heart disorders; (I), dec.	0.03	0.03
Salicaceae						
Salix alba L. (2659)	28	Söğüt	L. S.	For headache; (E), leaves are burnt, obtained ash applied on head. This application must cause wounds on head, otherwise it has no effect. For eczema on hand; (E), S. is burnt, obtained ash mixed with water, hands are washed with supernatant.	0.03	0.03
Santalaceae						
Viscum album L. (2630, 2657, 2676, 2719)	3, 27, 33, 46	Purç	L. W.p. Fr.	For stomachache, prostate disorders and kidney stone; (I), dec. For shortness of breath and hypertension; (I), dec. For leg pain; (I), dec. is drunk and (E), residue app. aff. For leg pain; (E), slivered Fr. is app. aff.	0.12	0.35

Table 1. continued

Family and scientific name (GUEF no)	Locality[a]	Local name	Used part[b]	Purpose of usage, preparation and application method[b]	UV	CI
Viscum album L. subsp. *austriacum* (Wiesb.) Vollm. (2727)	46	Purç	Fr.	For urinary tract inflammation, bronchitis and cough; (I), dec.	0.05	0.03
Scrophulariaceae						
Verbascum insulare Boiss. &Heldr. (2643, 2684, 2701, 2722)	12, 36, 42	Sığırkuyruğu	F.	For wounds on animals: (E), crushed and app. aff.	0.05	0.15
			A.p.	For wounds on animals: (E), wrapped on wounds, this application kills worms on wounds. For fishing: immersed to water. This application kills the fishes.		
Urticaceae						
Urtica dioica L. (2655, 2658, 2661, 2671, 2679, 2696, 2738, 2745)	25, 27, 32, 33, 36, 38, 46, 51	Dalağan otu, Isırgan otu	A.p.	For women's urinary tract disorders; (I), dec. with equal amount of A.p. of *Urtica dioica*, C. of *Pinus sylvestris* and leaves of *Cydonia oblonga* is drunk 3 times a day for 1-3 months. For prostate disorders; (I), inf., on empty stomach, in the evenings. For prostate cancer; (I), inf. is prepared with 500 g fresh A.p. and 4 L water, drunk on empty stomach. For prostate disorders and bronchitis; (I), inf. with leaves of *Tripleurospermum elongatum*. For rheumatism; (I), 1-2 glass of dec. was drunk every day. For shortness of breath; (I), A.p. (without F. and Se.) cooked as meal and eaten every day. For dandruff; (E), dec. of fresh A.p. is applied on scalp after shower. After waiting a while, hair is rinsed. This application is repeated three consecutive baths. For hemorrhoid; (I), inf. is drunk three times a day on empty stomach. Residue could be used to prepare inf. once more. As foodstuff: cooked as meal.	0.28	0.98
			A.p. with Se.	For urinary tract disorders, lung and urinary tract cancer; (I), a glass of dec. is drunk 3 times a day on empty stomach.		
			Se.	For cancer; (I), as dec./inf. or consumed with honey or eaten with meals.		
			L.	For knee pain or rheumatism; (E), knee was bitten to green thin hornet, and L. of *Urtica dioica* app. aff. Or fresh L. is wrapped on affected are for 5-10 minutes.		

[a]: Localities that corresponds the numbers are given in Figure 1, [b]: Abbreviations, *: Cultivated plants, A.p.: Aerial parts, app. aff.: Applied on affected area, C.: Cone, dec.: Decoction, E: Externally, e.d.: Eaten directly, F.: Flower, Fr.: Fruit, G.: Gall, I: Internally, inf.: Infusion, L.: Leaf, Lt.: Latex, Ph.: Phloem, po.: Poultice, po. m.: Poultice with milk, R.: Resin, Ro.: Root, S.: Stem, Se.: Seed, W.p.: Whole plant, UV: Use value, CI: Cultural importance index

During the field studies, diseases that local people tried to treat with folk remedies were classified into 11 groups (Table 3). The distribution of folk medicines according to pharmacological categories in Kızılcahamam is as follows: Respiratory system disorders, 18 medicines and 47 citations; gastrointestinal problems, 28 medicines and 44 citations, and urinary tract problems, 17 medicines and 29 citations (Table 3). Shortness of breath is the most referenced respiratory system disorder that is treated by folk medicines, while hemorrhoid and prostate disorders are illnesses that are attempted to be cured by folk medicines among gastrointestinal system and urinary tract problems. However, this order changes when FIC values are considered; dental diseases have the highest FIC value (1.00), followed by skeletomuscular system disorders (0.69) and respiratory system disorders (0.63) that ranked second and third, respectively. FIC is accepted as being the degree of agreement among the people interviewed concerning the use of a given taxon.[14] Thus, differences according to citation and FIC value were thought to be based on the disagreement among informants and the use of the same folk medicine for very different purposes. Dental disorders have the highest FIC value because of the citation of the same plant twice for dental illness, rather than being popular.

During our field study, a mushroom (*Morchella* sp.) was used for ethnobotanical purposes. It was named as "Kuzugöbeği, ayı mantarı" among local people and used for knee pain, inducing sleep, sedation, and foodstuff by cooking as meal. As it is a member of Kingdom Fungi, it was not listed in the table with other plant-sourced medicines.

DISCUSSION

The result of field studies conducted in the Kızılcahamam district provided important contributions to Turkish Folk Medicine literature. To the best of our knowledge, folk medicinal usage of *Beta lomatogona, Ballota larendana*, *Tripleurospermum elongatum,* and *Verbascum insulare* are recorded firstly within this research in Turkey (Table 1). In addition, four endemic plants, namely, *Arum euxinum*, *Ballota larendana*, *Sideritis germanicopolitana,* and *Verbascum insulare*, were used as folk remedies in the district.

Table 2. Distribution of folk medicines according to their preparation and application methods in Kızılcahamam

Application method	Preparation method	Number	Percentages (%)
Externally	a) Without processing, directly	14	8.0
	b) After process (as poultice, ointment, ...)	15	8.6
Internally	a) Without processing, directly	42	24.1
	b) Infusion	29	16.7
	c) Cooked as meal	9	5.2
	d) Decoction	59	33.9
	e) Other preparations (as jam, pill, ...)	6	3.5
Total		174	100.0

Table 3. Distribution of used plants by pharmacological categories and FIC values

Category of illness	Taxa	All taxa (%)	Use citation	All use citation (%)	FIC value
Respiratory system disorders	18	26.08	47	22.82	0.63
Gastrointestinal system disorders	28	40.57	44	21.36	0.37
Urogenital system disorders	17	24.63	29	14.08	0.43
Metabolic disorders	16	23.18	25	12.14	0.38
Dermatological system disorders	12	17.39	19	9.22	0.39
Skeleto-muscular system disorders	5	7.24	14	6.80	0.69
Cardiovascular disorders	9	13.04	11	5.34	0.20
Immunological disorders	8	11.59	9	4.37	0.13
Central nervous system disorders	3	4.34	3	1.45	0.00
Veterinary disorders	2	2.89	3	1.45	0.5
Dental disorders	1	1.44	2	0.97	1.00

FIC: Informant consensus factor

Folk medicines used in Kızılcahamam have similar usages in different parts of Turkey. The similarity is quite high with Çamlıdere, a border neighboring district of Kızılcahamam. Moreover, 27 of the 69 taxa used as folk medicines in Kızılcahamam have similar uses in Çamlıdere. For example, *Cota tinctoria* (urinary tract inflammation), *Juglans regia* (diabetes), *Viscum album* (shortness of breath and urinary tract disorders), *Malva neglecta* (hemorrhoid and rheumatism), *Cydonia oblonga* (shortness of breath, bronchitis, and cough), *Prunus spinosa* (diabetes) are used for same conditions in both districts. The utilization of *Salix alba* is an interesting example of this similarity, as the ash obtained from its root or leaf is applied to the head for the treatment of headache.[2] In Güdül, another border neighboring district of Ankara, *Paliurus spina-christi* was used for kidney stone similar to its use in Kızılcahamam.[7] Similar utilizations were also discovered with other remote districts of Ankara. For example, *Malva neglecta*, one of the most cited plants in our study area, is used for the treatment of hemorrhoids in Haymana and Beypazarı districts as in Kızılcahamam.[5,6] *Taraxacum scaturiginosum* was used for diabetes (or hyperglycemia) in both Beypazarı and Kızılcahamam districts.[5] *Echium italicum*, *Thymus leucostomus*, and *Sorbus domestica* were used with the same purposes in different districts of Ankara.[5] Similarities were observed with different provinces as well; for example, *Crataegus orientalis* is utilized for hypertension or hyperglycemia in different parts of Anatolia (e.g., Ağrı, Manisa) as in Kızılcahamam.[17-19] *Plantago major* is used for the treatment of abscess in almost every part of Turkey as in Kızılcahamam.[4-6,17,19-23] Furthermore, *Ajuga chamaepitys* subsp. *chia, Morus nigra*, *Centaurea solstitialis, Echium italicum*, and *Sanguisorba minor* were used in different provinces similarly as determined in our study area.[4,19,20,23-26] Similar usages in nearby districts/provinces are already expected. Having similar flora and the ease of idea exchange could be the reasons for this situation. The similar use of folk medicines at different locations may be considered an indication of the accuracy of the identified folk medicinal knowledge. Besides, domestic migration, development of communication, and transport facilities may contribute to the dissemination of information to remote areas.

Conversely, different species of some genera used in Kızılcahamam were detected to have similar usages in different parts of Turkey. When the border neighboring districts were examined, interesting similar utilizations were encountered; one of them is *Tripleurospermum callosum* (Boiss. & Heldr.) E. Hossain used for bronchitis in Çamlıdere, similar to *Tripleurospermum elongatum*. Similarities in the usage between *Prunus avium* (L.) L. and *Prunus cerasus* (diabetes and hyperglycemia), and between *Juniperus oxycedrus* L. and *Juniperus communis* var. *saxatilis* (for eczema) are among other examples.[2] Similarly, in Güdül, *Rumex tuberosus* L. is used for hyperglycemia just as *Rumex crispus* is used in Kızılcahamam.[7] Similar usages were also observed in remote districts of Ankara, such as between *Onopordum turcicum* and *Onopordum acanthium* L., and between *Salix alba* and *Salix babylonica,* which were used in Haymana.[6] The same situation applies for other provinces of Turkey: Some different *Hypericum* species (e.g., *Hypericum polyphyllum* Boiss. & Balansa and *Hypericum empetrifolium* Willd.) are utilized for the treatment of stomach disorders in Ağrı and Muğla, as in Kızılcahamam.[17,27] *Arum* species are other examples. The root of *Arum euxinum* is used for hemorrhoid treatment in Kızılcahamam, and different *Arum* species (e.g., *Arum balansanum* R. R. Mill. and *Arum italicum* Mill.) are also used for the same purpose in different regions of Anatolia.[4,28,29] This may result from local people's assumption that different species of a genus are the same because of morphological similarities. Moreover, different species of a genus are likely to show phytochemical similarity. Thus, based on the information obtained from different regions by information exchange, people may have tried to prepare the same medicine with morphologically similar plants that are grown nearby, seen the same effect, and continued to use them.

Contrary to the abovementioned similarities, quite different usages for the same species were also determined in different parts of Ankara. These differences were observed even in border neighboring districts; for instance, *Cirsium arvense* is used for urinary tract and prostate disorders in Kızılcahamam, while it is used for shortness of breath in Çamlıdere. *Sinapis arvensis* is used for kidney stones in our research area, but it is recommended for shortness of breath in Çamlıdere.[2] Another example is *Crataegus orientalis*, which is used for diarrhea, stomach disorders, hypertension, and hyperglycemia. Unlike the usages detected in Kızılcahamam, this plant is used for shortness of breath and heart disorders in the Güdül district.[7] *Arctium minus*, *Cichorium intybus*, and *Sinapis arvensis* are other examples, which were used for different purposes in different districts of Ankara.[8]

The Anatolian Peninsula has hosted quite different cultures throughout history, and this situation has been inevitably reflected in folk medicinal knowledge. Communities, migrating from different regions throughout history, have blended their folk medicinal knowledge, which was obtained in the region where they came from, with local folk medicine knowledge. Therefore, the usage of the same plant as a folk remedy may vary by region.

Folk medicines are mostly cited for the treatment of gastrointestinal system disorders (especially hemorrhoid and stomach ache), respiratory (especially shortness of breath, asthma, and common colds), and urogenital system problems (especially, prostate disorders) in Kızılcahamam. Considering these results, the aforementioned disorders could be considered the most common health problems in the study area.

When the cited folk medicines are examined, some plants attract attention with their usage in the treatment of disorders which could be hardly noticed by common people. For example, *Cirsium arvense, Citrullus lanatus*, *Malva neglecta, Tripleurospermum elongatum*, *Urtica dioica*, and *Viscum album* are used for the treatment of prostate disorders, but the recognition of this disorder and use of folk medicine for its treatment are interesting. Likewise, informants expressed that they use various folk medicines to treat some illnesses, such

as cancer (*Arum euxinum, Urtica dioica*, and *Portulaca oleracea*), hypercholesterolemia (*Juglans regia*), hyperglycemia (*Prunus spinosa, Rosa canina*, and *Juglans regia*), goiter (*Juglans regia*), and vascular occlusion (*Cyanus depressus*). Recently, these disorders are becoming increasingly widespread. In addition, at present, reaching a physician has become quite easy. Hence, these situations suggest that people start to search for solutions by themselves after being diagnosed by physicians. Moreover, this information could be obtained from various sources. Nevertheless, interviewees indicated that they learned these uses from their ancestors. Therefore, the mentioned usages were included in the present work. In addition, there may be some uses that may result from information pollution obtained from books, television, newspaper, etc., such as the use of *Citrullus lanatus* against prostate disorders. It is rich in lycopene[30] that reduces the risk of prostate disorders.[31] Methanol extract of seeds were demonstrated to cause a significant reduction in the size of the enlarged prostate.[32] However, this usage could be interpreted as a result of information pollution of folk medicine knowledge.

Some interesting folk remedies were encountered in the study area. For example, the phloem of *Pinus sylvestris* is consumed with honey as treatment of stomachache, or the knee is bitten by green thin hornets for the treatment of knee pain and rheumatism. *Echium italicum* is used for wound healing in Kızılcahamam, and according to previous bioactivity research, the ethanol extract of *Echium italicum* root increased wound tensile strength by 37%.[33] Similarly, *Hypericum perforatum, Phlomis* sp.*, Malva neglecta,* and *Rosa canina* are used as folk medicines in peptic ulcer symptoms, and in the literature, these plants have a strong *in vivo* anti-ulcerogenic effect.[34-36] In addition, *Pinus nigra* subsp. *pallasiana* and *Pinus sylvestris* are interestingly used for removing foreign objects from the skin. When their resins were applied to the affected area, foreign materials such as splinters could be removed. The usage of *Cota tinctoria* for weight loss is also interesting. To the best of our knowledge, no study has examined this effect. The investigation of the mentioned effect of *Cota tinctoria* can be a new research topic. It is possible to increase the number of these examples. This situation is a good example to unroll the possible high potential of folk medicines as the starting point for new drug discovery.

Conversely, *Onopordum turcicum* flower is used against hyperglycemia in the research area. In our previous study, this plant species was used to treat diabetes in Çamlıdere, a neighboring district, and local names of this plant are very similar in both districts (called as "galgan" in Kızılcahamam and "Kalkan" in Çamlıdere).[2] The usage of the same folk medicines for the same purposes in two neighboring regions increases the reliability of the data obtained in this study.

In Kızılcahamam, folk medicines are generally preferred if prepared from a single plant species; only 17 of 69 taxa are included in the mixtures. These mixtures are usually prepared as decoction/infusion (e.g., *Cota tinctoria*, *Cydonia oblonga*, and *Rosa canina*), but there are some ointment (e.g., *Echium italicum*) or poultice (e.g., *Malva neglecta*) formulations, as well. These results are consistent with the folk medicine data previously obtained in Turkey because folk remedies are generally prepared as simple formulations and used as a single component.

In this study, although investigations aimed at folk remedies, other ethnobotanical usages of plants rather than their usage in folk medicine were also detected and recorded on the field. For example, *Achillea* sp. is used as perfume by applied on hands; *Arum euxinum*, *Morchella* sp., *Polygonum cognatum, Rumex scutatus, Thymus longicaulis* subsp*. chaubardii,* and *Urtica dioica* are used as foodstuff, and *Verbascum insulare* is used for fishing.

As a result of interviews, the majority of informants in the research area preferred to use modern medicine for the treatment of their illnesses; by contrast, some of the informants expressed that they do not want to use modern medicines because of their side effects, so they try treating illnesses with herbal remedies. However, people who know about folk medicines are limited; in 51 villages, no knowledgeable people or folk medicine users were found in 21 villages (41% of visited locations). However, if this survey was conducted 20 years ago, ethnobotanical information could probably be obtained from all villages. This situation is a very important indicator of the loss of ethnobotanical knowledge owing to modern life.

CONCLUSION

To the best of our knowledge, this is the first study to explore folk medicines in the Kızılcahamam district. This study provided important contributions to the inventory of Turkish Folk Medicines. This study also highlights the rapid disappearance of folk medicine knowledge and urgent need for recording it in all parts of Turkey. Folk remedies are important resources, as they provide advantages on modern drug research. They could be considered as sources that have been shown to be effective on humans and tested for toxicity because they have been used for centuries, and useless or toxic ones were discarded throughout history. In previous bioactivity studies based on folk medicines conducted by our research group, many herbs used as folk remedies in different regions of Anatolia have been shown to be effective against the mentioned bioactivities *in vivo*.[34,35,37-40] Many drugs have been developed based on their traditional use worldwide; aspirin (*Salix* sp.), artemisinin (*Artemisia annua* L.), galantamine (*Galanthus woronowii* Losinsk.) are some examples of compounds developed from plants that were used traditionally.[41-43] Besides recording new folk medicines to the ethnobotanical heritage of Turkey, this study is a valuable source of data for future bioactivity studies and discovery of new drug candidate molecules.

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ORIGINAL ARTICLE

Neoteric Approach of Fluoxetine Laden Orodispersible Film for Non-compliant Pediatric Patients of Selective Mutism and Obsessive-compulsive Disorder

Seçici Mutizm ve Obsesif Kompulsif Bozukluğu olan Uyumsuz Pediatrik Hastalar için Fluoksetin Ladenin Ağızda Dağılan Filmi için Yeni Yaklaşım

Haseeb Anwar DAD[1,2], Asra Shanzeh SHABBIR[3,4], Saman ALI[2,4*], Tariq MAHMOOD[2]

[1]Zhejiang University College of Pharmaceutical Sciences, Department of Pharmaceutics, Hangzhou, China
[2]University of Central Punjab Faculty of Pharmacy, Department of Pharmaceutics, Lahore, Pakistan
[3]Punjab University College of Pharmacy, Department of Pharmaceutics, Lahore, Pakistan
[4]Lahore Medical and Dental College Lahore Pharmacy College, Department of Pharmaceutics, Lahore, Pakistan

ABSTRACT

Objectives: The objective of this research was to fabricate, characterize, and optimize fluoxetine laden orodispersible film (ODF), in enhancing dosage forms options for the pediatric population suffering from incapacitating psychotic disorders of selective mutism and obsessive-compulsive disorder, which will be ultimately beneficial in enhancing compliance factor and the quality of pharmacotherapy.

Materials and Methods: Solvent casting technique was used to formulate the ODF formed by natural hydrophilic polymers matrix of hydroxypropyl methylcellulose E15 and pullulan. Propylene glycol as plasticizing agent imparted satisfactory tenacity and flexibility to ODFs. Fourier transform infrared spectroscopy studies were performed to investigate any potential compatibility, and the results revealed no potential interaction between fluoxetine and excipients. Developed ODFs were evaluated for physicochemical properties, content uniformity, *in vitro* disintegration time, and *in vitro* dissolution time studies.

Results: The experimental data suggested that different polymer concentrations had a complex effect on content uniformity, *in vitro* disintegration time, and cumulative percentage drug release from the ODFs. TF7 was the most optimized formulation with a disintegration time of 10.66 sec and 99.37% drug release within 3 min. Additionally, the most optimized fluoxetine ODF was submitted to a Universal Testing Machine for tensile strength and percentage elongation determination. It was also further evaluated by thermogravimetric analysis, scanning electron microscopy and X-ray diffraction.

Conclusion: Fluoxetine ODFs of good pharmaceutical quality can be prepared on a small scale. Therefore, the perspective of using fluoxetine ODFs for individualized pharmacotherapy to ameliorate the compliance issues in selective mutism and OCD pediatric patients can be considered.

Key words: Fluoxetine, HPMC E15, obsessive-compulsive disorder, orodispersible film, pullulan, selective mutism

ÖZ

Amaç: Bu araştırmanın amacı, seçici mutizm ve obsesif-kompulsif bozukluğu gibi inkapasite edici psikotik bozuklukları olan pediatrik popülasyon için sonuçta uyum faktörünü ve farmakoterapinin kalitesini artırmada yararlı olabilecek fluoksetin yüklü ağızda dağılan filmi (ODF) imal etmek, karakterize etmek ve optimize etmektir.

Gereç ve Yöntemler: Hidroksipropil metil selüloz E15 ve pulluhanın doğal hidrofilik polimer matrisi tarafından oluşturulan ODF'yi formüle etmek için çözücü döküm tekniği kullanılmıştır. Plastikleştirici madde olarak propilen glikol, ODF'lere tatmin edici dayanıklılık ve esneklik kazandırmıştır.

***Correspondence:** saman.rizvi@outlook.com, **Phone:** +92-310-4875869, **ORCID-ID:** orcid.org/0000-0002-5962-3996

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Olası uyumluluğu araştırmak için fourier dönüşümü kızılötesi spektroskopisi çalışmaları yapılmış ve sonuçlar fluoksetin ile eksipiyanlar arasında potansiyel bir etkileşim olmadığını ortaya koymuştur. Geliştirilen ODF'ler fizikokimyasal özellikler, içerik homojenliği, *in vitro* parçalanma süresi ve *in vitro* çözünme süresi çalışmaları için değerlendirilmiştir.

Bulgular: Deneysel veriler farklı polimer konsantrasyonlarının, içerik tekdüzeliği, *in vitro* parçalanma süresi ve ODF'lerden kümülatif ilaç salımı yüzdesi üzerinde karmaşık bir etkiye sahip olduğunu göstermiştir. TF7, 10,66 saniyelik dağılma süresi ve 3 dakika içinde %99,37 ilaç salımı ile en optimize edilmiş formülasyon olarak belirlenmiştir. Ek olarak, en optimize fluoksetin ODF, gerilme mukavemeti ve uzama yüzdesi belirlemesi için bir Evrensel Test Makinesi'ne gönderilmiştir. Ayrıca termogravimetrik analiz, taramalı elektron mikroskobu ve X-ışını kırınımı ile de değerlendirilmiştir.

Sonuç: İyi farmasötik kalitede fluoksetin ODF'leri küçük ölçekte hazırlanabilir. Bu nedenle, seçici mutizm ve OKB pediatrik hastalarda uyum sorunlarını iyileştirmek için bireyselleştirilmiş farmakoterapi için fluoksetin ODF'lerin kullanılması perspektifi düşünülebilir.

Anahtar kelimeler: Fluoksetin, HPMC E15, obsesif-kompulsif bozukluk, ağızda dağılan film, pulluhan, seçici mutizm

INTRODUCTION

The pediatric population comprises of heterogeneous age bracket as it encompasses the entire population from neonate to adolescence. For decades, complications faced by the pediatric population during the administration of oral dosage forms are not taken into grave account.[1] About 90% of total marketed pediatric medicinal products are in liquid dosage forms. However, liquid dosage forms are replete with complications of erroneous dosing, instability, augmented contamination probability, and the requirement of a dedicated utensil for administration.[2,3] Pediatric patients with disabilities, mentally challenged states, and psychotic disorders experience many hindrances in the oral administration of the drugs.[4]

Focusing on psychiatric disorders, selective mutism, and obsessive-compulsive disorder (OCD), which are anxiety disorders according to the Diagnostic and Statistical Manual of Mental Disorders-IV, inflict pediatric population and sometimes exist concurrently.[5-7] Selective mutism, a complex incapacitating anxiety disorder with onset between two to five years of age, is typically delineated by perpetual failure to develop verbal communication in distinct social settings where verbal communication is required (e.g., school); however, such children can converse well in a domestic backdrop.[8,9] Similarly, OCD lands up the afflicted children in vitiated social and academic performance.[10,11]

Selective serotonin reuptake inhibitors, an anti-depressant class of drugs, have been recommended to treat selective mutism and OCD due to their significant efficacy.[12-14] Studies have reported the well-tolerated and effective treatment of OCD and selective mutism with fluoxetine, which diminishes the panic attacks and social phobias in a pediatric population.[15-17] It is a well-reported fact that the pediatric population experiences dysphagia while intake solid dosage forms.[18] One of the major factors, among others, might be the smaller dimensions of the children's pharynx and the developing oropharyngeal musculature.[19]

Therefore, this study aimed to fabricate the fluoxetine laden oral dispersible films to tackle the challenges. Orodispersible film (ODF) technology comprises a thin postage stamp-sized pliable strip manufactured of hydrophilic film-forming polymers and excipients, releasing active drugs within seconds on contact with saliva in the buccal cavity. Needless to mention that excipients incorporated in ODF, such as plasticizer, film stabilizer, saliva stimulating agent, taste masking agent, flavor, and super-disintegrant, must be Generally Regarded as Safe, i.e., GRAS listed according to Food and Drug Administration.[20-22] In addition, one of the most salient and crucial components for the formation of ODF is the utilization of polymers. The optimal polymer utilization is vital to impart the required critical characteristics to ODF, for instance, solubility, hydrophilicity, pliability, and a pleasant mouthfeel. Recently, ODF technology has garnered massive attention for the delivery of active drugs, prone to deterioration and GIT due to enzymes and pH variation. Additionally, ODF offers substantial benefits like widened surface area, which shortens the disintegration and dissolution time.[20] It ascertains the better accurate and precise dose per film compared to other dosage forms like syrup and drop. It is equipped with an oral absorbance feature, which leads to accelerated and enhanced bioavailability while offering less frequent dose schedule. Consequently, it led to enhanced clinical outcomes with minimized side effects.[23] On top of that, ODFs are more palatable than other dosage forms, which enhance patient compliance. Moreover, its pliable nature is less frangible than an orally disintegrating tablet (ODT).[24] It requires no water and no need for swallowing the whole film as it disintegrates in saliva. Further, it takes precedence over ODT among patients with fear of choking the tablets, making ODFs ideal for dysphagic patients.[23] While ODFs represent phenomenal advantages, a major obstacle to their widespread application is the limited drug loading capability of ODFs, which only permits the incorporation of low-dose high potency drugs into ODFs.[25]

The objective of the current research was to formulate, optimize, and characterize the ODFs to attain faster disintegration leading to faster dissolution and brisk drug absorbance. In addition, our work aims to widen the dosage forms options for pediatric patients with selective mutism and OCD and enhance patient compliance to achieve better therapeutic outcomes. Furthermore, the influence of formulation parameters like (polymer, plasticizer) concerning their concentrations on ODF's evaluation was also investigated.

MATERIALS AND METHODS

Materials

Fluoxetine was used as an active pharmaceutical ingredient (API) and was generously given by Wilshire Labs (Pvt) Ltd. Lahore, Pakistan. Pullulan, a film-forming polymer, was purchased from Sigma Aldrich. Hydroxypropyl methylcellulose (HPMC) low viscosity E15, a film-forming polymer, and polyvinylpyrrolidone (PVP) (crospovidone), a super-disintegrant, were purchased from Moringa Pharmaceutical Pvt. Ltd., Lahore, Pakistan. Propylene glycol (PG), an excellent plasticizer, was obtained from Sigma Aldrich. Citric acid used as a saliva stimulating agent and fructose as a sweetening agent were obtained from CCL Pharmaceutical Pvt. Ltd., Lahore, Pakistan. Phosphate-Buffer Saline pH 6.8 was purchased from the Pharmaceutical research Lab of the University of Central Punjab, Lahore, Pakistan. All the chemicals were of analytical grade.

Methods

The solvent casting evaporation method reported in previous literature was adapted with slight modification.[26] The procedure involved solubilization of required quantity of film-forming polymers HPMC E15 and pullulan into distilled water. Then, plasticizer PG was solubilized into the hydro-polymeric solution and stirred for 30 min. Required quantities of fluoxetine, crospovidone (super-disintegrant), citric acid, and fructose were solubilized with distilled water in a separate beaker and mixed by stirring for 30 min. The plasticizer and polymeric solution were poured into drug-excipients solution, and the final volume of the formulation was adjusted to 15 mL. The final solution was homogenized at 1000 rpm for 60 min by a highspeed homogenizer. The homogenized solution was kept aside for 1 h to remove all entrapped air bubbles. Next, the homogenized and almost air bubble-free solution was cast in petri dishes and placed in a hot air oven at 45˚C for 24 h. Subsequently, the dried film was meticulously peeled off, carved into 2 cm × 2 cm ODFs, wrapped in aluminum foil, and stored in a desiccator until further use. Trials design and composition are shown in Table 1.

Preformulation studies

Fourier transform infrared spectroscopy (FTIR)

FTIR was employed to determine the compatibility between fluoxetine and excipients. The FTIR of fluoxetine and each excipient was performed independently. API and all excipients were in a 1:1 blended ratio. Each chemical was individually admixed with KBr and then scanned over the frequency range of 400 to 4000 cm^{-1}.[27]

Post-formulation studies

Physical appearance

The physical appearance of all batches of ODFs was visually investigated considering multiple factors: Aesthetic appearance, color, ODF surface texture and its symmetry, peel ability of ODF from mold (degree of ease in removing ODF from the mold without cracks and punctures in its surface), stickiness, and its flexibility.

Table 1. Experimental trials design and composition

Trials	Pullulan	HPMC E15	Fluoxetine	PG	PVP	Citrc acid	Fructose	Water
TF1	15%	15%	10%	10%	10%	4%	4%	q.s
TF2	20%	15%	10%	10%	10%	4%	4%	q.s
TF3	25%	15%	10%	10%	10%	4%	4%	q.s
TF4	30%	15%	10%	10%	10%	4%	4%	q.s
TF5	15%	20%	10%	10%	10%	4%	4%	q.s
TF6	20%	20%	10%	10%	10%	4%	4%	q.s
TF7	25%	20%	10%	10%	10%	4%	4%	q.s
TF8	30%	20%	10%	10%	10%	4%	4%	q.s
TF9	15%	25%	10%	10%	10%	4%	4%	q.s
TF10	20%	25%	10%	10%	10%	4%	4%	q.s
TF11	25%	25%	10%	10%	10%	4%	4%	q.s
TF12	30%	25%	10%	10%	10%	4%	4%	q.s
TF13	15%	30%	10%	10%	10%	4%	4%	q.s
TF14	20%	30%	10%	10%	10%	4%	4%	q.s
TF15	25%	30%	10%	10%	10%	4%	4%	q.s
TF16	30%	30%	10%	10%	10%	4%	4%	q.s

All quantities are expressed in % w/v. HPMC: Hydroxypropyl methylcellulose, PG: Propylene glycol, PVP: Polyvinylpyrrolidone, q.s: Quanum satis

Mechanical characteristics

Weight variation

Five oral strips from each batch were carved into 4 cm^2 and weighed individually using an electronic weighing balance. The average weight of weighed ODFs was calculated and subtracted from individual strip weight. Less variation of resultant value suggests the efficiency of the method employed and uniform distribution of API and inactive ingredients across the surface of ODF.[28]

Thickness

Thickness determination of ODF holds great importance in revealing the drug's even distribution and appropriate thickness, which facilitates ODF's adhesion capability to the tongue. Consistency in thickness of the film validates the accurate dose embodied in the ODF.[28] ODF thickness was measured using calibrated micrometer screw gauge at all the four corners and center points of individual ODF. Mean and standard deviation (SD) were calculated.

Folding fortitude

Folding fortitude manifests the pliability, tenacity, and resilience of ODF. It depicts the capability of ODF to withstand the folding on one plane without rupturing and cracking.[29] This test was performed by repeatedly folding three ODF of each batch at a single plane until it cracked, and the value was expressed as folding endurance value.

Drug content uniformity

Drug content uniformity of fabricated ODFs was determined according to El-Setouhy and Abd El-Malak[30] with modifications. ODF was cut into 4 cm^2 and put into a beaker with 50 mL of phosphate buffer (pH 6.8) and stirred for 1 h. Then, the solution was filtered using a syringe filter of 0.45 µm pore size. Absorbance was read at λ max of 263 nm using a ultraviolet (UV) spectrophotometer.

Disintegration test

Currently, there is no method for disintegration test specified in pharmacopeias officially. Among multiple reasons, the major and most demanding solution is a high volume of disintegration medium used in conventional dosage forms. Because high media volume does not simulate the biological and physiological condition of a buccal cavity due to limited saliva volume retaining capacity. In this consideration, several disintegration test techniques have been reported in the literature.[31] By considering the popularity and feasibility, the petri dish method was chosen in which phosphate buffer of pH 6.8 was used as a solvent medium for the *in vitro* disintegration test.[32] ODF was cut into 4 cm^2 and placed in a petri dish, and previously heated 2.5 mL of phosphate buffer (pH: 6.8) at 37°C was poured in the petri dish and whirled every 10 sec. The time (seconds) when ODF started to disintegrate and the fragment, was noted as *in vitro* disintegration time.[33,34] Disintegration time for three ODFs of the same batch was determined, and the average was calculated.

In vitro dissolution test

There is no appropriate dissolution method for ODF mentioned in any of the pharmacopeias. Various published methods in previous research of ODF were taken into consideration due to the lack of any authentic official method prescribed and adapted.[35,36] Hence, *in vitro* drug release was determined using USP paddle apparatus type II with 500 mL phosphate buffer (pH 6.8) and temperature set at 37±0.5°C at 50 rpm. ODF of 4 cm^2 was cut and placed into the dissolution media. An aliquot of 5 mL was taken at 0 min, 0.5 min, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 5 min, 7 min, 9 min, and 11 min, and immediately replaced with fresh media. Then, the sample was filtered through a filter syringe of 0.45 µm. The absorbance was read at 263 nm by UV visible spectrophotometer.

Scanning electron microscopy (SEM)

Surface morphology and framework of optimized formulation were scanned and examined by SEM. ODF was cut into 2 cm × 2 cm, and its surface morphology was scanned with SEM (model JSM5910JEOL, Japan) at voltage acceleration of 10 kV with a resolving power of max 2.3 nm.

Tensile strength, percent elongation, and Young's modulus

Mechanical properties of tensile strength, percent elongation, and Young's modulus were evaluated using Universal Testing Machine (UTM) (100-500KN, Testometric Inc. UK).[37] The optimized formulation of 30 mm × 16 mm was placed between two clamps, situated at a distance of 2 cm. One clamp was immotile while another clamp moved in the opposite direction at the speed of 1.00 mm/min.[27] At the point of rupturing and cracking, force and elongation values were recorded. Tensile strength, percent elongation, and Young's modulus were computed by equations below:[27,38]

$$Tensile\ Strength\ (N/mm^2) = \frac{Force\ at\ rupture\ (N)}{Thickness\ X\ width\ (mm^2)}\ X\ 100$$

$$\%\ Elongation\ at\ break = \frac{Increase\ in\ length}{Initial\ length}\ X\ 100$$

$$Young's\ modulus\ (N/mm^2) = \frac{Force\ at\ corresponding\ strain\ (N)}{Cross\ sectional\ area\ (mm^2)}\ X\ \frac{1}{Corresponding\ strain}$$

X-ray diffractometry

The crystallinity of fluoxetine was estimated using X-ray diffractometry. X-ray diffraction (XRD) of optimized formulation, placebo formulation, and pure drug (fluoxetine) was performed. Drug powder and formulations were scanned in two thetas (Θ) in a range from 19° to 70° with a tube voltage of 30 kV and current of 10 mA and the usage of monochromatic copper radiation Cu Ka (k: 2 Å) with a nickel filter.

Thermogravimetric analysis (TGA)

TGA helps the researcher to construe the effect of temperature rise on the mass of the sample tested. The sample was subjected to heating, and consequent mass variation was detected by a sensitive balance in a controlled environment. Mass variation occurs due to multiple factors such as sample chemical atrophy and physical processes like drying, vaporization, and

sublimation. The TGA was performed for the thermal analysis of optimized ODF and pure drug (fluoxetine) by gradually increasing temperature from 25˚C up to 300˚C at the rate of 10˚C per minute while continuous exposure to heating by constant nitrogen flow (20 mL/min).

Statistical analysis

All calculations were performed using Microsoft Excel® (Microsoft Office 2019, USA). All calculations are expressed as mean ± SD except dissolution studies, which were expressed as mean of the percentage of the drug release.

RESULTS AND DISCUSSION

The FTIR spectrums of fluoxetine and blended fluoxetine with polymers and other excipients was observed, and no interaction was detected (not shown).

Physical appearance

Visual inspection revealed that trial formulations with polymer concentration cumulatively up to 40% were considered as failed batches. The trial number of TF1, TF2, and TF3 were unable to be detached from the petri dish. These trial formulations displayed significant adhesiveness presumably because of the low percentage of polymer and higher PVP concentration (Table 2). This pronounced adhesiveness can be attributed to the hydrophilic and hygroscopic nature of PVP, which ultimately imparts tackiness that could also be seen in TF5 and TF13.[39] ODF batches with relatively higher pullulan content than HPMC rendered stickiness/tackiness and translucency. ODF batches with low pullulan and HPMC content were semi-transparent as the polymer content accumulated, it imparted the translucency to the formulation. ODF batches with lower content of polymer exhibited wrinkles and curves in ODF

Table 2. Physical appearance of trial formulations

Trial no.	Visibility	Surface texture	Wrinkles around edges	Flexibility & brittleness	Tackiness	Peelability
TF1	Semitransparent	Coarse & punctured	-	-	Extremely tacky	0 ++
TF2	Semitransparent	Bubbly, smooth, fragile, punctured	Wrinkled and bent edges	Brittle	Extremely tacky	0 ++
TF3	Translucent	smooth, bubbly, thin fragile,punctured	Slightly wrinkled edges	Brittle	Extremely tacky	0 +
TF4	Translucent	Smooth, intact surface	Wrinkleless straightened edges	Flexible	Non-tacky	1 +
TF5	Translucent	Smooth, bubbly, and intact surface	Inconspicuous wrinkles around edge	Flexible	Tacky	1 +
TF6	Translucent	Coarse and intact surface	Wrinkleless straightened edges	Flexible	Non-tacky	1 +
TF7	Translucent	Extremely smooth and intact	Wrinkleless straightened edges	Flexible	Non-tacky	1 ++
TF8	Translucent	Smooth and intact	Wrinkleless straightened edges	Flexible	Slightly tacky	1 +
TF9	Translucent	Punctured surface	Wrinkleless straightened edges	Flexible	Slightly tacky	0 +
TF10	Translucent	Smooth bubbly and intact	Wrinkleless straightened edges	Flexible	Non-tacky	1 +
TF11	Translucent	Very smooth and intact	Wrinkleless straightened edges	Flexible	Non-tacky	1 ++
TF12	Translucent	Very smooth and intact	Wrinkleless straightened edges	Flexible	Non-tacky	1 ++
TF13	Translucent	Extremely bubby, coarse, and intact	Wrinkleless straightened edges	Flexible	Tacky	0 +
TF14	Translucent	Smooth and intact	Wrinkleless straightened edges	Flexible	Non-tacky	1 ++
TF15	Translucent	Slightly coarse thick and intact	Wrinkleless straightened edges	Partial brittleness	Slightly tacky	0 +
TF16	Translucent	Coarse, thick, and intact	Wrinkleless straightened edges	Brittle	Slightly tacky	0 +

1 +: Good, 1 ++: Very good, 0 +: Poor, 0 ++: Very poor

batches. Formulation trials TF5, TF7, TF11, TF12, and TF14 exhibited the exceptional characteristics of having a smooth and intact surface with minimum or no wrinkled edges and phenomenal detachability from petri dish mold. Almost similar physical appearance parameter outcomes were observed in the films of levocetirizine, in which ODF formed with low viscous polymer grades and the ODF with low polymer percentage content were difficult to be peeled from mold. Hence, it can be established that ODFs with low polymer content lacks detachability. Moreover, in levocetirizine ODFs, films with higher pullulan content were translucent in appearance.[40]

Weight variation and thickness

Weight variation and thickness tests were performed on five ODFs of all retrieved batches. TF1, TF2, TF3, and TF9 batches were unable to be detached and retrieved from mold. Therefore, these batches were not included in both tests. All ODF batches qualified for the % weight variation according to USP pharmacopeial limit of ± 10% for dosage form with 130 mg or less weight. Weight variation was within the range of 72.67 mg±0.12-102.42 mg±1.41 (Table 3). The thickness of ODFs was recorded in the range of 0.11 mm±0.0089-0.63 mm±0.0549. These findings were in accordance with Nair et al.[28] where the thickness range of the typical film must be within the range of 50 µm-1000 µm. The optimum and homogenous thickness of the film is requisite for its uniform drug distribution, which ultimately has a profound effect on its content uniformity.[41,42] Uniform drying of ODF is a crucial stage in providing the uniform thickness of the ODF batch. The recorded thickness values were almost uniform in all trials suggesting the homogenous distribution of all ingredients in the ODF. In addition, findings also highlighted the validity of uniform drying in a hot air oven at 45˚C. The ODF with higher thickness values contributes toward diminished pliability and consequently low values in folding fortitude evaluation.[43]

Folding fortitude

Folding fortitude was observed by rotating the ODF at a single plane of 180˚ until it broke. A plasticizer is supposed to impart major pliability properties along with main polymers. In this context, plasticizer plays its role by entrapping itself into the polymer matrix and consequently rupturing and weakening the polymer-polymer linkages and augmenting the motility of polymer strands.[44] The folding fortitude of all retrieved ODF trial formulations was within the scale of 81.66±7.63-372.33±1.57 (Table 3). It was deduced that ODF with a relatively high pullulan content is more pliable and records higher folding fortitude value. In contrast, the ODF with relatively higher HPMC E15 tended to show diminished flexibility and exhibited lowered folding endurance. This deduction was supported by analyzing the recorded folding endurance values of TF5, TF13, TF14, and TF15. All mentioned trial formulations were formulated with relatively higher HPMC E15 than pullulan, which imparted the diminished pliability to ODFs and consequently poor folding endurance. This finding corroborated with ElMeshad and El Hagrasy[45], who found that mosapride ODFs formed with HPMC are stiff and lack pliability.

The influence of plasticizer concentration was also observed in the study of clobazam ODFs formation, where the increase in plasticizer concentration imparts enhanced pliability to ODF.[46] There is no authentic, official pharmacopeial value range for folding endurance; hence the ODF with more than 250 folding fortitude values were regarded as ODF with good folding fortitude attribute.[31]

Content uniformity

Drug content homogeneity was examined to ascertain the homogenous and precise distribution of fluoxetine in all successfully retrieved ODF trial batches. Three ODFs out of each trial formulation batch were evaluated using a UV spectrophotometer. Drug content must be within 85-115% to be

Table 3. Weight variation, thickness, folding fortitude, content uniformity, and disintegration time of trial formulations

Sr. no.	Trial no.	Weight variation (mg) ± standard deviation (n=5)	Thickness mean (mm) ± standard deviation (n=5)	Folding fortitude mean ± standard deviation (n=3)	Drug content uniformity (%) mean ± standard deviation (n=3)	Mean disintegration time (sec) ± standard deviation (n=3)
1	TF4	82.55±0.17	0.16±0.01	276.33±1.52	80.09±0.07	20.33±0.57
2	TF5	72.67±0.12	0.11±0.01	140.33±4.16	81.33±5.04	19.66±0.53
3	TF6	76.68±0.13	0.14±0.00	232.66±3.05	113.58±0.45	26.66±1.15
4	TF7	83.66±0.51	0.12±0.01	372.33±1.57	98.23±0.10	10.66±1.15
5	TF8	88.84±0.07	0.21±0.02	294.66±1.63	73.28±0.15	39±2
6	TF10	83.97±0.05	0.17±0.01	287.33±2.30	90.73±0.62	20.33±0.57
7	TF11	88.27±0.12	0.13±0.00	310.33±1.57	96.68±0.43	22±1
8	TF12	92.85±0.13	0.27±0.07	320.33±0.57	82.47±0.22	41.33±0.57
9	TF13	82.23±0.18	0.20±0.01	180.33±1.52	106.85±2.28	14±2
10	TF14	88.39±0.85	0.22±0.01	201.66±2.08	118.82±0.57	30.33±1.52
11	TF15	95.05±1.25	0.52±0.03	81.66±7.63	89.41±0.25	44.66±2.08
12	TF16	102.42±1.41	0.63±0.05	161.66±10.40	84.39±0.06	48.33±2.57

regarded as a successful batch.[20] Drug load of 10 mg per ODF of 4 cm^2, which corresponds to 2.5 mg/cm^2, was targeted to be achieved in all ODF trial batches, whereas the ranges of drug content in fluoxetine ODFs were found to be from 73.28%±0.15 to 118.82%±0.57 (Table 3).

Disintegration time

As European pharmacopeia publishes that ODF must disintegrate as placed in a buccal cavity. However, it does not declare any authentic method and maximal acceptable time for disintegration. Centre of drug evaluation and research states that the disintegration time range should be within the limits of 0-30 sec, so the same criterion was selected for ODF.[47,48]

PVP was employed as the super-disintegrant in a concentration of 160 mg per ODF batch. The PVP performs rapid disintegration by absorbing water through capillary action and expands, ultimately increasing hydrostatic pressure, which is required to disintegrate ODF readily.[43] Notably, the disintegration time ascended with the aggravation of polymer content in the ODF trials. Furthermore, the constant concentration of super-disintegrant in all ODFs was sparse to induce the disintegration in ODF of such high polymer content. Therefore, it can be assumed that the concentration of super-disintegrant must be commensurate with polymer content in ODF, and its higher concentration will lead to faster disintegration. Moreover, high polymer content could also be the cause to seal the capillary pores and ultimately block the influx of liquid into ODF, resulting in delayed disintegration time.[49]

ODF trial batches recorded the disintegration time equal to or less than 30 sec were regarded as the successful ODF in terms of disintegration time characterization (Table 3). TF7 manifested itself with an exceptional and phenomenal disintegration time of just 10.66 sec. After keen analysis of disintegration time correlated to polymer percentage, it was inferred that ODFs formulated with 45% of polymer content revealed themselves with exceptionally short disintegration time up to 19 sec. The recorded data showed that PVP proved itself as a competent super-disintegrant for the ODF formulation, although its efficacy is contingent on incorporating polymer concentration.

In vitro dissolution studies

No official drug release method of ODF is prescribed in pharmacopeias to be referred to for *in vitro* drug release characterization. For drug release, large volumes of media were used, whereas it did not conform to the limited saliva volume capacity of the buccal cavity.[23]

Additionally, the media used is not a bio-relevant medium and does not conform to physiological conditions of the buccal cavity. Due to the unavailability of artificial saliva in the research lab, phosphate buffer of pH 6.8 was used in dissolution apparatus type II, and absorbance was recorded at 263 nm. The dissolution apparatus used only permits the sampling to be taken at the minimum intervals of 30 sec, and it is not possible to withdraw the samples less than 30 sec. All the ODF batches exhibited a gradual increase in the percentage of drug release over the 11-min assay runtime. The percentage of drug release of ODF also varied along the consecutive 11 min of assay runtime (Table 4). It was due to various concentration combinations of polymers, pullulan, and HPMC. The fastest drug dissolution was observed in TF7, which almost completely released (99.37%) of fluoxetine within 3 min (Figure 1). It was deduced from the recorded data that ODF with lower polymer content tended to exhibit fast drug release. ODF formulated with total polymer content between 35% and 45% faster releasing the fluoxetine than other ODF batches, reaching their maximum drug release in 5 min (Figure 1a).

ODFs formulated with a higher polymer concentration (50-60%) combination of pullulan and HPMC E15 showed a slow drug release pattern (Figure 1b). Similar outcomes were observed in the levothyroxine ODF formulation, where films formed with a higher percentage of polymer content tended to slow down the release of levothyroxine.[26] It was concluded that

Table 4. Cumulative percentage of drug release of fluoxetine orodispersible film batches (*in vitro* dissolution studies)

Time (min)	0	0.5	1	1.5	2	2.5	3	5	7	9	11
TF4	0	15.65	26.98	39.31	55.51	66.30	76.01	80.14	80.71	-	-
TF5	0	13.71	30.15	42.15	50.01	59.81	72.15	83.91	84.42	-	-
TF6	0	7.30	18.95	32.33	45.12	56.13	68.31	86.15	103.21	-	-
TF7	**0**	**23.13**	**41.15**	**66.15**	**82.15**	**96.75**	**99.37**	-	-	-	-
TF8	0	5.15	12.31	22.15	38.01	57.60	68.91	80.15	81.12	81.16	81.19
TF10	0	16.71	29.15	41.65	59.36	73.12	80.12	98.15	-	-	-
TF11	0	9.16	18.15	25.79	38.29	52.76	65.90	83.15	97.61	-	-
TF12	0	4.9	9.53	18.15	26.15	39.56	50.25	67.53	85.93	98.47	-
TF13	0	19.45	37.26	54.57	71.77	86.89	98.35	102.4	-	-	-
TF14	0	12.17	20.65	30.45	43.25	57.48	67.38	84.74	98.30	113.15	-
TF15	0	7.80	14.16	22.15	33.67	49.56	63.21	75.5	84.56	87.75	87.81
TF16	0	5.90	9.40	12.45	18.45	22.56	32.76	49.65	62.43	78.57	89.35

CPDR slowed down in ODFs with higher polymer content. This was presumably due to higher polymer quantity, which could cause the generation of robust matrix layer by close and in-depth contact among the swollen matrix's molecular structures of, which possibly participated in the slow drug release phenomenon.[42]

Analysis and selection of optimized formulation

The most optimized formulation was selected by evaluating the characterization data of all ODF batches. The selection was based on the criteria of ODF with instant detachability/peel ability from petri dish mold, rapid maximum drug release in a minimum period, the briefest disintegration time, acceptable folding endurance, and content uniformity. The results showed that TF7 possessed the most desired properties. TF7 was then reformulated with the same concentration and procedure and further evaluated for mechanical properties such as SEM, XRD, and TGA.

Tensile strength, percent elongation, and Young's modulus

The main objective of the tensile test was to evaluate the fortitude and plasticity of TF7. The TF7 of 30 mm×16 mm was tested in UTM by mounting it between two tensioning clamps, and the motile clamp was set at the speed of 1.0 mm/min. Then load at break was observed, which ruptured the ODF at 0.1200 N. It was noted that the sample was ruptured at the middle of ODF instead of ODF at clamps.

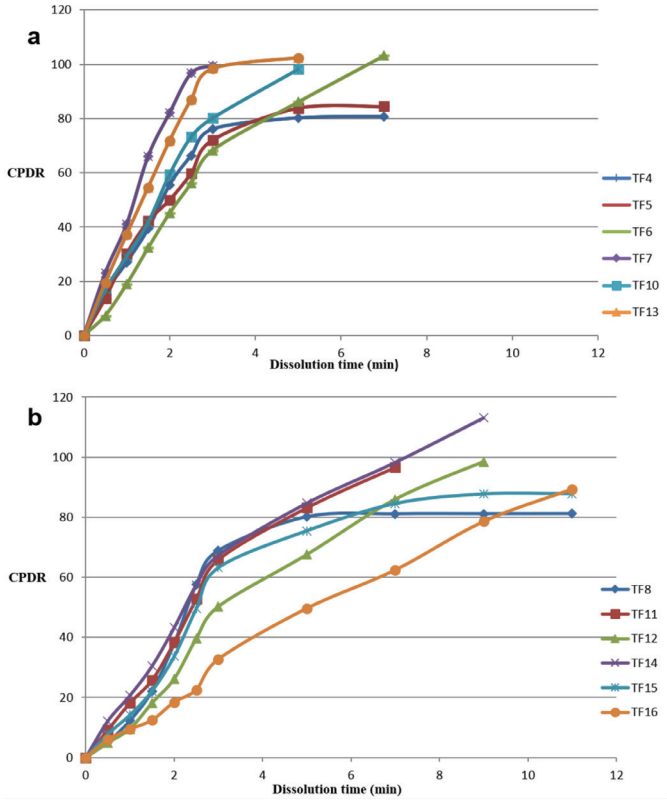

Figure 1. Dissolution profile of all trial ODFs: (a) Dissolution profile of ODFs with 35-45% content of polymer and (b) dissolution profile of ODFs with 50-60% content of polymer

ODFs: Orodispersible films

It is essential for the finished product to meet the requisite parameter criteria for its required competent functioning. Hence, to achieve this aspect, critical quality attributes (CQA) are supposed to be specified.[50] Classical ODF must be physically robust and pliable. These traits can be interpreted as classical ODF must possess high tensile strength and high percent elongation at rupture and low value of Young's modulus. According to CQA of mechanical properties of ODF, corresponding values should be as tensile strength >2 N/mm^2, % elongation >10%, Young's modulus <550 N/mm^2.[51] The mechanical properties of TF7: Tensile strength was 5.76 N/mm^2, % elongation was 38.85%, and Young's modulus was 280.37 N/mm^2. The results indicated the robustness of the TF7 structure (see detailed results in Table 5).

Therefore, TF7 is robust, flexible, and tough. The concentration of incorporated plasticizer (PG) has positive effects on tensile strength, and % elongation could be due to bonds formation between PG (plasticizer) and polymer (pullulan & HPMC E15), thereby imparting adequate flexibility and fortitude to ODF to endure and resist the rupture. However, it was found that plasticizer concentration has a negative effect on disintegration time.[26] It is also to be considered that much higher elongation is not desirable because it could generate the problem of elongation at edges while cutting the ODF batches, which could yield inhomogeneous ODFs, and diversify drug load. Therefore,

Table 5. Universal Testing Machine test results of optimized formulation of TF7

Area (mm^2)	16.000
Diameter (mm)	16.000
Elongation @ break (mm)	11.657
Elongation @ peak (mm)	0.5470
Elongation @ yield (mm)	0.4910
Energy @ break (N.m)	0.0016
Energy @ peak (N.m)	0.0024
Energy @ yield (N.m)	0.0020
Load @ break (N)	0.1200
Load @ peak (N)	7.1700
Load @ yield (N)	7.0100
Plastic strain @ break (%)	38.877
Strain @ break (%)	38.857
Strain @ peak (%)	1.8233
Strain @ yield (%)	1.6367
Stress @ break (N/mm^2)	0.0577
Stress @ peak (N/mm^2)	3.4471
Stress @ yield (N/mm^2)	3.3702
Young's modulus (N/mm^2)	280.37

optimal incorporation of appropriate plasticizer concentration holds key importance in the formation of classical ODF with adequate physical attributes.[52]

X-ray diffractometry

The XRD patterns investigation of pure fluoxetine powder showed clear and sharp peaks, implying that it is pure and crystalline. Peak signals were distinctively manifested at two theta (ϴ): 20.30˚, 21.94˚, 23.79˚, 27.94˚, which substantiated the high crystallinity of fluoxetine. The findings of Childs et al.[53] also corroborated the XRD pattern of the current investigation. XRD patterns of placebo formulation (TF0) showed no signal of fluoxetine peak as it was devoid of fluoxetine. TF0 gave no distinct peak in its XRD analysis pattern, verified its incorporated excipients' amorphous nature and lack of crystallinity. XRD investigation of TF7 manifested the peak signals of fluoxetine, which substantiated the efficient loading of fluoxetine into the TF7 and indicated the fluoxetine recrystallization phenomena (Figure 2). However, peak signal intensities of TF7 were less pronounced and showed minor aberration compared to peak intensities of pure fluoxetine. Due to the already detected 98.23% content uniformity of TF7, it can be conclusively affirmed that fluoxetine did not decompose, but rather fluoxetine presumably recrystallized in another refitting.

ODF technology is typically focused on water-soluble drugs, and the ideal case dictates that after solvent evaporation, the drug retains its dissolved form in the ODF matrix and does not crystallize. Whereas during experimentation, it usually differs from the ideal situation.[54] Fluoxetine is a white powder drug with crystalline form, which exists in multiple polymorph crystalline forms. The results indicated that fluoxetine stays in crystalline form in ODFs, which could exert its multi-faceted effects. On the one hand, this crystallinity refers to the enhanced inherent stability of the drug in a dosage form. On the other hand, a stable crystal form of the drug may display inadequate solubility, dissolution rate, and effects on pharmacokinetics.[55] Here it is noteworthy that recrystallization of fluoxetine in TF7 can exert its excessive influence on the disintegration properties, and dissolution, which may lead to problematic bioavailability.[23]

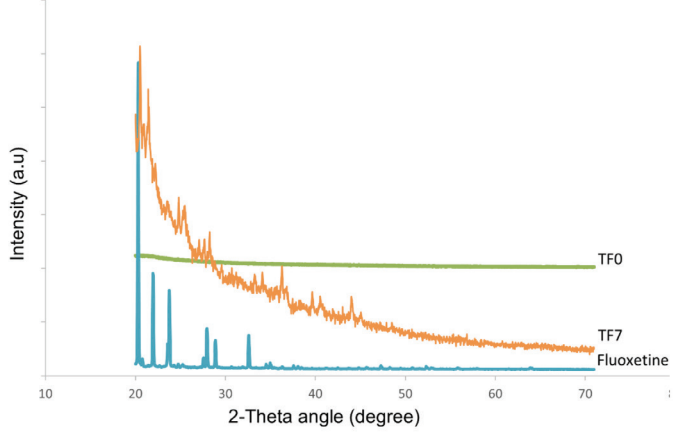

Figure 2. X-ray diffraction patterns of fluoxetine, TF7, and TF0

However, these influences need to be explored further in future studies, especially concerning its *in vivo* effects.

SEM

The SEM was performed for the morphological comparison of TF7 with TF0. Naked eye visual evaluation of TF7 and TF0 ostensibly reveal not much difference in their surface morphology; both ODFs exhibit relatively smooth surfaces. However, it was observed that the surface structure of TF7 and TF0 varied from each other. Both samples showed coarse surfaces on higher resolution images of SEM. TF7 showed drug particles on its surface, but TF0 had no crystal-like drug particles presented on it (Figure 3).

It was observed in SEM images that fluoxetine recrystallized and formed a needle-like structure in TF7. In comparison, TF0 was devoid of such needle-like crystal structures. The XRD pattern of TF7 can also validate the recrystallization phenomena of fluoxetine in SEM of TF7. Fluoxetine recrystallization could contribute to the coarseness or roughness of the TF7 surface observed under high resolution. Previous studies suggest that the crystalline nature of drugs may severely influence the mechanical characteristics of ODF by imparting more brittleness and less transparency.[56] However, as per our results, recrystallization of fluoxetine in TF7 could not negatively display its impact on final dosage, presumably owing to excellent polymer selection and plasticizer role.

TGA

The optimized formulation of TF7 and pure drug fluoxetine were subjected to TGA. It was found that fluoxetine showed initial weight loss at 165˚C and ensued by frequent weight loss around 208˚C, which elucidated the initiation of fluoxetine decomposition. As the temperature reached 228˚C, an abrupt and massive plummet in the mass of fluoxetine was spotted, which conspicuously correspond to the decomposition of fluoxetine.

The commencement of decline in the mass of TF7 can be observed in the initial stage of heating exposure. This mass decline can be presumably attributed to the evaporation of its entrapped wat er molecules and not the degradation of polymers in ODF. The degradation profile of TF7 is also attributed to

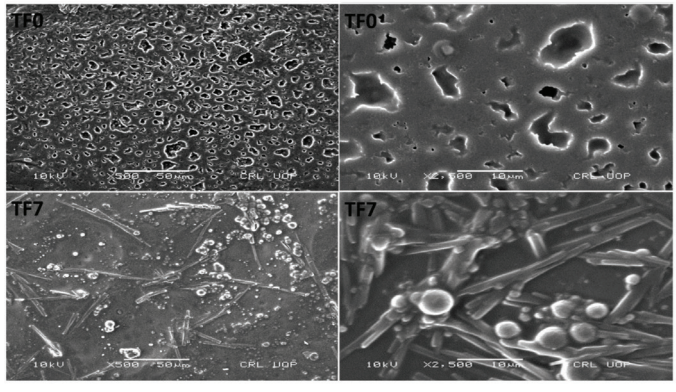

Figure 3. Scanning electron microscopy images of TF0 and TF7

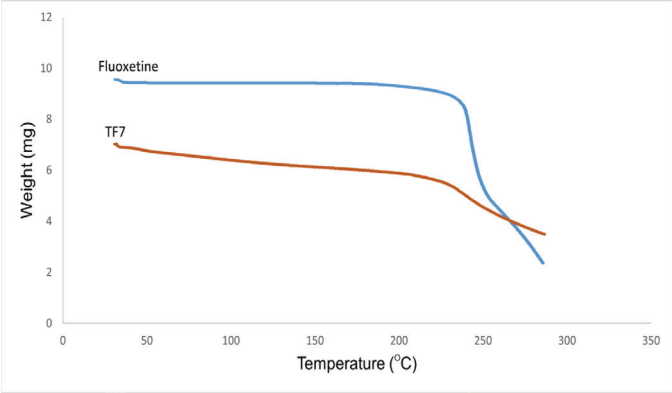

Figure 4. Thermogravimetric analysis curve of fluoxetine and TF7

the incorporated excipients. As ODF are highly susceptible to moisture uptake and regarded as hygroscopic, it is crucial to attribute the initial weight loss up to 100°C due to evaporation of water molecules. According to previously reported data, the TGA profile of PG suggested that PG degradation initiated at low temperature.[57]

It can be inferred from the data that TF7 comprised a higher percentage of polymers, and the ability of both polymers to retain water molecules in their matrices cannot be ignored while inspecting the TGA profile of formulation. Initial weight loss can be attributed to drying and water evaporation, followed by PG'searly degradation tendency. In addition, considerable mass decomposition spotted after 220°C can certainly be ascribed to fluoxetine decomposition as elucidated in the TGA of fluoxetine (Figure 4).

CONCLUSION

ODFs fabricated with cumulative percentage content of hydrophilic film-forming polymers (pullulan and HPMC E15) within the range of 35-45% resulted in shorter disintegration time and faster percentage drug release. PG exerts excellent plasticizing characteristics in formulating ODFs, and it imparted satisfactory tenacity and flexibility to ODFs. Conclusively, as the formulation of fluoxetine ODF was successful and has been thoroughly characterized with unobjectionable results, this dosage form sets a precedent and future prospect for further uptake of its *in vivo* evaluation by the scientific community and ensuing compliance studies in pediatrics patients of selective mutism and OCD. It is hypothesized that the convenience will persuade pediatric psychiatric patients in the need of fluoxetine of ODF. There is also an imperative necessity of standardized methods and official guidelines for the evaluation techniques of ODF for efficient quality analysis.

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ORIGINAL ARTICLE

The Financial Status of Community Pharmacies: Çorum Province

Toplum Eczanelerinin Mali Durumu: Çorum İli Örneği

Sıdıka Öznur SAKINÇ[1], Emrah BİLGENER[2]*

[1]Hitit University Vocational School of Social Sciences, Department of Management and Organization, Çorum, Turkey
[2]Hitit University Faculty of Health Sciences, Department of Health Economics, Division of Healthcare Management, Çorum, Turkey

ABSTRACT

Objectives: Community pharmacies have financial difficulties due to reasons such as economic crises in recent years, increased competition, reimbursement cuts, decreasing drug prices and profit ratio, and increasing operation costs. The main purpose of this study was to evaluate the financial status of community pharmacies in Çorum and to guide future sectoral financial studies.

Materials and Methods: One of the most commonly used method to evaluate the financial status and performance of companies is the "ratio analysis method". This ratio analysis has been conducted by accounting that data of 51 of the 93 community pharmacies operating in Çorum city.

Results: Considering the results of the ratio analysis in general, the pharmacies in Çorum have liquidity problems. More than half of the pharmacies could not pay their short-term debts with their current assets in the years mentioned in the study. Community pharmacies take more debit/credit to pay their short-term debts. The debit burden of the pharmacies has increased in the years mentioned in the study. Pharmacies have a low stock turnover ratio, and their inventory managements are insufficient.

Conclusion: It is seen that the main reason for the financial problems in community pharmacies is the low efficiency and productivity loss in resource management. It is thought that it would be beneficial to provide financial management training at the undergraduate level in the faculties of pharmacy and to provide vocational trainings within the Turkish Pharmacists' Association (TEB). In addition, an effective accounting system for community pharmacies should be prepared and implemented together by the TEB and relevant institutions.

Key words: Community pharmacy, Çorum, financial ratios, pharmacy management, Turkey

ÖZ

Amaç: Toplum eczaneleri son yıllarda yaşanan ekonomik krizler, artan rekabet, geri ödeme kesintileri, artan işletme maliyetleri, düşen ilaç fiyatları ve kar oranları gibi nedenlerden dolayı finansal zorluklarla karşı karşıyadır. Bu çalışmanın temel amacı, Çorum'daki toplum eczanelerinin mali durumunu değerlendirmek ve gelecekteki sektörel mali çalışmalara rehberlik etmektir.

Gereç ve Yöntemler: Şirketlerin finansal durumu ve performansı hakkında değerlendirmede en yaygın olarak kullanılan yöntemlerden biri "oran analizi yöntemi"dir. Bu analiz, Çorum ilinde faaliyet gösteren 93 toplum eczanesinin 51'ine ait muhasebe verileriyle gerçekleştirilmiştir.

Bulgular: Genel olarak oran analizi sonuçları dikkate alındığında, Çorum'daki toplum eczanelerinde likidite sorunları bulunmaktadır. Eczanelerin yarısından fazlası kısa vadeli borçlarını mevcut varlıkları ile ödememiştir. Toplum eczaneleri kısa vadeli borçların ödenmesinde daha fazla borç/kredi kullanmaktadır. Çalışmada belirtilen yıllarda eczanelerin borç yükü artmıştır. Eczanelerin stok devir hızı düşüktür ve stok yönetimi yetersizdir.

Sonuç: Toplum eczanelerinin mali sorunlarının temel nedeninin kaynak yönetimindeki düşük verimlilik ve verimlilik kaybı olduğu görülmektedir. Eczacılık fakültelerinde lisans düzeyinde ve Türk Eczacıları Birliği (TEB) bünyesinde mesleki eğitimlerde finans yönetimi eğitimi verilmesinin yararlı olacağı düşünülmektedir. Ayrıca, TEB ve ilgili kurumlar tarafından Toplum Eczaneleri için etkili bir muhasebe sistemi hazırlanmalı ve uygulanmalıdır.

Anahtar kelimeler: Toplum eczanesi, Çorum, finansal oranlar, eczane yönetimi, Türkiye

***Correspondence:** ebilgener@yahoo.com, **Phone:** +90 364 223 07 30, **ORCID-ID:** orcid.org/0000-0001-9916-3800

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INTRODUCTION

Community pharmacies have excellent medical facilities that are mostly accessed by patients.[1] Literature evidence over the past 30 years has shown that pharmacy performance is a critical factor in the success of the healthcare system and that it creates a significant improvement in the health outcomes of the community. These are direct pharmaceutical service providers that offer a wide range of services and simultaneously use many limited healthcare resources.[2,3] Healthcare services are one of the largest and fastest growing industries in the world. In developed countries, an average of 15% of health spending is allocated to drug expenditures.[4] The increasing demand of community pharmacy customers has strengthened the health system all over the world. Therefore, it seems necessary to evaluate the financial performance of this industry to ensure that limited resources are spent for the best benefits.[5] For community pharmacies, financial management is a dynamic process that requires adapting to the changes of the economic environment and other organizations. Thus, they could be more effective in the future and reach the strategic goals of the organization.[6,7] At present, community pharmacy is going through a very difficult process as it is exposed to daily financial constraint and economic pressures.[8] A community pharmacy must have adequate funds and manage them, ensuring its financial stability to fulfill its obligations and normally survive. Inputs make up most of the costs in providing pharmaceutical care in a community pharmacy. The difference between costs and revenues is the profit of the community pharmacy. Working order is required to ensure proper functioning of the community pharmacy.[9,10] Despite the difficulties it faces, it should continue its community pharmacy operations and survive in the future.[11]

For pharmacies to survive in a healthier way, their financial structure must be strong. One of the most commonly used methods in the financial structure analysis is ratio analysis. Ratio analysis is calculated using the data in the financial statements of the companies. In this method, the relationships between the data forming the financial statements are examined.[12] Financial ratios allow companies to compare both their status over the years and to other companies in the sector.[13-17] While interpreting the results obtained from the ratio analysis calculation, the situation of the company and the sector should be considered.

There are four subgroups in the ratio analysis method (Table 1). The first group is liquidity ratios. The liquidity ratio analysis examines the liquidity status of the companies, the adequacy of the company's capital, and the power to pay the company's short-term debits.[14] There are turnover ratios in the second group. The turnover ratios examine how effectively the business uses its assets.[15] The third group is leverage ratios. In this group, the source structure of the businesses is examined. The effectiveness of the businesses in resource use is investigated.[16] Profitability ratios are included in the fourth and last group. With profitability ratios, it is examined whether the profits obtained from all operations of the business are sufficient or not.[17]

The biggest customer of the community pharmacies in Turkey is Social Security Institution (SGK). Since most community pharmacies have contracts with SGK, they are less affected by economic crises than other retail sectors are. SGK payments are made regularly within the period specified in the contract. However, environmental factors, such as gradual profit ratio applied in the recent years, decreasing profitability, and price decreases in stock products and increasing costs, pose financial problems for community pharmacies. In addition, factors such as the fact that pharmacists do not know financing management and how to use their cash and capital in other sectors weakens the financial structures of these companies. When community pharmacies manage financial assets and resources effectively and efficiently, they may continue their operations and look to the future with confidence.

MATERIALS AND METHODS

In the study, 16 ratios covering five-year (2015-2019) liquidity, turnover, leverage, and profitability ratios were analyzed in four groups. Community pharmacies' accounting data were used which are operating in the central district of Çorum. Community pharmacies that do not want to give data (22 pharmacies), whose data were not reliable (six pharmacies), and that have been operating for less than five years (14 pharmacies) were not included in the study. For this reason, 51 of 93 pharmacies, from which we could obtain reliable data, were used in the study. Absence of a sector with a similar structure to make comparisons in Istanbul Stock Exchange, the absence of a similar study to this study conducted in other provinces or in Turkey, in general, and the fact that the accounting system of the community pharmacies is not effective, are the limitations of this study. These restrictions constitute an obstacle to the comparison of results obtained in the study and to comment on the overall situation of Turkey. The comments and the suggestions made were based on the data of pharmacies included in the study. Ethics committee approval was received from Non-Interventional Research Ethics Committee of Hitit University (05.11.2020/2020-113). No statistical analysis was used in the study.

RESULTS AND DISCUSSION

According to the data obtained from the 51 community pharmacies included in the study, "Average Liquidity Ratio of the Sector" is given by years in Table 2.

According to the data obtained from Table 3, most pharmacies cannot pay their short-term debits with their current assets for the year 2015. When the current ratios of the year 2015 were analyzed, it was determined that 18 pharmacies had values above the average, five pharmacies were close to the average, and the remaining 28 pharmacies had below-average values. The results for the year 2016 are like those for 2015. In 2017, there was an increase in the number of pharmacies whose current situation deteriorated. The number of pharmacies, which remained below the average in 2018, increased in 2017. Although there was a decrease in the number of pharmacies

that remained below the average in 2019, it was determined that, as in previous years, more than half the pharmacies could not pay their short-term debits with their current assets.

When the acid test ratio was analyzed, it was determined that in the year 2015, 18 pharmacies had values above average, 8 pharmacies were close to the average, and 25 pharmacies had values below average (Table 3). Acid test ratio is a more sensitive ratio compared to the current ratio. Short-term debt payment power is investigated by reducing inventories that take time to sell from current assets.[18] According to these results, it is seen that more than half of the pharmacies do not have the power to pay the short-term debt. This situation may be explained by the fact that pharmacies switched their purchases from long-term to short-term. In 2016, there were not many changes compared to 2015. In 2017, there was an increase in the number of pharmacies that remained below the average compared to the previous year. In 2018, there was a decrease in the number of pharmacies in a poor condition compared to the year 2017. However, an increase in the number of pharmacies below the average was determined in 2019.

When the liquid ratio of the pharmacies was analyzed by years, it was determined that in 2015, 18 pharmacies had values above average, 7 pharmacies were close to the average, and 26 pharmacies had values below average (Table 3). Liquid ratio is the ratio that determines the most sensitive situation among the liquidity ratios. This ratio shows the short-term debt payment power only with cash and similar assets.[19] It was determined that more than half of the pharmacies had cash problems while paying their short-term debts in 2015. In 2016, there was an increase in the number of pharmacies having cash shortage compared to 2015. In 2017, there was a decrease in the number of pharmacies with an average ratio, while there was an increase in the ones above average. In 2018, there was an increase in the number of pharmacies that had cash problems. In 2019, the situation remained almost the same compared to the year 2018.

According to the data obtained from the 51 community pharmacies included in the study, "Sector Average of Operating Ratio" is given by years in Table 4.

When the accounts receivable turnover ratio was examined, it was determined that 13 pharmacies had values above the average, 8 pharmacies were close to the average, and 30 pharmacies had values below average in 2015 (Table 5). The higher the turnover ratio, the better it is for a company.[20] Since the maturity given to its customers gradually decreases, it may collect more in a year. According to these data, more than half of the pharmacies make more deferred payment sales than average. This may be explained by the fact that cash sales of

Table 1. Ratios used in ratio analysis method[17]

Liquidity ratios	Turnover ratios	Leverage ratios	Profitability ratios
Current ratio	Account receivable turnover ratio	Financial leverage ratio	Gross profit ratio
Acid test ratio	Account receivable days	Equity to debt ratio	Net profit ratio
Liquid ratio	Stock turnover ratios	Shor-term debt to debt ratio	Return on assets
	Stock days		Return on equity
	Debtor turnover ratios		
	Debtor days		

Table 2. Average liquidity ratio of pharmacies by years

Liquidity ratios	2015	2016	2017	2018	2019
Current ratio	4,65637037	4,132142857	3,547741935	3,957096774	3,736451613
Acid test ratio	3,410740741	3,350357143	2,902258065	2,483870968	2,168709677
Liquid ratio	1,435888889	1,080357143	0,728064516	0,649677419	0,669548387

Table 3. Distribution of pharmacy liquidity ratios by years

Years	Pharmacy distribution by current ratios			Pharmacy distribution by acid test ratios			Pharmacy distribution by liquid ratios		
	< A	A	A <	< A	A	A <	< A	A	A <
2015	28	5	28	25	8	18	26	7	18
2016	27	4	20	25	9	17	29	4	18
2017	31	0	20	31	2	18	30	1	20
2018	33	2	16	28	5	18	33	0	18
2019	30	1	20	31	3	17	33	1	17

A: Average

pharmacies that make deferred payment sales are lower than the other pharmacies. Pharmacies increasing the cash sales levels will decrease average collection times. In 2016, there was a serious decrease in the number of pharmacies below the average compared to the year 2015. In 2017, pharmacies remained almost the same as in the previous year. It is believed that the cash sales of the pharmacies included in the study increased in 2016 and 2017. While there was an increase in the number of the pharmacies below the average in 2018, it is thought that there was a serious decrease in the cash sales of pharmacies in 2019.

When the account receivable day was analyzed, it was determined that 25 pharmacies had above-average values, 10 pharmacies were close to the average, and 16 pharmacies had below-average values (Table 5). While there was a small increase in the number of pharmacies below the average in 2016 compared to the year 2015, there was a significant increase in the pharmacies below average in 2017. As in previous years, the increase in the number of pharmacies below average continued in 2018. In 2019, unlike previous years, there was a significant increase in pharmacies above the sector average, and there was a decrease in those below the sector average. It is believed that the ratio of pharmacies' credit sales to total sales affects the debt collection periods.

When the stock turnover ratios of 2015 were examined, it was determined that 17 pharmacies had values above average, 9 pharmacies were close to average, and 25 pharmacies had values below average (Table 5). The stock turnover ratio shows how many times the business renews its stocks in a year.[21] In 2015, most pharmacies had a low stock turnover ratio. In 2016, there was a little increase in the number of pharmacies below average compared to the year 2015. In 2017, the situation remained the same compared to 2016. In 2018, the number of pharmacies below average decreased slightly, while those above average increased. In 2019, almost the same results were achieved compared to 2018. Pharmacies with a low stock turnover ratio are thought to bear an unnecessary inventory cost and do not behave carefully when buying goods.

When the stock days of 2015 were analyzed, it was determined that 16 pharmacies had values above average, 12 pharmacies were close to the average, and 23 pharmacies had values below average (Table 5). In 2016, there was a slight increase in the number of pharmacies, which remained below average compared to 2015. The situation like 2016 occurred in 2017. In 2018, there was an increase in the number of the pharmacies above average compared to 2017. In 2019, there was a decrease in pharmacies above the sector average compared to 2018. The stock days and the stock turnover ratio of the pharmacies in 2015-2019 are in line with each other. Pharmacies' inventory management is thought to be insufficient.

When the debtor turnover ratio of 2015 was examined, it was determined that 14 pharmacies had values above average, 6 pharmacies were close to average, and 31 pharmacies had values below average (Table 5). The high ratio of this is an indication that the company may have trouble while paying debt.[21] According to the data of 2015, most pharmacies did not

Table 4. Sector average of pharmacies' turnover ratios by years

Turnover ratios	2015	2016	2017	2018	2019
Account receivable turnover ratio	647	4.595	4.643	5.131	6.417
Account receivable days	77.532	79.781	95.467	98.805	82.246
Stock turnover ratios	8.501	14.428	12.141	7.452	6.277
Stock days	71.901	83.784	99.468	71.363	88.841
Debtor turnover ratios	26454.236	6435.872	14.504	28.707	9.247
Debtor days	59.967	59.576	72.903	63.529	67.466

Table 5. Distribution of pharmacy turnover ratios by years

Years	Account receivable turnover ratio			Pharmacy distribution by account receivable days			Pharmacy distribution by stock turnover ratios			Pharmacy distribution by stock days			Pharmacy distribution by debtor turnover ratios			Pharmacy distribution by debtor days		
	< A	A	A <	< A	A	A <	< A	A	A <	< A	A	A <	< A	A	A <	< A	A	A <
2015	30	18	13	16	10	25	25	9	17	23	12	16	31	6	14	27	5	19
2016	18	15	18	19	10	22	28	5	18	28	8	15	33	6	12	25	6	20
2017	19	15	17	24	8	19	29	4	18	31	6	14	31	5	15	24	4	23
2018	23	11	17	31	5	15	26	3	22	25	5	21	35	1	15	26	1	24
2019	35	2	14	19	4	28	25	5	21	26	7	18	28	4	19	26	4	21

A: Average

encounter this problem. In 2016, the situation was like that of 2015. In 2017, there was a decrease in the number of the pharmacies below average and an increase in the number of those above average compared to 2016. There was an increase in the number of pharmacies below average in 2018 compared to 2017. In 2019, compared to 2018, the number of pharmacies below the sector average decreased, and the number of pharmacies above it increased.

When debtor day - that is, due dates, one of the main activities of the pharmacy - of 2015 was analyzed, it was determined that 19 pharmacies had values above average, 5 pharmacies were close to the average, and 27 pharmacies had values below average (Table 5). While 2015 and 2016 show similarities, in 2017, there was an increase in the number of pharmacies above average, and a decrease in those below average. In 2019, there was a small decrease in the number of pharmacies above the sector average compared to 2017 and 2018.

According to the data obtained from the 51 community pharmacies included in the study, "Leverage Ratios Sector Average" is given by years in Table 6.

When financial leverage ratios of 2015 were analyzed, it was determined that 31 pharmacies had values above average, 3 pharmacies were close to the average, and 17 pharmacies had values below average (Table 7). The financial leverage ratio is a ratio that shows the debt burden in the funds of the business. The fact that it is high indicates that the debts of the business increased.[22] It is believed that more than half of the pharmacies use debit/credit. In 2016, the number of pharmacies below average increased compared to 2015. In 2017 and 2018, there was an increase in the number of pharmacies above average. There was a decrease in the number of the pharmacies above average in 2019 compared to 2018. It is an expected situation that pharmacies who have a cash shortage (Table 3) in the payment of short-term debts will use more debt.

When the equity to debt ratio of 2015 was analyzed, it was determined that 29 pharmacies had values above average, 8 pharmacies were close to the average, and 14 pharmacies had values below average (Table 7). Equity to debt ratio is found by dividing the debt by equity. The higher this ratio, the more the company's debt burden.[21] The debt burden of more than half of the pharmacies increased. The number of pharmacies above average continued to increase in 2016, 2017, 2018, and 2019. These data are compatible with the financial leverage ratios.

When the short-term debt to debt ratio of 2015 was examined, it was determined that 29 pharmacies had values above average, 6 pharmacies were close to the average, and 16 pharmacies had values below the average (Table 7). The weight of the short-term part of the debts is calculated at the short-term debt to debt ratio. This high ratio indicates that the company may face liquidity shortage. A short-term debt burden of more than half of the pharmacies is high. This situation explains the cash shortage in the payment of short-term debt and the use of debit/credit. In 2016, there was a decrease in the number of pharmacies below average compared to 2015. The number of pharmacies with a high debt burden increased in 2017 and 2018 compared to 2016. In 2019, the number of the pharmacies with high short-term debt burdens decreased compared to 2018. It is thought that the burden of short-term debts was reduced with the use of more debit/loans. In a survey conducted by Turkish Pharmacists' Association (TEB) in 2019, in Turkey, 57% of pharmacists stated that they used credit.[23]

According to the data obtained from the 51 community pharmacies included in the study, "Profitability Ratios Sector Average" is given by years in Table 8.

When the gross profit ratio of 2015 was analyzed, it was determined that 16 pharmacies had values above average, 23 pharmacies were close to the average, and 12 pharmacies had values below the average (Table 9). High gross profit ratio indicates that the company's purchase cost has decreased.[24]

Table 6. Leverage ratio sector averages of pharmacies by years

Leverage ratios	2015	2016	2017	2018	2019
Financial leverage ratio	2.315	1.946	2.350	2.925	2.602
Equity to debt ratio	2043.093	1968.811	4.771	5.363	4.580
Shor-term debt to debt ratio	1045.392	798.924	4244.861	4142.701	4727.976

Table 7. Leverage ratio distribution of pharmacies by years

Years	Pharmacy distribution by financial leverage ratio			Pharmacy distribution by equity to debt ratio			Pharmacy distribution by short-term debt to debt ratio		
	< A	A	A <	< A	A	A <	< A	A	A <
2015	17	3	31	14	8	29	16	6	29
2016	16	5	30	15	5	31	14	7	30
2017	14	4	33	13	4	34	12	5	34
2018	11	2	38	11	5	35	12	3	26
2019	11	5	35	12	3	36	20	4	27

A: Average

Most pharmacies have an average and above-average gross profit ratio. While the number of pharmacies with low profit margins increased slightly in 2016 and 2017, this situation improved in 2018 and 2019. It is thought that the profit ratio varies due to the discount ratio in the sales of the pharmacies and the distribution of imported/domestic drugs.

When the net profit ratio of 2015 was examined, it was determined that 12 pharmacies had values above average, 25 pharmacies were close to average, and 14 pharmacies had values below average (Table 9). High net profit ratio indicates that the productivity of companies has increased.[25] In this case, most pharmacies have a net profit ratio close to the sector average. In 2016 and 2017, the number of pharmacies above average increased compared to 2015. This situation may also be explained by the distribution of imported/domestic drugs in sales. In 2018 and 2019, there was not much change in the above-average and near-average pharmacies. Although most pharmacies continue their operations with an average and above-average net profit ratio, the fact that they experience cash and short-term debt payment difficulties and that they use debit/loans suggest that they are not financially well managed.

When return on assets (ROA) of 2015 was examined, it was determined that 25 pharmacies had values above average, 12 pharmacies were close to the average, and 14 pharmacies had values below the average (Table 9). ROA shows how effective the assets of companies are in generating profit.[26] More than half of the pharmacies manage their assets successfully. While there was not much change in 2016 compared to 2015, there was an increase in the number of below-average pharmacies in 2017. In 2018, there was an increase in the number of pharmacies close to the average. In 2019, with 13 pharmacies below-average, a better situation was found out than those in previous years.

When return on equity (ROE) of 2015 was examined, it was determined that 13 pharmacies had values above average, 24 pharmacies were close to the average, and 14 pharmacies had values below the sector average (Table 9). The ROE shows how much profit the partners have made in return for the capital they put in.[22] If this ratio is high, the profit of the business is high too. In this case, half of the pharmacies achieve the same profitability as the average. While the number of pharmacies close to the average and above average increased in 2016, the number of pharmacies close to average and below average increased in 2017. In 2018 and 2019, there was a decrease in the number of pharmacies with a below-average condition. It is noteworthy that although more than half of the pharmacies have a good picture in terms of gross profit margin, net profit margin, ROA, and ROE, they are experiencing financial difficulties. Increasing costs are thought to cause financial difficulties.

Reasons such as absence of a sector to make comparisons in İstanbul Stock Exchange, the absence of a similar study to this study, and the fact that the accounting system of pharmacies is not effective constitute obstacles to compare the results. Because of these reasons, general comments cannot be made about the financial status of community pharmacies in Turkey.

CONCLUSION

Community pharmacies want to survive like other businesses. They strive to maintain and increase their current market share while surviving. For a business to increase its competitiveness, its financial structure must be strong.

Table 8. Profitability ratios sector averages of pharmacies by years

Profitability ratios	2015	2016	2017	2018	2019
Gross profit ratio	0.920	0.877	0.761	0.676	0.823
Net profit ratio	0.712	0.484	0.484	0.320	0.403
Return on assets	0.902	1.043	0.935	0.953	0.828
Return on equity	1.939	2.030	2.469	2.057	2.939

Table 9. Profitability ratio distribution of pharmacies by years

Years	Pharmacy distribution by gross profit ratio			Pharmacy distribution by net profit ratio			Pharmacy distribution by return on assets			Pharmacy distribution by return on equity		
	< A	A	A <	< A	A	A <	< A	A	A <	< A	A	A <
2015	12	23	16	14	25	12	14	12	25	14	24	13
2016	14	22	15	13	24	14	15	10	26	12	25	14
2017	15	22	14	13	23	15	17	9	25	14	22	15
2018	11	24	16	10	25	15	15	14	22	13	24	14
2019	11	22	18	11	24	16	13	15	23	10	25	16

A: Average

As a result of the study based on the data obtained from community pharmacies, it is seen that these businesses prefer short-term debit/credit. This situation negatively affects the resource structure and cash situation of the business. It causes pharmacies to face liquidity shortage while paying their debts. When turnover ratios of the pharmacies are analyzed, the fact that the maturity given by them to their customers and the maturity given to them by the sellers are almost the same is increasing the importance of liquidity management for these businesses. For an effective financial structure to be formed, the existing financial structure needs to be changed. It is seen that most pharmacies in Çorum province are not effective in liquidity and resource management. This negatively affects the efficiency of commercial activities. The profitability situations of pharmacies generally move in the same direction.

It is thought that it would be beneficial to provide finance management training at the undergraduate level in the Faculties of Pharmacy and in vocational trainings within the TEB. In addition, an effective accounting system for community pharmacies should be prepared and implemented together by the TEB and relevant institutions.

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ORIGINAL ARTICLE

Salacia pallescens Oliv. (Celastraceae) Scavenges Free Radicals and Inhibits Pro-inflammatory Mediators in Lipopolysaccharide-activated RAW Cells 264.7 Macrophages

Lipopolisakkarit ile Aktive Olan RAW Hücreleri 264.7 Makrofajlarda *Salacia pallescens* Oliv. (Celastraceae) Serbest Radikalleri Uzaklaştırması ve Pro-inflamatuvar Mediatörlerini İnhibe Etmesi

Oyindamola Olajumoke ABIODUN[1,2*], Tolulope Aderinola OKE[2], Faridah Oyinkansola ADEYEMI[1], Adedunke Omobolanle OSHINLOYE[3], Adesewa Omolola AKANDE[1]

[1]Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria
[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Federal University Oye-Ekiti, Oye-Ekiti, Nigeria

ABSTRACT

Objectives: *Salacia pallescens* has folkloric anti-inflammatory claims, with little scientific investigation. Hence, the antioxidant and anti-inflammatory effects along with phytochemical components of the plant were investigated.

Materials and Methods: The antioxidant property of *S. pallescens* leaf (SPL) methanol extract was evaluated using 1,1-diphenyl-2-picrylhydrazyl and nitric oxide inhibition assays. The anti-inflammatory property of SPL in lipopolysaccharide-stimulated RAW 264.7 macrophages was determined. The cytotoxicity of SPL was assessed in brine shrimp lethality assay (BSL) and against RAW 264.7 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide based assay. Gas chromatography-mass spectrometry was employed to identify SPL phytochemical compounds.

Results: SPL significantly scavenged free radical generated in the antioxidant assays and inhibited nitrite production in stimulated RAW 264.7 cells. Similarly, there was a 9-fold reduction in interleukin-6 produced in RAW 264.7 cells when exposed to the highest concentration of SPL. In addition, 50% lethal concentration of SPL was 455.58±82.35 µg/mL while cyclophosphamide gave 16.3±0.15 µg/mL in BSL test. Moreover, cell viability was not affected by SPL. Sixteen compounds were identified from SPL where thymol (29.79%), 3-carene (15.97%), and p-cymene (12.19%) are the most abundant.

Conclusion: Methanol extract of SPL showed antioxidant and anti-inflammatory activities by free radicals and cytokines inhibition. The activity observed may be related to the polyphenolic compounds in the plant.

Key words: Anti-inflammation, antioxidant, *Salacia pallescens*

ÖZ

Amaç: *Salacia pallescens*, çok az bilimsel araştırma ile folklorik anti-inflamatuvar iddialara sahiptir. Bu nedenle bitkinin fitokimyasal bileşenleri ile birlikte antioksidan ve anti-inflamatuvar etkileri araştırılmıştır.

Gereç ve Yöntemler: *S. pallescens* yaprağı (SPL) metanol ekstresinin antioksidan özelliği, 1,1-difenil-2-pikrilhidrazil ve nitrik oksit inhibisyon deneyleri kullanılarak değerlendirilmiştir. Lipopolisakkarit ile uyarılan RAW 264,7 makrofajlarda SPL'nin anti-inflamatuvar özelliği belirlenmiştir. SPL'nin sitotoksisitesi, tuzlu su karides ölümcül tahlilinde (BSL) ve 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür bazlı bir tahlilde RAW 264,7 hücrelerine karşı değerlendirilmiştir. SPL fitokimyasal bileşiklerini tanımlamak için gaz kromatografisi-kütle spektrometrisi kullanılmıştır.

*Correspondence: oyindamolaabiodun1@gmail.com, Phone: +2347030964774, ORCID-ID: orcid.org/0000-0002-6629-3016
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Bulgular: SPL, antioksidan deneylerde üretilen serbest radikali önemli ölçüde uzaklaştırmış ve uyarılmış RAW 264,7 hücrelerinde nitrit üretimini inhibe etmiştir. Benzer şekilde, en yüksek SPL konsantrasyonuna maruz bırakıldığında RAW 264,7 hücrelerinde üretilen interlökin-6'da 9 kat azalma olmuştur. Ek olarak, BSL testinde SPL'nin lethal konsantrasyon 50'si 455,58±82,35 µg/mL iken, siklofosfamid için bu değer 16,3±0,15 µg/mL olarak belirlenmiştir. Ayrıca, hücre canlılığı SPL'den etkilenmemiştir. SPL'den on altı bileşik tanımlanırken, bunların aralarında en çok olanları timol (%29,79), 3-karen (%15,97) ve p-simenindi (%12,19).

Sonuç: SPL'nin methanol ekstresi, serbest radikaller ve sitokinlerin inhibisyonu ile antioksidan ve anti-inflamatuvar aktiviteler göstermiştir. Gözlenen aktivite, bitkideki polifenolik bileşiklerle ilgili olabilir.

Anahtar kelimeler: Anti-inflamatuvar, antioksidan, *Salacia pallescens*

INTRODUCTION

Inflammation is the host defensive immune response to tissue damage or infection.[1] Inflammation aims at localizing, eliminating, removing infecting agent, or repairing the injured tissue. In order to achieve these goals, the innate immune system tissue-resident cells discover the toxic insult and alert circulating neutrophils, which then proceed to the inflamed tissue.[2] Thus, promoting inflammatory monocyte recruitment and potentiating pro-inflammatory mediators such as cytokines and chemokine to handle the situation appropriately.[3] This process can cause reactive oxygen species (ROS) formation, vital signaling molecules that play a crucial part in the initiation, progression, and resolution of inflammatory response.[4] An increased ROS production by polymorphonuclear neutrophils at the inflammation area leads to tissue injury and endothelial dysfunction.[1] However, neutrophils undergo apoptosis under normal conditions after executing their roles.[5] The removal of apoptotic neutrophils prompts a change from a pro- to an anti-inflammatory macrophage phenotype.[6,7] Nonetheless, when inflammation is unresolved, it can progress to chronic inflammation. The persistence of chronic inflammation can result in cardiovascular, neurodegenerative, and respiratory diseases, including cancer.[8]

Drugs for inflammation treatment are effective but have serious side effects when used for prolonged time. For this reason, it is crucial to search for new and safe anti-inflammatory agents. Medicinal plants are valuable source of novel molecules and efficient alternative strategy for newer therapeutics development.[9] Many plants have been documented in traditional medicine to ameliorate various inflammatory disorders.[10] *Salacia pallescens* is a plant commonly known as Elewekan in the Yoruba ethnomedicine. It is used in folk medicine as an ingredient for a decoction to treat children,[11] particularly fever and pain. However, pharmacological activities of this plant are not readily available except for antioxidant activity.[12] Antidiabetic, anti-inflammatory, antioxidant, anticancer, nephroprotective, or hepatoprotective activities of other species of *Salacia* such as *S. chinensis, S. oblonga, S. reticulata, S. reticulate, Salacia parviflora, S. lehmbachii, S. senegalensis*, and *S. crassifolia* have been reported.[13-20] Therefore, this is the first report on *S. pallescens* anti-inflammatory activity and chemical composition.

MATERIALS AND METHODS

Plant material

Salacia pallescens leaf (SPL) was collected from Idi-Ayunre, Ibadan. A sample was taken for identification and authentication at the Forestry Research Institute of Nigeria (110527).

Extraction of the leaf of S. pallescens

The leaf of *S. pallescens* was air-dried and coarsely ground. Then, 206 g of SPL was macerated in 50% methanol for 72 h. Subsequently, the methanol extract was concentrated at reduced temperature and pressure. The extraction process was done thrice to increase the yield. The SPL methanol extract was stored at 4°C for further use.

Determination of total flavonoid content (TFC)

The method previously reported was used to determine TFC.[21] Briefly, 0.6 mL of SPL (1 mg/mL), 6.8 mL of 30% methanol, 0.30 mL of 0.5 M sodium nitrite, and 0.30 mL of 0.3 M aluminum chloride hexahydrate were mixed. Five minutes later, 2 mL of 1 M sodium hydroxide was added into the mixture, and the absorbance was read at 506 nm. TFC was reported as milligrams of rutin equivalents (RE) per gram of dried plant sample.

Determination of total phenolic content (TPC)

The TPC of SPL was estimated by a spectrometric method.[22] An equal volume (0.1 mL) of SPL (1 mg/mL) and Folin and Folin-Ciocalteuphenol reagent were mixed. After 5 min incubation, 1.3 mL of distilled water and 1 mL of 7% Na_2CO_3 were added into the mixture. Absorbance at 750 nm was read after 90 min. TPC was reported as milligrams of gallic acid equivalents (GAE) per g of the dried sample.

Antioxidant assays

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of SPL

SPL antioxidant activity by DPPH assay was estimated using a method previously reported.[23] Briefly, gradient concentrations of SPL (6.25-400 µg/mL) or standard ascorbic acid (0.25-16 µg/mL) were prepared in a 96 microtiter well plate, incubated for 30 min at 29°C in the dark after the addition of a freshly prepared solution of DPPH (0.04 mg/mL). The absorbance at 517 nm was read against the blank, and values obtained were expressed as the percentage of the control.

Nitric oxide scavenging activity of SPL

Antioxidant activity of SPL by nitric oxide inhibition assay was determined following a modified method of Panda et al.[24] Sodium nitroprusside solution (40 mM) was mixed with graded concentrations (50-800 µg/mL) of SPL (1:4 v/v) and incubated at 29°C for 2 h in the dark. Then, an equal volume of the incubated test solution and Griess reagent (1% sulphanilamide and 0.1% N-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) were added into a 96-well plate in duplicate and kept for an additional 15 min at 29°C in the dark. Absorbance at 550 nm was read. The amount of nitric oxide generated was extrapolated from the sodium nitrite curve.

Cell viability testing

RAW 264.7 cell line from the American Type Culture Collection (TIB-71; Rockville, MD, USA) maintained in cultured Dulbecco's modified eagle medium, 10% fetal bovine serum supplemented, 2 mM L-glutamine, and 100 IU/mL of penicillin-100 µg/mL streptomycin, at 37°C in 5% carbon dioxide incubator was used. SPL effect on cell viability was determined following a previously published method.[25] RAW 264.7 cells (5×10^5 cells/mL) were placed in a 96 microtiter well plate for 18 h prior to exposure to a graded concentration of SPL for 2 h. The cells were subsequently stimulated with 100 ng/mL liposaccharide from *Escherichia coli* 055: B5 for 24 h. Thereafter, the cultured medium was substituted with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in culture medium and further incubated for 2 h. The formazan blue formed due to the addition of MTT was dissolved with DMSO. Absorbance was read at 540 nm.

Anti-inflammatory testing

The SPL effect on nitrite and interleukin-6 (IL-6) produced in liposacharride (LPS) stimulated RAW 264.7 cell was determined. RAW 264.7 cells were seeded at 5×10^5 cells per well in a 24-well plate and permitted to grow into confluence before exposure to a graded concentration of SPL (50-400 µg/mL) for 2 h. The cells were subsequently stimulated with 100 ng/mL LPS, and the plate was incubated for 24 h. The nitrite content in the supernatant was estimated using the Griess reagent.[26] The IL-6 level in the supernatant was determined using m*ouse IL-6* ELISA MAX™ deluxe kit according to the manufacturer's instruction.

Brine shrimp lethality assay (BSL)

The SPL cytotoxicity was evaluated using BSL according to the method previously reported.[27] *Artemia salina* (brine shrimp eggs) was purchased at an Aquarium shop in the UK. The nauplii (larvae) were hatched by placing the eggs of *A. salina* in a tank containing seawater at 29°C. One part of the tank was exposed to light, and the other was covered with aluminum foil. The eggs were hatched after 48 h to nauplii which were attracted to light. Ten nauplii were transferred into graded concentrations of SPL extract (1.0-1.000 µg/mL) in plain test tubes. Cyclophosphamide drug was a positive control.

Chemical composition of SPL

The SPL phytochemical compounds were identified using an Agilent technologies 7890 gas chromatography system with a 5975 mass spectrometry (MS) following a previously described method.[28] Helium, 99.99% purity, was the mobile phase. The column (HP5 MS) had a thickness of 0.25 µm, an internal diameter of 0.320 mm, and length of 30 m. Compounds were identified by comparing retention time and fragmentation pattern against the NIST mass spectra library.

Statistical analysis

The antioxidants, anti-inflammatory, and BSL assays were performed in duplicates and repeated in three independent experiments. The 50% inhibitory concentration (IC_{50}) or lethal concentration (LC_{50}) values were expressed as mean ± standard error of three independent data. The mean IC_{50} or LC_{50} comparison of the SPL with the standard drug was made with the Mann-Whitney U test. P value <0.05 was taken as significant.

RESULTS

SPL percentage yield extracted with 50% methanol was 29.3%. The TFC and TPC of SPL extract were 190.29±1.43 mg RE/g and 609.5±0.42 mg GAE/g, respectively.

Antioxidant activity and BSL of SPL

The SPL antioxidant activity was concentration-dependent. Fifty percent IC_{50} in the DPPH and nitric oxide scavenging assays were 21.47±1.96 and 49.49±1.24 µg/mL, respectively (Table 1). Ascorbic acid gave an IC_{50} of 4.31±0.26 and 48.74±1.41 in DPPH and nitric oxide scavenging assays, respectively (Table 1). In the BSL test, LC_{50} of SPL was 455.58±82.35 µg/mL while cyclophosphamide was 16.3±0.15 mg/mL (Table 1).

Anti-inflammatory activity

The graded concentration effect of SPL on cell viability in LPS stimulated RAW 264.7 cells was assessed using MTT based assay. The concentration of SPL ranging from 50 to 400 µg/mL showed no effect on cell viability (Figure 1). Since SPL appeared non-toxic to RAW 264.7 cells at the tested concentration, its effect on nitrite production in LPS stimulated RAW 264.7 cells were assessed. SPL pre-treated LPS stimulated cells released a lower level of nitrite in the medium than the untreated control.

Table 1. IC_{50} and LC_{50} of *Salacia pallescens* leaf (SPL)

IC_{50} or LC_{50} (µg/mL)	*S. pallescens*	Standard drug
*IC_{50} DPPH inhibition	21.47±1.96#	4.31±0.26*
IC_{50} nitric oxide inhibition	49.49±1.24	48.74±1.41*
LC_{50} brine shrimp lethality test	455.58±82.35#	16.3±0.15**

*Ascorbic acid, **Cyclophosphamide, #SPL vs. control p<0.05, IC_{50}: 50% inhibitory concentration, LC_{50}: 50%$_{lethal}$ concentration, DPPH: 1,1-diphenyl-2-picrylhydrazyl

The SPL graded concentration (50-400 µg/mL) significantly inhibited nitrite production caused in LPS stimulated macrophages with inhibition ranging from 19.2 to 83.5% (Figure 2). Similarly, there was a 9-fold reduction in LPS induced IL-6 production in RAW 264.7 cells pre-treated with 400 µg/mL of SPL while pre-treatment with 50 µg/mL SPL resulted in a 1.4-fold decrease in IL-6 production (Figure 3).

Phytochemical constituents of SPL

The retention time, abundance, and m+1 values of SPL are presented in Table 2. Sixteen compounds were identified from the SPL extract. The most abundant compound is thymol (29.79%), followed by 3-carene (15.97%), p-cymene (12.19%), caffeine (8.28%), hexadecanoic acid (6.19%), bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl) (5.92%), and caryophyllene (5.17%). The mass spectra data depicted the fragmentation patterns and structures of the compounds in SPL are shown in Figure 4a-e.

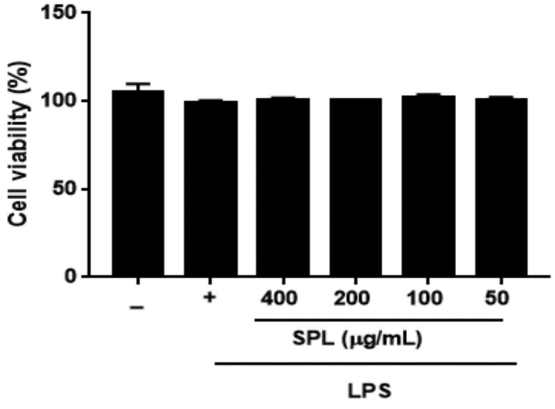

Figure 1. Effects of methanol extract of leaf of *S. pallescens* on cell viability in LPS stimulated RAW 264.7 cells. Data are expressed as means ± SEM

SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean

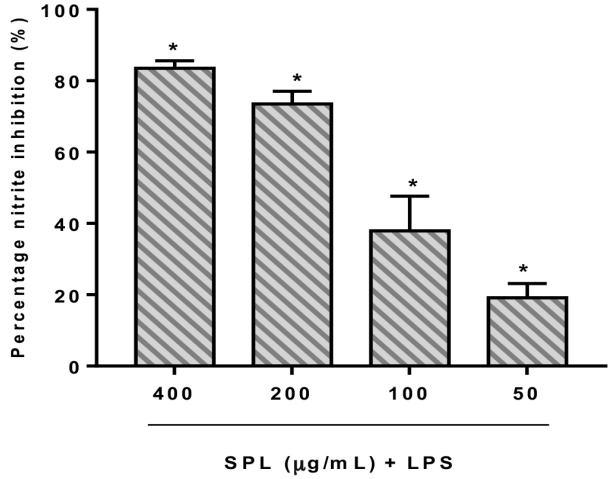

Figure 2. Inhibition of nitrite production in LPS stimulated RAW 264.7 cells by methanol extract of leaf of *S. pallescens*. Data are expressed as means ± SEM

*Significant percentage reduction in nitrite production, SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean

Table 2. Chemical compounds of ethyl acetate fraction of *Salacia pallescens* using GC-MS

GC peak	Compound names	GC-MS-RT (min)	Relative abundance %	M+1
1	p-Cymene	6.58	12.19	134
2	1R)-2,6,6-Trimethylbicyclo[3.1.1] hept-2-ene	7.10	1.82	136
3	3-carene	7.83	15.97	136
4	Methyl m-tolyl carbinol	9.12	1.99	136
5	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	9.91	1.44	164
6	Thymol	10.88	29.79	150
7	2-(Thiazolylazo)-p-cresol	12.25	1.06	219
8	Caryophyllene	12.70	5.17	204
9	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	12.83	5.92	204
10	Humulene	13.17	0.97	204
11	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]	13.62	4.08	204
12	3,6-Nonadien-5-one, 2,2,8,8-tetram ethyl-	13.72	1.43	190
13	Formamide, N-(4-benzofurazanyl)-	17.93	2.24	101
14	Caffeine	19.21	8.28	194
15	Hexadecanoic acid, methyl ester	19.70	6.19	270
16	9-Hexadecenoic acid	22.59	1.47	254

GC-MS: Gas chromatography-mass spectrometry, RT: Retention time

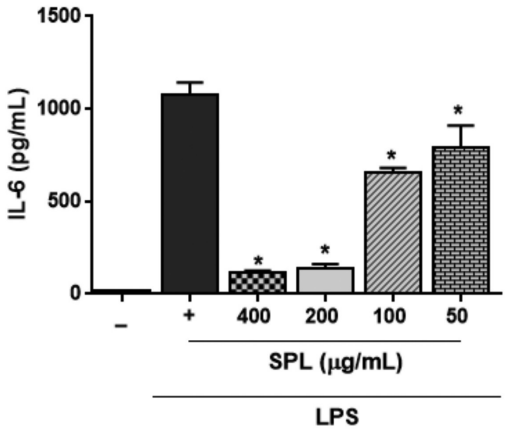

Figure 3. Effects of methanol extract of the leaf of *S. pallescens* on IL-6 production in LPS stimulated RAW264.7 cells. Data are expressed as means ± SEM

*Significant percentage reduction in IL-6 production, SPL: *Salacia pallescens* leaf, LPS: Liposacharride, SEM: Standard error of mean, IL-6: Interleukin-6

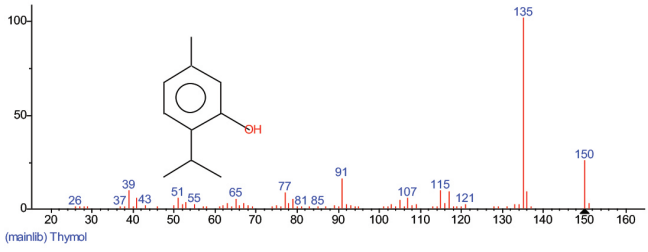

Figure 4a. Mass spectra and structure of thymol

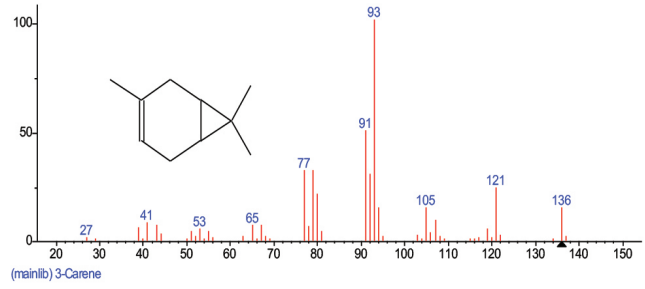

Figure 4b. Mass spectra and structure of 3-carene

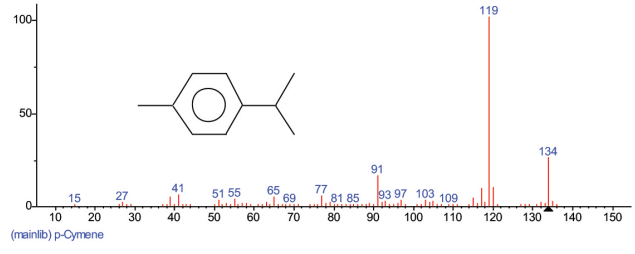

Figure 4c. Mass spectra and structure of p-cymene

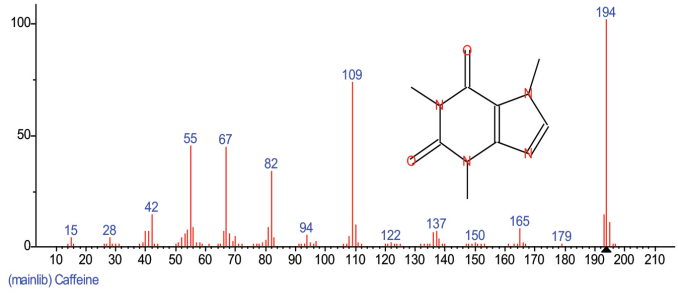

Figure 4d. Mass spectra and structure of caffeine

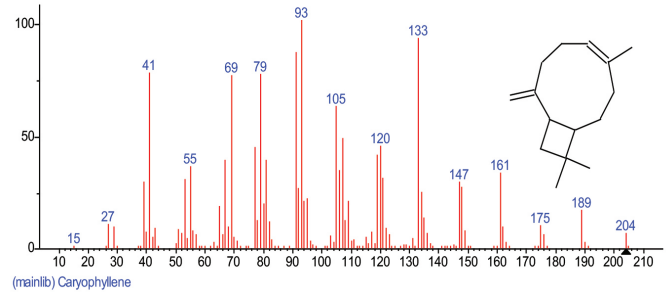

Figure 4e. Mass spectra and structure of caryophyllene

DISCUSSION

Findings in this study revealed that SPL possesses significant antioxidant activity. It scavenged free radicals generated by DPPH and nitric oxide in the DPPH- and sodium nitroprusside-based chemical assays. The high antioxidant property in plants is linked to its flavonoids and phenolic constituents, especially polyphenols,[29,30] found in SPL. Previous studies have reported antioxidant activity of *S. pallescens* and other species such *S. chinensis* and *S. oblonga*.[12,31,32] Oxidative stress occurs due to the high production of free radicals like alkoxy, hydroxyl, superoxide, and nitric oxide and non-radical species such as peroxynitrite, hydrogen peroxide, and singlet oxygen. These free radical and non-radical species cause redox system alteration, induction of DNA damage, and procarcinogens activation, which are linked to pathological conditions' development and progression.[33] Interestingly, the nitric oxide scavenging activity of SPL was comparable to ascorbic acid, a standard antioxidant drug, suggesting the benefit of the plant in controlling diseases associated with oxidative stress.

Liposaccharide stimulated RAW 264.7 cell is an anti-inflammatory model for screening agents with anti-inflammatory properties. Overproduction of nitrite and accumulation of cytokines which amplifies the inflammation cascade are the main characteristics of this model.[34] SPL significantly inhibited the overproduction of nitrite caused by liposaccharide and remarkably reduced the IL-6 level measured in this study. With the significant reduction of nitrite and IL-6 production, the leaf of *S. pallescens* demonstrated anti-inflammatory activity. To the best of our knowledge, this is the first anti-inflammatory activity report of *S. pallescens*. However, species of Salacia

with *in vivo* anti-inflammatory activity include *S. oblonga* and *S. lehmbachii*.[13,35]

Furthermore, the cytotoxicity of SPL was evaluated to determine its safety profile. The SPL cytotoxicity was evaluated in the BSL test. SPL was not toxic on *A. salina* nauplii. LC_{50} value of less than 100 µg/mL was toxic,[36] and SPL produced LC_{50} >400 µg/mL. Also, SPL was 28 times less toxic than cyclophosphamide, the standard drug. The BSL assay is used as a model for primary toxicity screening;[37] additional complementing tests might be needed before drawing a safety conclusion. Additionally, it was observed that SPL does not affect the RAW 264.7 cells viability.

Besides that, 13 compounds were identified in SPL, where thymol had the highest relative abundance. Thymol, a monoterpene, possesses antifungal, antidepressant, and anti-inflammatory, and cicatrizing properties.[38-40] The second most abundant compound was 3-Carene, a bicyclic monoterpene. 3-carene is one the major components of *Bupleurum gibraltaricum* essential oil with anti-inflammatory activity in carrageenan-induced paw edema in rats.[41] Antibacterial activity of 3-carene against *B. thermosphacta* and *P. fluorescens* resulted in morphological, genomic damages, and eventual cell death to the bacterial has been reported.[42] Also, 3-Carene exhibited a hypnotic effect in mice.[43]

Anti-inflammatory, anti-nociceptive, and antioxidant of p-Cymene, a monoterpene, the third most abundant in SPL, has been reported.[44-46] Caffeine, a CNS and metabolic stimulant, is the fourth most abundant in SPL. Caffeine is a xanthine alkaloid present in 60 plant species.[47] It has effects on smooth muscle, mood, memory, alertness, and physical and cognitive performance.[47] Hexadecanoic acid, methyl ester, is also known as palmitic acid. *Annona muricata* L. seeds fixed oil contains palmitic acid and other fatty acids showing free radical scavenging activity.[48] The antioxidant, anticancer, and anti-inflammatory activities of palmitic acid have been documented.[49-51]

Similarly, bicyclo [3.1.1] hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- found in SPL has been reported to be present in *Cinnamomum zeylanicum* with antibacterial and anti-fungi activities.[52] Likewise, antioxidant, anti-obesity, and antidiabetic activities of bicyclo [3.1.1] hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl) in *Ocimum basilicum* has been documented.[53] A bicyclic sesquiterpene, caryophyllene, also identified in SPL possesses anti-inflammatory activity by inhibiting pro-inflammatory cytokines, cyclooxygenase 1 and 2, and inducible nitric oxide synthase.[54] Other pharmacological activities of caryophyllene include neuro-protective, anti-apoptosis, antioxidant, and analgesia.[54-56] Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR (4a.alpha.7. alpha, 8a.beta.)] also known as β-selinene, is another compound found in SPL. β-selinene a component of essential oils in *Artemisia annua* has antioxidant activity.[57,58] Also, *Callicarpa macrophylla* β-selinene rich essential oils showed antioxidant anti-inflammatory, antipyretic, and analgesic properties.[58]

CONCLUSION

Methanol extract of SPL showed antioxidant and anti-inflammatory activities by free radicals and cytokines inhibition. The extract appears to be non-toxic. The activities observed may be related to the polyphenolic compounds in the plant.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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ORIGINAL ARTICLE

Discovery of Novel Pyruvate Kinase Inhibitors Against *Leishmania major* Among FDA Approved Drugs Through System Biology and Molecular Docking Approach

Sistem Biyolojisi ve Moleküler Yerleştirme Yaklaşımı Yoluyla FDA Onaylı İlaçlar Arasında *Leishmania major*'a Karşı Yeni Piruvat Kinaz İnhibitörlerinin Keşfi

Nasrin AMIRI-DASHATAN[1], Mostafa REZAEI-TAVIRANI[1], Mohammad Mehdi RANJBAR[2], Mehdi KOUSHKI[3], Seyed Dawood MOUSAVI NASAB[4], Nayebali AHMADI[1,5]*

[1]Shahid Beheshti University of Medical Sciences, Faculty of Paramedical Sciences, Proteomics Research Center, Tehran, Iran
[2]Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
[3]Zanjan University of Medical Sciences School of Medicine, Department of Clinical Biochemistry, Zanjan, Iran
[4]Pasteur Institute of Iran, Production and Research Complex, Department of Research and Development, Tehran, Iran
[5]Shahid Beheshti University of Medical Sciences, Faculty of Paramedical Sciences, Department of Medical Lab Technology, Tehran, Iran

ABSTRACT

Objectives: Leishmaniasis is one of the common forms of neglected parasitic diseases that cause a worldwide disease burden without any effective therapeutic strategy. Control of the disease currently relies on chemotherapy because most of the available drugs have toxic side-effects and drug-resistant strains have emerged. Therefore, the development of new therapeutic strategies to treat patients for leishmaniasis has become a priority. The first step in drug discovery is to identify an effective drug target by methods such as system biology. Protein kinases are a promising drug target for different diseases. Due to lack of a functional krebs cycle in *Leishmania* species, they use glycolysis as the only source of ATP generation. Pyruvate kinase is the enzyme involved in the last step of glycolysis and considered as essential enzyme for the *Leishmania* survival.

Materials and Methods: This study sought to discover FDA approved compounds against the leishmanial pyruvate kinase protein. Our approach involved using quantitative proteomics, protein interaction networks and docking to detect new drug targets and potent inhibitors.

Results: Pyruvate kinase was determined as the potential drug target based on protein network analysis. The docking studies suggested trametinib and irinotecan with high binding energies of -10.4 and -10.3 kcal/mol, respectively, as the potential chemotherapeutic agents against *L. major.*

Conclusion: This study demonstrated the importance of integrating protein network analysis and molecular docking to identify new anti-leishmanial drugs. These potential inhibitors constitute novel drug candidates that should be tested *in vitro* and *in vivo* to determine their potential as an alternative chemotherapy in the treatment of leishmaniasis.

Key words: *Leishmania major*, pyruvate kinase, protein network, drug target, docking

ÖZ

Amaç: Leishmaniasis, herhangi bir etkili tedavi stratejisi olmadan dünya çapında bir hastalık yüküne neden olan ihmal edilmiş paraziter hastalıkların yaygın formlarından biridir. Mevcut ilaçların çoğu toksik yan etkilere sahip olduğundan ve ilaca dirençli suşlar ortaya çıktığından, hastalığın kontrolü şu anda kemoterapiye dayanmaktadır. Bu nedenle, leishmaniasis hastalarını tedavi etmek için yeni terapötik stratejilerin geliştirilmesi bir öncelik

***Correspondence:** nayebalia@sbmu.ac.ir, **Phone:** +98-021-22714248, **ORCID-ID:** orcid.org/0000-0002-8870-7267
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haline gelmiştir. İlaç keşfinde ilk adım, sistem biyolojisi gibi yöntemlerle etkili bir ilaç hedefi belirlemektir. Protein kinazlar, farklı hastalıklar için umut verici bir ilaç hedefidir. *Leishmania* türlerinde fonksiyonel bir krebs döngüsü olmaması nedeniyle, ATP üretiminin tek kaynağı olarak glikoliz kullanırlar.

Gereç ve Yöntemler: Bu çalışma, leishmania piruvat kinaz proteinine karşı Gıda ve İlaç İdaresi onaylı bileşikleri keşfetmeyi amaçlamıştır. Yaklaşım, nicel proteomikler, protein etkileşim ağları ve yeni ilaç hedeflerini ve güçlü inhibitörleri tespit etmek için yerleştirmeyi içermiştir.

Bulgular: Protein ağı analizine dayalı olarak potansiyel ilaç hedefi olarak piruvat kinaz belirlenmiştir. Docking çalışmaları, *L. major*'a karşı potansiyel kemoterapötik ajanlar olarak sırasıyla -10,4 ve -10,3 kcal/mol'lük yüksek bağlanma enerjilerine sahip trametinib ve irinotekanı önermiştir.

Sonuç: Bu çalışma, yeni anti-leishmanial ilaçları tanımlamak için protein ağı analizini ve moleküler yerleştirmeyi entegre etmenin önemini göstermiştir. Bu potansiyel inhibitörler, leishmaniasis tedavisinde alternatif bir kemoterapi olarak potansiyellerini belirlemek için *in vitro* ve *in vivo* olarak test edilmesi gereken yeni ilaç adaylarını oluşturmaktadır.

Anahtar kelimeler: *Leishmania major*, piruvat kinaz, protein ağı, ilaç hedefi, docking

INTRODUCTION

Leishmaniasis is a complex infectious disease caused by various species of the genus *Leishmania, and* is a serious public health problem in many tropical and subtropical regions of the world.[1,2] The disease currently occurs in 12 million people and is threatening about 350 million in 98 countries worldwide.[3] At least 20 different species of *Leishmania* are responsible for a wide spectrum of human leishmaniasis forms.[4] This infectious disease is spread by the bites of the female *Phlebotomus* infected with metacyclic (infective) forms. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis in humans mainly caused by *L. major* and *L. tropica*.[1]

Currently, despite worldwide efforts to develop new drugs and vaccines, there are not effective vaccines against leishmaniasis. The main line of leishmaniasis treatment is chemotherapy. Pentavalent antimonials are the first choice for leishmaniasis treatment but have many limitations such as toxicity and emergence of drug resistance.[5,6] Alternative chemotherapeutic drugs such as amphotericin B and paromomycin are limited due to their high toxicity and cost.[7] The challenges of toxicity, drug resistance and cost make it difficult to design an effective drug system. Therefore, the main challenge in the treatment of leishmaniasis is to discover highly effective drugs. Drug target identification is the first step in the complex drug discovery process.[8] An ideal target in a pathogen should be one that is necessary for survival of the pathogen, and should not be present in the mammalian host or must differ from its host homolog. Protein kinases are the main regulators of several cellular processes and have attracted much attention as potential drug targets to treat a wide range of infectious diseases. Although trypanosomatids generally use glycolysis as a primary source of ATP, *Leishmania,* in their amastigote form, use both glycolysis and aerobic oxidation. They are just less dependent on oxygen than their other form.[9] In addition, *Leishmania* grow without glucose *in vitro* indicating that glucose is not their primary energy substrate. Therefore, inhibiting the glucose pathway may not have an effect on parasite growth.[10] Energy metabolism during *Leishmania* growth and metacyclogenesis is affected by regulated enzymes that probably respond to changes in glucose and amino acid levels in the culture medium.[10]

The unique location of glycolytic enzymes in glycosomes in *Leishmania* provides them with specific features.[7] Pyruvate kinase, an enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis, plays a role in regulating cell metabolism. In these organisms pyruvate kinase plays a key regulatory role, and is unique in responding to fructose 2,6-bisphosphate as an allosteric activator.[11] Given the importance of the energy production cycle and the important role of pyruvate kinase in this cycle, this enzyme could be considered as a potentially important pharmaceutical target. Also, this enzyme is necessary for survival of the parasite in the host and is different from the host homolog.[7,12]

Computational techniques increase the chance of success in drug discovery and in the design of desirable lead compounds. There are several computational approaches such as protein-protein interaction network analysis to select novel promising drug targets in different diseases.[13-15] In this work, we profiled the proteomic pattern of L. *major* metacyclic (infective stage) by using a label-free quantitative proteomic technique (sequential window acquisition of all theoretical fragment ion spectra mass spectrometry), and predicted the protein network of L. *major* in the STRING database. We analyzed the predicted protein network with centrality metrics to identify essential proteins as potential drug targets using the Cytoscape software. We identified drug targets evaluated with molecular docking to present new anti-leishmanial compounds (Figure 1). The objectives of this study were to: 1) identify appropriate target protein for discovery of drugs against leishmaniasis by system biology approach, and 2) identify potential lead compounds [Food and Drug Administration (FDA) compounds] for inhibition of the target enzyme by the docking method for studying protein- ligand binding interactions.

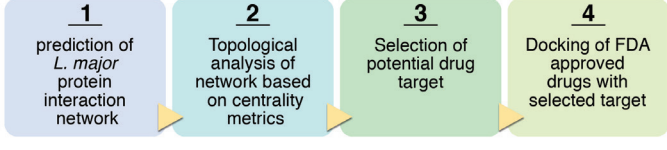


Figure 1. The framework used in the protein network and target prediction of Iranian isolate of *L. major*

FDA: Food and Drug Administration

MATERIALS AND METHODS

Prediction of protein network (system biology) and selection of target

The protein profile of L. *major* metacyclic was used for construction of protein-protein interactions (PPIs) through text mining, co-occurrence and co-expression in the STRING database. We calculated power law fit through Network Analyzer for predicted protein network. Topological analysis of predicted protein network, based on degree and betweenness centrality, were used to identify potential drug targets using CytoHubba plugin tool in Cytoscape software.

Selection of ligand dataset

The 1948 FDA approved compounds data were retrieved from the DrugBank database (www.drugbank.ca). Refinements of the 3D structure of compounds were done by performing energy minimization by the HyperChem software using the molecular mechanics approach (Hypercube Inc. Gainesville, FL, USA).

Target and ligand preparation

As the protein target chosen in our study was pyruvate kinase, the most suitable 3D we used was *Leishmania* pyruvate kinase enzyme in interaction with Suramin drug [Protein Data Bank (PDB) ID: 3PP7] downloaded from the PDB database (www.pdb.org). The resolution and R-value of this structure were 2.35 A˚ and 0.23, respectively. Energy optimization and correction of target protein and ligands were done using Chimera and HyperChem softwares, respectively. Briefly, water molecules and heteroatoms including ligand, cofactor, and carbohydrates were removed from the structure. Polar hydrogen atoms were then added before Kollman charges were assigned. The active sites of the target protein were evaluated based on prediction servers, inspection of 3D structure surface and literature review. These estimations provide the probable pocket for the binding of the substrate, which plays critical and significant roles in the enzyme function. Achieved residues in the binding pocket included: Arg_{49}, Thr_{26}, Pro_{29}, Tyr_{59}, Lys_{335}, His_{54}, and Asn_{51}.

Docking studies

In the docking study phase, compounds were searched for leishmanial pyruvate kinase inhibition activity. After the target protein and ligands preparation had been performed, docking was performed by the AutoDockVina software.[16] The ligands and protein format were required to convert to the PDBQT as input format of AutoDockVina. The 3D structures of ligands were docked with the 3D structure of the pyruvate kinase enzyme and their affectivity was ranked on the basis of their binding energy and hydrogen bond interactions. The docking approach uses an energy-based scoring method in which low binding energy scores represent premier protein-ligand bindings.[17] Grid box parameters were set as follows: (center_x = 27, center_y = 18, center_z = 40, size_x = 28, size_y = 32, size_z = 3), which cover the whole protein. The Ligplot+ software was used for automatic generation of schematic diagram of protein- ligand interaction.[18] LigPlot+ is a successor to the original LIGPLOT program for automatic generation of 2D ligand-PPI diagrams. It is run from an intuitive java interface which allows on-screen editing of the plots via mouse click-and-drag operations. The results showed some ligands which can be taken for further study on drug discovery.

RESULTS AND DISCUSSION

Target selection

We constructed a PPI map, from the results generated by mass spectrometry of L. *major* metacyclic protein profiling. From 144 detected proteins (results not published) in the Iranian isolate of L. *major* metacyclic (infective stage), we predicted a protein network with 135 nodes and 1051 interactions using the STRING database (Figure 2). Based on analysis of the topology of the network with connectivity (node degree) and betweenness centrality, we predicted potential drug targets. A measure of these centrality values has also been proposed as a new way for investigating potential drug targets. Proteins with high connectivity and betweenness centrality values are called hubs and bottlenecks, respectively. In protein networks, the proteins with high betweenness centrality value are referred to as scaffold proteins and are associated with protein essentiality more than connectivity. It has been reported that connectivity[19] and betweenness centrality[20] are important indices for the identification of essential proteins in protein- protein interaction networks.[21] In the L. *major* network, we have provided a putative list of five essential (hub-bottleneck) proteins with high connectivity and betweenness centrality. Among the top five hub-bottlenecks on the basis of its major role in ATP generation in *Leishmania* pathogen, we selected pyruvate kinase as target for a docking study (Table 1). The 3D structure of the pyruvate kinase is available in the PDB database. Kinases are of special

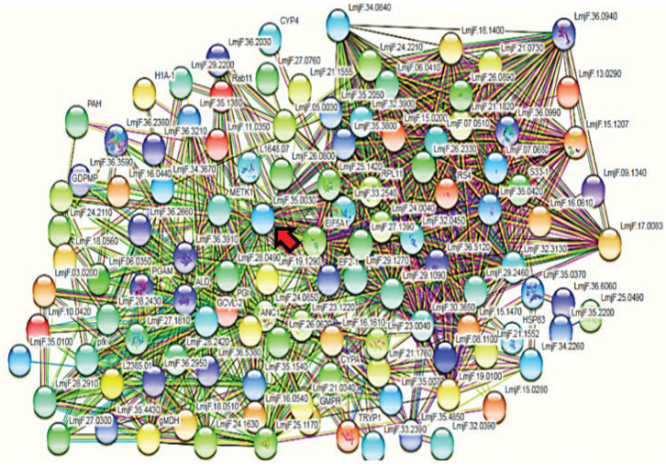


Figure 2. Protein-protein interaction network obtained by STRING v10.5. Nodes depict proteins and PPI are represented by edges in the network; interaction source of the PPI's are represented by various colors. The pyruvate kinase protein locus in PPIN is shown by arrows

PPI: Protein-protein interaction, PPIN: Protein-protein interaction network

interest as an important category of *Leishmania* proteins to be considered as sources of new drug targets.[12] In addition, several investigations compared the PyK crystal structure of the parasite with the PyK of other organisms. These studies reported important differences between these enzymes especially at the level of its effector site indicating distinctive regulatory attributes for the parasite enzyme, which could be a potential anti-leishmanial drug target.[11,22] For example Rebollo et al.[23] also selected pyruvate kinase along with other enzymes as a target protein for discovery of a new agent with potential leishmanicidal activity by virtual screening of chemical databases that all of them considered essential for the survival of *Leishmania*.[24] In fact, the parasite needs glycolysis enzymes because they are essential for the amastigote form of parasite. So the target would stop the intracellular parasite development in the host.

Docking studies

Structure-based virtual screening was carried out to predict the binding affinity of 1388 FDA approved compounds in order to identify new inhibitors of pyruvate kinase from *Leishmania*. This approach involved computational docking of ligands with a receptor, followed by scoring and ranking of ligands to discove potential leads. In previous studies, docking studies of some *Leishmania* enzymes such as Tryparedoxin peroxidase,[25] cysteine protease A,[26] N-myristoyltransferase,[27] 6-phosphoglucono-lactonase,[23] and trypanothione reductase[28] were done. Tryparedoxin peroxidase and trypanothione reductase as antioxidant enzymes play an important role in parasite survival, therefore, they can be suitable options for drugs against *Leishmania*. For example, Kothandan et al.[28] conducted a toxicology and docking study, and introduced Emetine as a potential lead compound against leishmaniasis. In the aforementioned studies, the several virtual screening databases have included Pubchem, ChemBridge and Pubchem BioAssay database. The various tools and softwares included Dock[29], Gold[30], Molegro Virtual Docker[17], SwissDock server[31], LibDock algorithm inbuild in DSv.5,[32] Sybyl 8.0 and AutoDockVina. In this study, we performed the docking assay for the first time by FDA approved compounds by AutoDockVina in order to identify pyruvate kinase inhibitors. After the compounds were screened against pyruvate kinase protein, the results were ranked based on their docking scores and the top 10 high scoring compounds are listed in Table 2. These compounds with high affinity to receptors could be considered as potential leads, which must be tested *in vitro* or *in vivo* experiments to confirm their real potential as anti-leishmanial drugs. Our results indicated that trametinib (DB08911) and irinotecan (DB00762) had the highest binding affinity to the target with -10.4 and -10.3 kcal/mol, respectively, and could provide a scaffold for further drug design efforts. Hydrogen-bonds play a crucial role in determining the specificity of ligand binding. In potential drugs from our findings, trametinib has one hydrogen bonding with a length of 2.88 A° and Irinotecan has also two hydrogen bonds, one with a length of 2.99 A° and the other one 3.14 A°. The number of hydrogen bonds of other compounds are shown in Table 2. Also, among these potential inhibitors, no side-effects have been reported for nilotinib (DB04868, Dock score: -10.1), netupitant (DB09048, Dock score: -10.1) and conivaptan (DB00872, Dock score: -9.9) in DrugBank database, rendering them valuable candidates for drug designing in future.

The protein-ligand interaction plays a crucial role in structure-based drug discovery. Based on our *in silico* docking analysis we suggest the following leishmanial pyruvate kinase potential drugs (trametinib, irinotecan, nilotinib, netupitant, naldemedine, eltrombopag, vumon, conivaptan, valstar and lomitapide). In this study, we took the pyruvate kinase essential protein (target) that plays a significant role in leishmaniasis and proposed the potent drugs that were used against leishmaniasis to evaluate its efficacy. These drugs can be tested *in vivo* and *in vitro* and as a lead for further validation in clinical trials. The study results would help to develop the new drugs for the leishmaniasis treatment.

This study has potential limitations. The anti-leishmanial effect of FDA-approved ligands against pyruvate kinase enzyme estimates are based only on computational methods and molecular interaction mechanism (i.e., protein interaction network and docking study). Further research involving experimental screening of compounds with high affinity and their analogues, optimization, and validation could lead to discovery of novel drugs for leishmaniasis treatment.

Table 1. Putative drug target ranked according to connectivity and betweenness centrality values in Hubba plugin in Cytoscape software. Pyruvate kinase selected in order to perform docking assay (grey color)

Hub-bottleneck proteins		
Rank	Gene name	Protein name
1	*LmjF.28.2780*	Putative heat-shock protein hsp70
2	*ENOL*	Enolase
3	*LmjF.35.0030*	Pyruvate kinase
4	*LmjF.36.2030*	Chaperonin HSP60, mitochondrial
5	*LmjF.25.1170*	ATP synthase subunit beta

Table 2. List of docking results of the FDA approved compounds with high binding affinity to the leishmanial pyruvate kinase inhibition

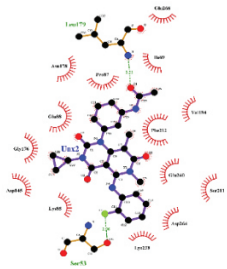
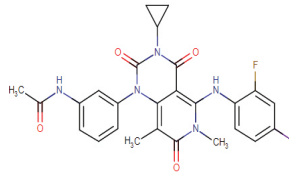
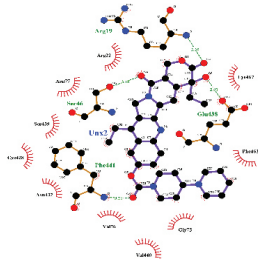
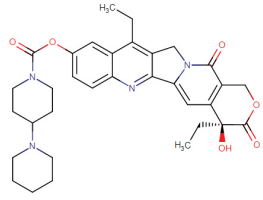
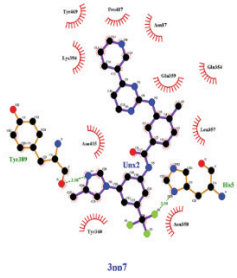
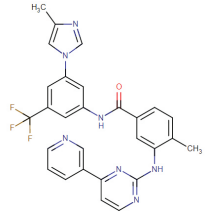
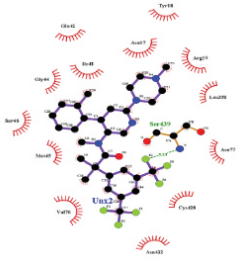
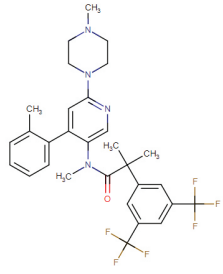
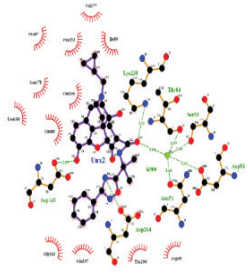
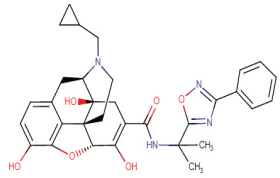
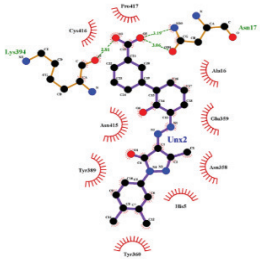
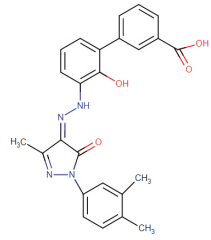
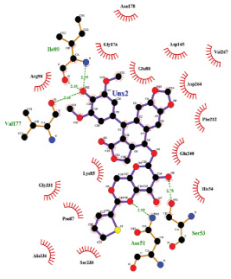
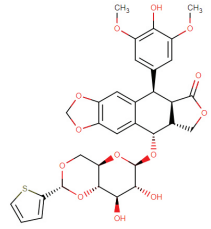
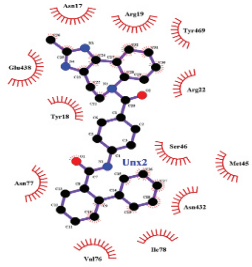
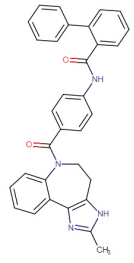
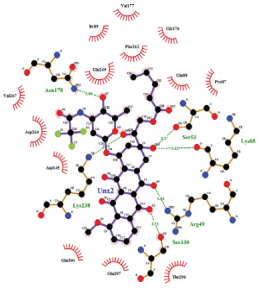
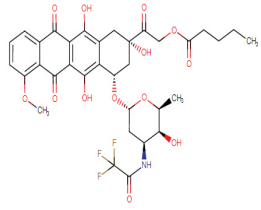
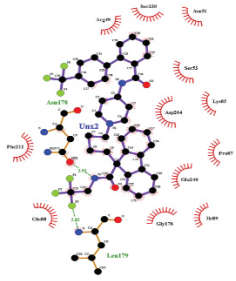
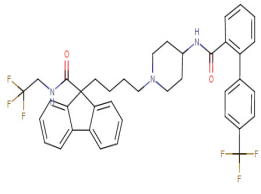
No.	Compound ID	Compound name	Binding energy (kcal/mol)	H-bond number (length:A°)	Target residues	Ligplot+ analysis	Drug structure
1	DB08911	Trametinib	-10.4	1 (2.88)	Glu268, Il89, Val184, Phe212, Glu240, Ser211, Asp264, Lys238, Lys85, Asp145, Gly176, Glu88, Asn178, Pro87		
2	DB00762	Irinotecan	-10.3	2 (2.99, 3.14)	Lys467, Phe463, Gly75, Val440, Val76, Asn432, Cys428, Ser439, Asn77, Arg22		
3	DB04868	Nilotinib	-10.1	-	Lys394, Tyr469, Pro417, Asn17, Glu359, Gln354, Leu357, Asn358, Try360, Asn415		
4	DB09048	Netupitant	-10.1	3 (3.11, 3.12, 3.23)	Asn17, Tyr18, Arg19, Leu351, Asn77,Cys428, Asn432, Val76, Met45, Ser46, Gly44, Ile41, Gln42		
5	DB11691	Naldemedine	-10.1	1 (3.09)	Arg49, Thr296,Gln297, Gly263, Glu88,Glu240, Leu186, Asn178,Pro87, Phe212, Val177, Ile89		

Table 2. contiuned

No.	Compound ID	Compound name	Binding energy (kcal/mol)	H-bond number (length:A°)	Target residues	Ligplot+ analysis	Drug structure
6	DB06210	Eltrombopag	-10.0	1 (3.12)	Pro417, Ala16, Glu359, Asn358,His5, Tyr360, Try389, Asn415, Cys416		
7	DB00444	Vumon	-9.9	-	Asn178, Gly179, Glu88, Asp145, Val267, Asp264, Phe212, Glu240, His54, Ser330, Ala334, Pro87, Gly331, Lys85, Arg90		
8	DB00872	Conivaptan	-9.9	3 (2.81, 3. 26, 3.23)	Asn17,Arg19, Tyr469, Arg22, Ser46, Met45, Asn432, Ile78, Val76, Asn77, Tyr18, Glu438		
9	DB00385	Valstar	-9.9	2 (3.01, 3.20)	Val177, Gly176, Ile89, Phe212, Glu240, Glu88, Pro87, Thr296, Gln297, Glu300, Asp145, Asp264, Val267,		
10	DB08827	Lomitapide	-9.8	1 (2.80)	Arg49, Ser330, Asn51, Ser53, Lys85, Asp264, Pro87, Glu240, Gly176, Glu88, Phe212		

CONCLUSION

In this study, we used molecular docking to identify the top potent pyruvate kinase inhibitors among different FDA approved compounds. Structure-based drug designing and lead discovery were used effectively for computer-aided drug discovery. We screened *in silico* a large library of FDA approved compounds against pyruvate kinase protein, a vital enzyme for *Leishmania* survival. Ligands bounded with higher affinity to the target protein ranked by AutoDockVina score. The results presented here require clinical evaluation before the identified therapeutic agents can be used as anti-leishmanial drugs. The identified leads with the higher possibility of binding to the target protein would reduce the cost of biological testing.

Code availability

Data were analysed using Cytoscape and AutoDock softwares.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

AQbD Driven Development of an RP-HPLC Method for the Quantitation of Abiraterone Acetate for its Pharmaceutical Formulations in the Presence of Degradants

Degradantların Varlığında Farmasötik Formülasyonları için Abirateron Asetat Miktarının Belirlenmesi için AQbD Güdümlü Bir RP-HPLC Yönteminin Geliştirilmesi

Bikash Ranjan JENA[1,2*], Siva Prasad PANDA[2], Umasankar KULANDAIVELU[2], Rajasekhar Reddy ALAVALA[2], G.S.N Koteswara RAO[2], Suryakanta SWAIN[3], Gurudutta PATTNAIK[1], Debashish GHOSE[4]

[1]School of Pharmacy and Life Sciences, Centurion University of Technology and Management, Odisha, India
[2]KL College of Pharmacy, Koneru Lakshmaiah Education Foundation, Andhra Pradesh, India
[3]Department of Pharmacy, School of Health Sciences, The Assam Kaziranga University, Koraikhowa, Assam, India
[4]Department of Pharmaceutics, Roland Institute of Pharmaceutical Sciences, Odisha, India

ABSTRACT

Objectives: Abiraterone acetate is a well-known anticancer drug and a steroidal derivative of progesterone for treatment of patients with hormone-refractory prostate cancer. Chemometrics-assisted reverse phase high performance liquid chromatography (RP-HPLC) development of the drug abiraterone acetate has been employed in this study using an analytical quality by design (AQbD) approach.

Materials and Methods: Drug separation was performed using a Princeton Merck-Hibar Purospher STAR (C18, 250 mm × 4.6 mm) i.d., 5 µm particle size) with ultraviolet detection at 235 nm. A Box-Behnken statistical experimental design with response surface methodology was executed for method optimization and desired chromatographic separation from its formulation with a few numbers of experimental trials. The impact of three independent variables, namely, composition of the mobile phase, pH, and flow rate, on response retention time and peak area was studied by constructing an arithmetic model from these variables.

Results: Optimized experimental conditions for the proposed work include the mobile phase acetonitrile and phosphate buffer (10 mM KH_2PO_4) (20:80 %v/v). At the concentration range of 2-100 µg/mL, a linear calibration curve was found. Recovery was performed at three concentrations and was foun to be between 98% and 102%. The 3D response surface curves revealed that mobile phase composition and flow rate were the most substantial critical factors affecting desired responses.

Conclusion: An attempt has been made to develop and validate an economical, precise, robust, stability-indicating AQbD-based RP-HPLC method that can be employed successfully for the routine analysis of abiraterone acetate in quality control labs.

Key words: Precision, accuracy, ICH guidelines, method validation, experimental design

***Correspondence:** bikashranjan.jena97@gmail.com, **Phone:** +91 (0674) 2596228, **ORCID-ID:** orcid.org/0000-0001-9722-4454
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ÖZ

Amaç: Abirateron asetat, iyi bilinen bir antikanser ilacıdır ve hormona dirençli prostat kanseri olan hastaların tedavisi için progesteronun steroidal bir türevidir. Bu çalışmada abirateron asetat ilacının kemometri destekli ters fazlı yüksek performanslı sıvı kromatografisi (RP-HPLC) ile geliştirmesi, analitik kalite tasarım (AQbD) yaklaşımı kullanılarak gerçekleştirilmiştir.

Gereç ve Yöntemler: İlaç ayrımı, 235 nm'de ultraviyole saptamalı bir Princeton Merck-Hibar Purospher STAR (C18, 250 mm x 4,6 mm) i.d., 5 um partikül boyutu) kullanılarak yapılmıştır. Tepki yüzeyi metodolojisine sahip bir Box-Behnken istatistiksel deney tasarımı, yöntem optimizasyonu ve birkaç deneysel deneme ile formülasyonundan istenen kromatografik ayırma için uygulanmıştır. Üç bağımsız değişkenin, yani mobil fazın bileşimi, pH ve akış hızının, yanıt tutma süresi ve tepe alanı üzerindeki etkisi, bu değişkenlerden bir aritmetik model oluşturularak incelenmiştir.

Bulgular: Önerilen çalışma için optimize edilmiş deneysel koşullar, mobil faz asetonitril ve fosfat tamponunu (10 mM KH_2PO_4) (20:80 %h/h) içerir. 2-100 µg/mL konsantrasyon aralığında doğrusal bir kalibrasyon eğrisi bulunmuştur. Geri kazanım, üç konsantrasyonda gerçekleştirilmiş ve %98 ile %102 arasında bulunmuştur. 3B yanıt yüzey eğrileri, mobil faz bileşimi ve akış hızının, istenen yanıtları etkileyen en önemli kritik faktörler olduğunu ortaya çıkarmıştır.

Sonuç: Kalite kontrol laboratuvarlarında abirateron asetatın rutin analizi için başarıyla kullanılabilecek ekonomik, kesin, sağlam, stabiliteyi gösteren AQbD tabanlı RP-HPLC yöntemini geliştirmek ve doğrulamak için bir girişimde bulunulmuştur.

Anahtar kelimeler: Kesinlik, doğruluk, ICH yönergeleri, yöntem validasyonu, deneysel tasarım

INTRODUCTION

The potent anticancer drug abiraterone acetate {[(3*S*,8*R*,9*S*,10*R*,13*S*,14*S*)-10,13-dimethyl-17-pyridin-3-yl-2,3,4,7,8,9,11,12,14,15-decahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl]acetate} is an acetyl ester and a significant prodrug of its active metabolite abiraterone.[1-3] It is a well-built receptor blocker of androgen with potent bioavailability, especially in oral administration.[4] Abiraterone acetate is found to be more intense and effective than ketoconazole and liarozole in CYP17A1 inhibition; which is a rate-limiting enzyme for the biosynthesis of androgens.[4,5] Principally, abiraterone acetate is specified for use in combination with prednisone for treatment of men with metastatic castration-resistant prostate cancer who have already received prior chemotherapy comprising docetaxel.[5] Abiraterone acetate is poorly soluble over an extensive pH range. However, it is faintly soluble in HCl as well as in organic solvents (Figure 1). Abiraterone acetate can be detected by liquid chromatography-tandem mass spectrometry,[6-8] spectrofluorimetric for measuring fluorescence emission, and absorption spectroscopy in cancer patients.[9-11] Furthermore, the drug has been thoroughly recognized by other chromatographic techniques for the quantification of metabolites in biological samples bioanalytically.[8,9,12] However, there is scant research using QbD Paradigm, MODR Concepts, design space, and systematic development by chemometrics-assisted statistical optimization of critical method parameters (CMPs) and critical method attributes.[13] These optimization techniques with analytical quality by design (AQbD) are efficient, cost-effective, and innovative methodologies, which signify the "finest solution" to a meticulous "complexity" or problems raised in method development but deliver consistent quality output where other general High-performance liquid chromatography (HPLC) methods have been unable to achieve.[13-16] Notably, more relatively efficient optimization techniques such as Box-Behnken design (BBD) than other designs (central composite, d-optimal, and mixture design) have been applied for analytical method development and optimization of pharmaceutical drugs. BBD is a second-order three-level factorial design with advantages of user-friendly, economical, simple, and limited experimental runs.[17,18] Apart from that, such BBD optimization is a sequential design empowered with a feasible design matrix showing the quadratic model with a straightforward assessment of CMPs or critical material attributes. It also requires limited variables or responses, mainly three coded levels such as [low (-1), middle (0), and high (+1)], for the experimental design to fit the statistics or lack of fit values of the distinctive quadratic model.[17-19] Therefore, the developed method of validation, optimization, and stability-indicating reversed-phase (RP)-HPLC by employing the AQbD approach for the quantification of the bulk drug and its pharmaceutical formulations of abiraterone acetate is more economical, robust, and less expensive and reduces the heat and trial methods of optimization as well as the revalidation process as compared to one factor-at-a-time approach.

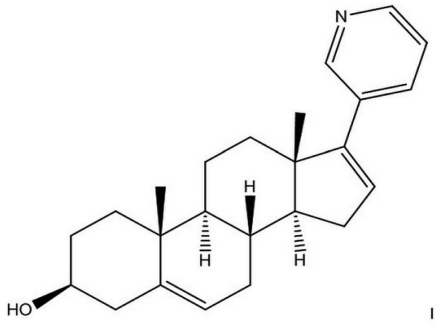

Figure 1. Chemical structure of abiraterone acetate

MATERIALS AND METHODS

A pure standard of the drug abiraterone acetate was obtained from Sun Pharmaceutical Industries Ltd., Halol, Gujarat, India. Abiraterone acetate was obtained commercially [XIBRA (Cipla Ltd, India), ZYTIGA (Janssen-Cilag Ltd., India), and ABIRAPRO (Glenmark Pharmaceuticals, India)] with a labeled claim of 250 mg. Phosphate buffer and acetonitrile (HPLC grade) were procured from Merck Laboratories, Mumbai.

Instrumentation

A Shimadzu HPLC system (LC-2010C HT) with a ultraviolet (UV)-visible detector, ultra-sonicator from Remi Instruments, Mumbai, and nylon filter (0.45 µm) from Millipore, Mumbai, India, was used.

Chemicals and reagents

Orthophosphoric acid (OPA) (AR grade) and acetonitrile (HPLC grade) were obtained from Merck Laboratories Pvt. Ltd., Mumbai. Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Fischer Scientific, Mumbai, India.

Methods

Method optimization was performed with Box-Behnken statistical design comprising the CMPs, which include three significant factors (the composition of the determined mobile phase, flow rate, and pH of buffer), encompassing three levels. Seventeen experimental runs were established with five center points. The flow rate was tested at 1.0, 1.2, and 1.5 mL/min; the pH was measured at 4, 5, and 6; and the concentration of the mobile phase was monitored at 20%, 50%, and 80%. The responses considered were retention time (Rt) and peak area, which were designated as critical analytical attributes (CAAs). The data were analyzed, and the model was validated with Design-Expert software. The quadratic model revealed a virtuous correlation with the experimental data executed for design space navigation. The 2D & 3D response surface techniques and perturbation sequential plots were scrutinized to assess the indicative critical factors' impact upon the observed responses or CAAs found within the predicted range. The predicted values from the practical responses were found to be satisfactory, and it was confirmed that it was acquired within the design space of the optimized results.[19,20]

Statistical analysis

Design-Expert (Version 12), Stat-Ease Inc., Minneapolis, MN, USA, was utilized for method optimization and estimation of its CMPs and randomization of the runs.[14] Microsoft Excel 2007 (Microsoft, USA) was used for the remaining calculations.

Preparation of solvent

Phosphate buffer (pH 4): Disodium hydrogen phosphate (5.04 g) and KH_2PO_4 (3.01 g) were dissolved in water to a volume of 1000 mL. The pH was adjusted to 4.0 with OPA. The resulting solution was passed through (0.45 µm) filter paper, filtered by vacuum filtration, and sonicated for about 15 min.[15] While preparing the mobile phase, only the phosphate buffer was filtered using (0.45 µm) nylon membrane filters. Acetonitrile was not filtered and was used as provided by the supplier.

Procedure for the stock standard solution

Standard stock solution of the drug was made by accurately weighing 10 mg of the drug and mixed with 10 mL of acetonitrile to acquire a concentration of 1000 µg mL^{-1}. Serial dilutions of the stock solution were prepared by diluting with the mobile phase to obtain concentrations of 1 to 100 µg mL^{-1} and filtered through a 0.45 µm syringe filter before chromatography. Calibration curves were constructed as depicted in Figures 2a-c. Here, a mixture of acetonitrile and phosphate buffer (10 mM KH_2PO_4) (20:80, %v/v) was selected as a mobile phase with a flow rate of 1 mL min^{-1} and UV detection at 235 nm. The limit of quantification (LOQ) and limit of detection (LOD) were estimated based on the linearity plot (concentration vs. peak area).

Chromatographic conditions

A Merck-Hibar Purospher STAR Analytical column (C18, 250 mm × 4.6 mm × 5 µm) was used for chromatographic separation due to its advantage of having the highest carbon loading for better separation of the desired analyte, minimizing the variabilities of the mode of selection. A mobile phase of acetonitrile and phosphate buffer (10 mM KH_2PO_4) (20:80 %v/v) was used. The flow rate was 1 mL min^{-1} with an injection volume of 10 µL. The oven temperature of the chromatographic column was 30°C, and the sample temperature was ambient.

Optimization of the chromatographic method using the analytical QbD approach

Initially, trial and error practices were executed to obtain rigorous data for the selected chromatographic method's performance and its finding of vital independent variables with its considerable effect upon the dependent variables. The utmost significance of developing the RP-HPLC method mostly separates the drug from its excipients and the degradants. Statistical analysis was accomplished by implementing a suitable experimental design by response surface methodology (RSM) through BBD principles. The statistical design intensifies ANOVA principles for establishing the optimized experimental conditions of the method.[16,21,22] A simple, accurate, and AQbD-based RP-HPLC method was developed, which is also stability indicating. This was further subsequently validated as per the International Conference on Harmonization (ICH) recommended stability guidelines for quantification of abiraterone acetate in its various pharmaceutical formulations (tablets). A mixture of phosphate buffer (10 mM KH_2PO_4 and acetonitrile) (20:80, %v/v) was used as the mobile phase with a 1 mL min^{-1} flow rate.

Optimized chromatographic conditions

The optimized conditions are as follows: Optimized trail concentration: 10 µg mL^{-1}; flow rate: 1mL min^{-1}; mobile phase: Acetonitrile/phosphate buffer [10 mM KH_2PO_4, (20:80) %v/v]. The optimized chromatograms of the blank, standard, and mixture of excipients (10 µg mL^{-1}) of the developed analytical method are shown in Figure 2a-c, respectively.

Method validation

Method validation was performed to substantiate that the analytical procedure employed for a definite experiment is appropriate for use.[19] The outcomes of method validation parameters can be highly requisite to judge the reliability, quality, and steadiness of analytical results. Validation of a method for parameters linearity, accuracy, precision, and robustness was done according to recommendations of ICH guidelines (ICH Q2) (R1).[23-25]

Linearity

The linearity plot of the proposed method was constructed as per the stated ICH guidelines.[26-28] The linearity chart of abiraterone acetate was found to be within the concentration range of 2-100 µg mL^{-1}; further, the calibration plot was constructed using the peak area versus the concentration.

Accuracy

A series of solutions were spiked with known standard concentrations of abiraterone acetate of 50%, 100%, and 150% (5, 10, and 15 µg/mL) in triplicate, performed as per recommendations of ICH guidelines.[26-28]

Precision

The precision of the developed technique, expressed in % relative standard deviation (RSD), was calculated by performing repeatability and intermediate precision studies.[21] The developed analytical QbD-based method was validated by the precision studies (both intraday and interday).

LOD and LOQ

The LOD and LOQ of the current investigation were evaluated from the baseline noise of abiraterone acetate through comparisons of measured signals of samples with known analyte concentrations with that of the blank by (signal-to-noise) S/N ratios of 3:1 (LOD) & 10:1 (LOQ) as per guidance and protocol recommended by ICHQ2B.[29]

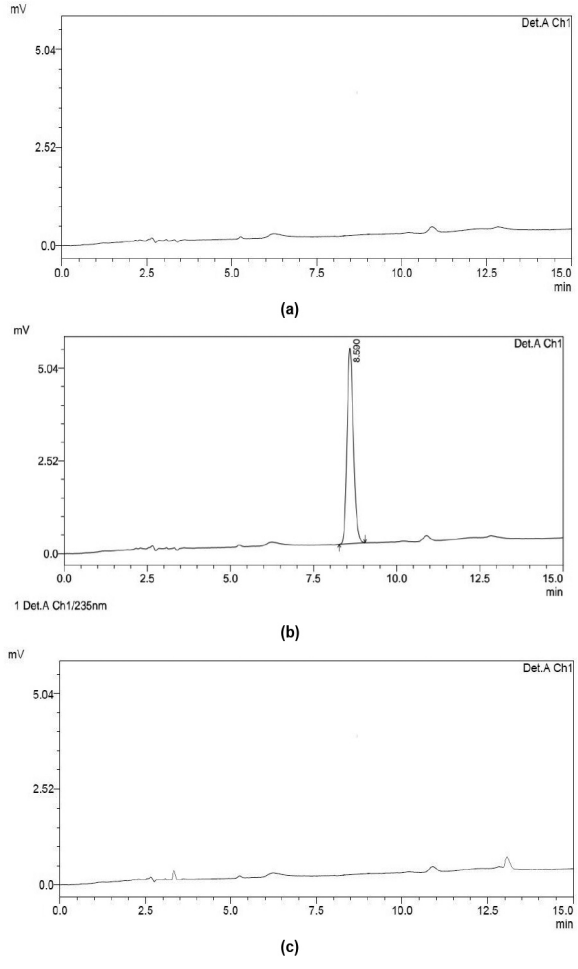

Figure 2. Optimized chromatograms of blank (a), standard 10 µg mL^{-1}, and the mixture of excipients (c)

Robustness

A robustness study was performed to recognize and evaluate the toughness of an analytical method.[30] To check the ability of the projected method, different factors were deliberately altered such as alternation in mobile phase composition, change in flow rate, etc.

Specificity

Specificity was carried out to evaluate the analyte noticeably in the occurrence of components that may be expected to be present during development. Specificity was established by representing no interference from the excipients and the degradation products.[28,29]

Forced degradation studies

Forced degradation generally includes exposure of drug substances to a range of stress conditions to demonstrate the stability profile and possible degradants of developed analytical methods.[26-28] Acid degradation was performed by adding 1 mL of 0.1 N HCl, then heating at 60˚C for 30 min, cooling to room temperature, and neutralizing. Alkali degradation was performed by adding 1 mL of 0.1 N NaOH, heating at 60˚C for 30 min, cooling to room temperature, and neutralizing. The degradative process through oxidation was performed by exposing the drug to 1 mL of 3% H_2O_2 and heating at 60˚C for 30 min. The degradation study was achieved by heating the drug content solutions at 105˚C on a thermostatically regulated water bath for half an hour. Photolytic degradation was done by subjecting the drug solutions to UV light in a UV chamber at 365 nm for 3 h. The degradation samples were accurately prepared through appropriate aliquots of the drug and in the solution form of their drug products, instructed by the stress testing protocol.[26,29] After a definite time, the treated solutions were adjusted with the mobile phase.

Stability of analytical solution

The solution stability study was effectively performed by observing the standard and sample solutions to determine the stability potential of the drug substance. This factor was analyzed by injecting the drug sample and standard solutions at distinctive intervals. This evaluation parameter is intended for the evaluation of the chemical stability of the drug and sample solution whether any significant changes occurred at varied time intervals.[29,31,32]

Assay of formulations

Twenty tablets of commercial brands of abiraterone acetate were chosen arbitrarily, and their average weight was determined.[31] The tablets were crushed to fine powder, 250 mg was weighed, and the powder was dissolved in 200 mL of acetonitrile (in a 250 mL volumetric flask). Then, it was shaken for 20 min and ultrasonicated for 20 min. After that, it was allowed to cool at room temperature, and the solution was diluted up to mark with diluents (1000 mcg mL^{-1}). The final obtained solution was

then diluted to 10 mcg mL^{-1} with acetonitrile/phosphate buffer (20:80 %v/v) and subsequently injected to an HPLC system for estimation in triplicate.

RESULTS AND DISCUSSION

Method development and optimization Using Box-Behnken experimental design

In the current investigation, trials were proposed and conducted using Box-Behnken experimental design.[21] The proposed BBD desires 17 experimental runs of examination to acquire data and model the response surface for a reliable analysis. A 3^2 level with 17 experimental runs was executed to identify the optimized design space for detection of the predicted response. The 3^2 BBD with RSM was executed.[16,21] The statistical design with optimization methodology and its data analysis through BBD were statistically evaluated using Design-Expert-Ver.12 software.[21,22] The three most influential factors (CMPs), the composition of the mobile phase (X1), flow rate (X2), and pH (X3), were selected; the peak area (Y1) and Rt (Y2) were used as observed responses. The design matrix of the statistical BBD and experimental runs are shown in Table 1.[21] The contour plots illustrate that the effect of both the responses stand in need about factors X1 (acetonitrile and phosphate buffer %) and X2 (flow rate), while the X3 (pH) is found to produce a nullified effect upon the obtained responses. The outline of 3^2 factors and ANOVA results, through its calculated mean and standard deviation values, are summarized in Table 2.[22] The statistically constructed model was suitably validated by interaction studies using the effect of various factors upon the found responses. The 2D counter plot analysis of peak area and Rt with the observed responses (R1) & (R2) are depicted in Figure 3a, b, respectively. Similarly, the 3D counter plot analysis of peak area (R1) response and Rt (R2) response is depicted in Figure 3c, d, respectively. The statistical model signifies that predicted values for both the responses (Rt and peak area) are a bit closer to the actual values representing superior accuracy and precision values for the obtained responses. The 2D and 3D counter plots of predicted versus actual values for peak area

Table 1. Box-Behnken design experimental runs by selecting 3^2 factors

Serial no	Factor 1: Mobile phase composition (%v/v)	Factor 2: Flow rate (mL/min)	Factor 3: pH	Response 1 peak area	Response 2 retention time
1	50	1.5	6	32909	8.59
2	50	1.25	5	109912	10.124
3	50	1.25	5	329581	11.457
4	50	1	4	699272	8.59
5	80	1.5	5	139884	12.321
6	80	1.25	6	559463	9.125
7	80	1	5	745612	12.581
8	50	1	6	411153	10.235
9	20	1.5	5	325114	7.235
10	50	1.25	5	451231	6.512
11	20	1.25	6	521231	5.236
12	50	1.25	5	425851	6.458
13	50	1.25	5	456875	7.126
14	50	1.5	4	612578	8.127
15	20	1	5	661257	9.245
16	80	1.25	4	457812	8.736
17	20	1.25	4	489254	7.984

Table 2. ANOVA by selecting 3^2 factors

Factors	Name	Units	Type	Minimum	Maximum	Coded low	Coded high	Mean	Standard deviation
A	Mobile phase composition	% v/v	Numeric	20.00	80.00	1-20.00	1-80.00	50.00	21.21
B	Flow rate	mL/ min	Numeric	1.0000	1.50	1-1.00	1-80.00	1.25	0.1768
C	pH	Moles/ltr	Numeric	4.00	6.00	1-4.00	1-80.00	5.00	0.7071

(R1) and Rt (R2) are depicted in Figure 4a, b, respectively. The perturbation plot displays the impact of all the influential factors (mobile phase and flow rate) at a particular point within the design space for the selected responses peak area and Rt. The representative plots of perturbation analysis for the observed responses peak area (R1) and Rt (R2) are depicted in Figure 4c, d, respectively. Here, for 2D and 3D surface numerical optimization, the Rt (R2) and peak area (R1) are depicted in Figure 5a-d, respectively. Figure 6 elucidates the parameters intended for numerical optimization for desirability and optimized data for factors indorsed by design. Finally, the optimized apparent chromatographic conditions can be well predicted from the arithmetical model, and it has strongly been recommended for the developed analytical method (Table 3).

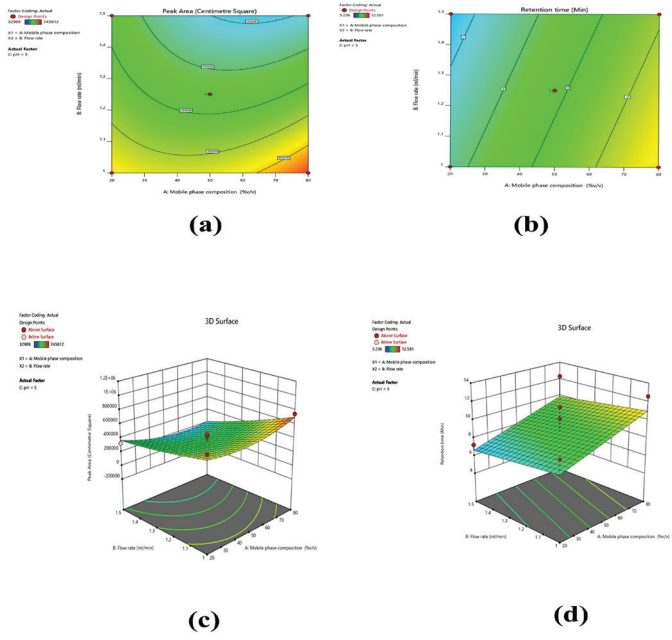

Figure 3. 2D surface contour plots of peak area (R1) response (a) and retention time (R2) response (b); 3D surface contour plots of peak area (R1) response (c) and retention time (R2) response (d)

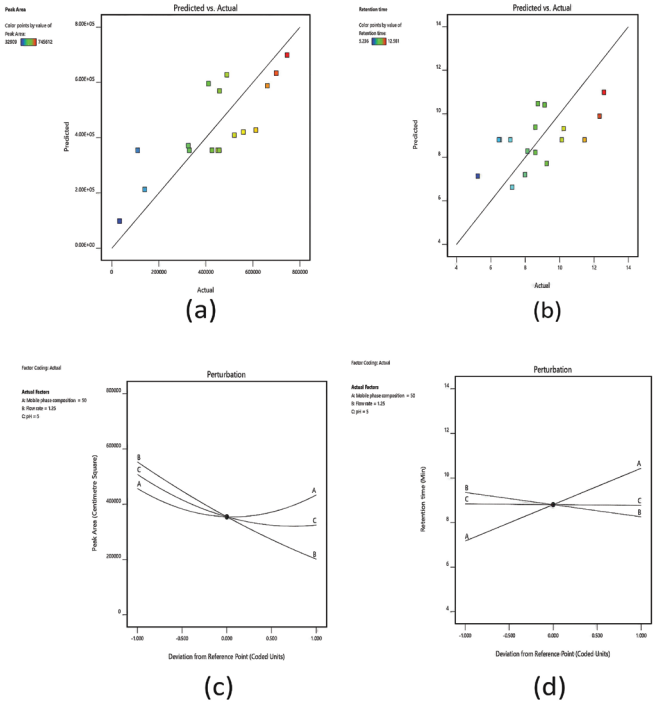

Figure 4. Predicted versus actual values for peak area (R1) (a) and predicted versus actual values for retention time (R2) (b). Representative plots of perturbation for (c) response peak area (R1) and (d) retention time (R2)

Results of validation studies

The optimized feasible chromatographic conditions were aggrandized and effectively implemented to validate the QbD-based method by a range of validation parameters, linearity, accuracy, precision, LOD, LOQ, robustness, etc., as per recommendations of ICH guidelines.

Linearity

Six concentrations of the abiraterone acetate working standard ranging from 2 to 100 ppm were prepared and analyzed for the linearity study. The calibration plot was constructed by plotting the chromatographic peak areas versus the known predicted concentrations in µg mL^{-1}, and values of observed concentrations were determined. The regression equation was found to be Y= 73399x - 71154, and the regression coefficient r^2 is 0.998. The detail of the calibration plot and its residual plot data analysis are depicted in Figure 7a, b, respectively.

Accuracy

The accuracy of the developed method was studied to make sure the agreement between the true and reference values in

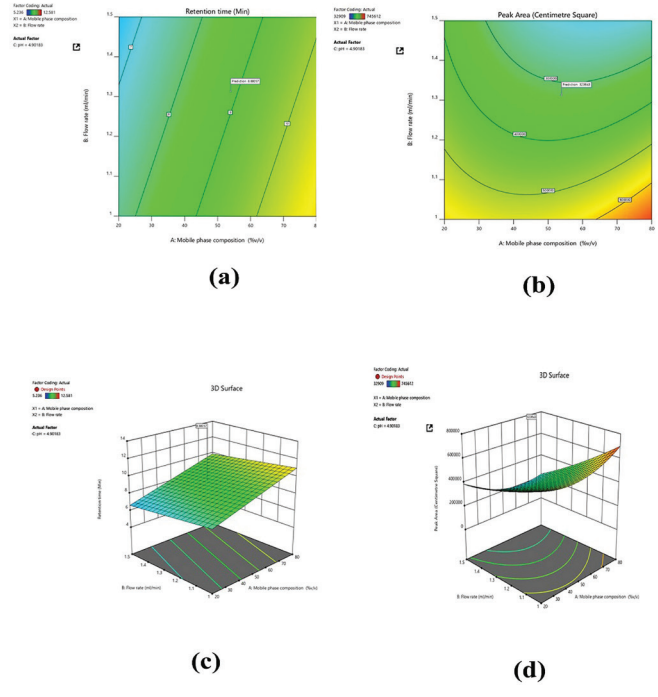

Figure 5. 2D surface numerical optimization for retention time (R2) (a) and peak area (R1) (b); 3D surface numerical optimization for retention time (R2) (c) and peak area (R1) (d)

three significant levels of 50%, 100%, and 150%. The percentage recoveries of three various concentrations were found to be within the range of 98% to 102%, and % RSD was obtained within the acceptance limit, that is, NMT 2.0%. The data of all the recovery studies are shown in Table 4.

Precision

Precision studies of the developed method were carried out by the system, method, and intermediate precision studies. Precision of the system suitability test for six replicate standard injections was calculated, within the acceptance criteria, that is, NMT 2%, and both intraday and interday precisions through three replicate injections of standards were calculated. Data of % RSD are reported in Table 5.

LOD and LOQ

The detection and quantification limits (LOD & LOQ) of the current investigation were actively quantified as per the recommendation of ICHQ2B guidelines for validation of analytical methodology. The detection limit was derived as a signal-to-noise (S/N) ratio of 3:1, whereas the quantification limit was indicated as a S/N ratio of 10:1, as an effect of the response by the detector. The estimated value for LOD was found to be 0.45 ppm, and the estimated value for LOQ was 1.35 ppm. The calculated values are shown in Table 3.[23]

Robustness

Robustness is the capacity to replicate the analytical method in diverse labs or under varied conditions without the manifestation of unanticipated differences in the obtained results. The estimated values of mobile phase composition and flow rates were taken, and changes in flow rate and mobile phase data are summarized in Table 6. The results show the calculated % RSD within the acceptance limit, that is, less than 2%.

Specificity

The specificity of the method has been studied to determine the interference from the degradation products as per ICH through a forced degradation study. The results denote no sign of peak formation at the Rt of abiraterone acetate and the case of degradation products since the peak purity passed. The data reveal that the purity angle was less than the purity threshold, presenting no specific interference (Table 7).

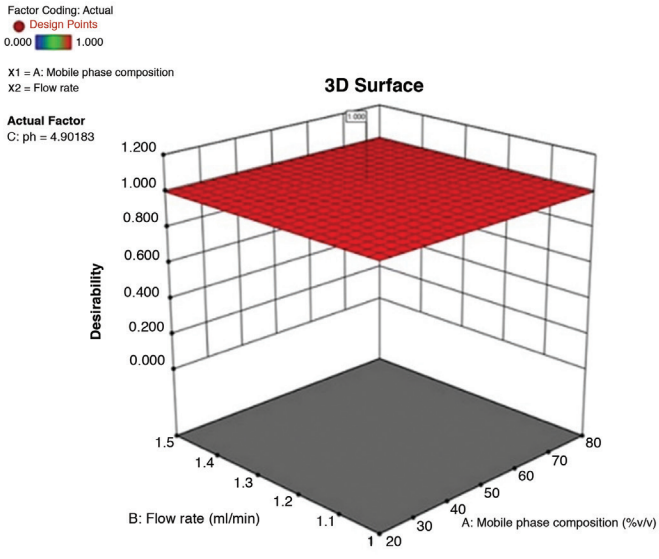

Figure 6. Numerical optimization for desirability data

Table 3. Optimum chromatographic conditions of abiraterone acetate

Optimum chromatographic conditions	
Run time	15. 0 minute
Retention time	8.59 minutes
Flow rate	1 mL/min
Linearity range	(2-100) µg/mL (r^2=0.998)
Accuracy	% recovery: Within (98-102%) (RSD <2%)
Precision	(RSD <2%)
LOD	0.45 µg/mL
LOQ	1.35 µg/mL

RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification

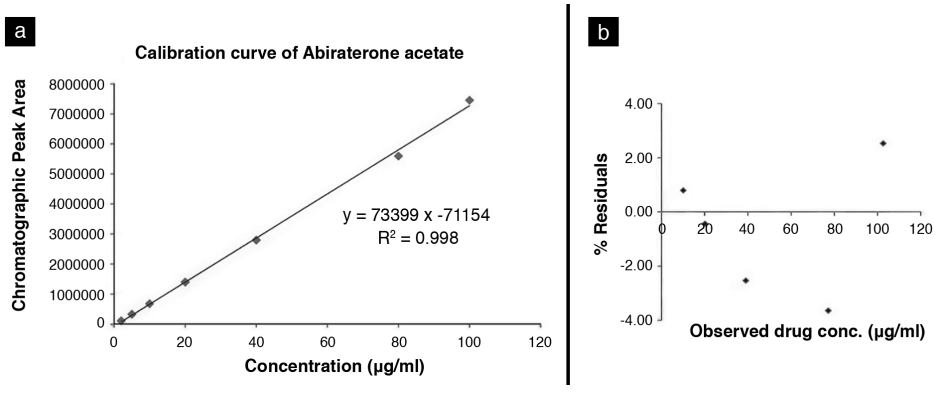

Figure 7. Schematic diagram of calibration plot (a) and residual plot (b) of abiraterone acetate

Table 4. Accuracy data of abiraterone acetate

Amount added (µg/mL)	Levels	Sl. no.	Chromatographic area	Mean area	SD	Amount recovered (µg/mL)	% recovery	% RSD
5	50%	1	337854	339294	1239.41	4.983	99.66 %	0.365
-	-	2	339816	-	-	-	-	-
-	-	3	340147	-	-	-	-	-
10	100%	1	697482	697500	341.86	9.971	99.71%	0.049
-	-	2	697168	-	-	-	-	-
-	-	3	697851	-	-	-	-	-
15	150%	1	1050226	1049216	1415.78	14.968	99.78%	0.134
-	-	2	1047598	-	-	-	-	-
-	-	3	1049825	-	-	-	-	-

RSD: Relative standard deviation, SD: Standard deviation

Table 5. Standard injection of abiraterone acetate peak response by system, intra, and interday precision tests

System precision				Intra-day			Inter-day			
Conc. (µg/mL)	Peak area	USP tailing	USP plate count	Conc. (µg/mL)	Peak area of at different time intervals (day 1)			Conc. (µg/mL)	Peak area of at different time intervals (day 2)		
					10 a.m	2 p.m	5 p.m		10 a.m	2 p.m	5 p.m
10	695445	1.01	7486	5	329851	326885	327519	5	326511	326578	326899
10	695872	1.02	7453	5	329557	326699	327157	5	326789	326576	326858
10	695234	1.01	7359	5	329259	326544	327556	5	326724	326544	326891
10	695982	1.03	7422	**Average**	329556	326709.3	327411	**Average**	326675	326566	326883
10	695971	1.02	7356	**SD**	296.00	170.73	220.46	**SD**	145.418	19.079	21.733
				% RSD	0.09	0.05	0.07	**% RSD**	0.045	0.006	0.007
				10	695272	698777	697651	10	696565	694872	697981
10	695332	1.03	7287	10	695773	698874	697236	10	696834	698671	697123
				10	695859	698349	697987	10	696785	698956	697855
Average	695639			**Average**	695635	698667	697625	**Average**	696728	697500	697653
SD	340.02			**SD**	317.01	279.35	376.19	**SD**	143.27	2280.08	463.30
% RSD	0.0489			**% RSD**	0.05	0.04	0.05	**% RSD**	0.02	0.33	0.07
				20	1373567	1398971	1385569	20	1398171	1373267	1385669
				20	1373776	1398885	1386981	20	1398985	1373976	1385781
				20	1373962	1398934	1385856	20	1398734	1373562	1385113
				Average	1373768	1398930	1386135	**Average**	1398630	1373602	1385521
				SD	197.61	43.14	746.30	**SD**	416.85	356.16	357.75
				% RSD	0.01	0.03	0.05	**% RSD**	0.03	0.03	0.03

RSD: Relative standard deviation, SD: Standard deviation

Table 6. Robustness data of abiraterone acetate

Robustness	Peak area	Average	SD	% RSD
Flow rate (1+0.2 mL/min) 10 (µg/mL)	689234	689558	384.448	0.05575
	689458	-	-	-
	689983	-	-	-
Flow rate (1-0.2 mL/min) 10 (µg/mL)	699764	699770	85.675	0.01224
	699859	-	-	-
	699688	-	-	-
Amount of (ACN +2% v/v) 10 (µg/mL)	699272	699641	323.013	0.04617
	699874	-	-	-
	699776	-	-	-
Amount of (ACN -2 %v/v) 10 (µg/mL)	685761	686455	748.890	0.1090
	686357	-	-	-
	687249	-	-	-
Detector wavelength 235 nm (+2 nm)	689071	689068	301.506	0.0437
	689369	-	-	-
	688766	-	-	-
Detector wavelength 235 nm (-2 nm)	683866	684357	687.714	0.10049
	684062	-	-	-
	685143	-	-	-

RSD: Relative standard deviation, SD: Standard deviation

Table 7. Forced degradation and solution stability data of abiraterone acetate

Stress conditions	Peak area	*Drug recovered (%)	*Drug decomposed (%)	Theoretical plates	Tailing factor	Purity angle	Purity threshold
Abiraterone acetate standard (control)	698252	100	-	7526	1.08	0.301	0.425
Acidic degradation 1 mL 0.1N HCl, 60˚C, 30 minutes	513215	73.5	26.5	6326	1.29	0.118	0.192
Alkaline degradation 0.1 mL 0.1N NaOH, 60˚C, 30 mins	612367	87.7	12.3	6823	1.24	0.356	0.526
Oxidative degradation 1 mL 3% H_2O_2, 60˚C, 30 minutes	686381	98.3	1.7	7067	1.21	0.319	0.423
Thermal degradation 105˚C, 30 minutes	692666	99.2	0.8	7236	1.12	0.238	0.469
Photolytic degradation 365 nm, 3 hours	695459	99.6	0.4	7468	1.17	0.278	0.568

Solution stability data

	Area counts		% Deviation from mean: ±3.0%	
Time (hrs)	Standard	Sample	Standard	Sample
Initial	695773	686747	0.0	0.0
7 hrs	694105	686875	0.23	0.01
16 hrs	697297	689303	0.21	0.37
25 hrs	698567	689473	0.40	0.39
32 hrs	699875	692615	0.58	0.85
40 hrs	699948	698543	0.60	1.71

*With respect to assay value of control

Forced degradation studies

Forced degradation studies provide knowledge on practical degradation pathways and degradation products of the active ingredients and help expound the configuration of the degradants as per ICH recommendations.[27] The following conditions were applied for degradation: Acid (1 mL of 0.1 N HCl, 60°C for 30 min), alkali (1 mL of 0.1 N NaOH, 60°C for 30 min), peroxide (1 mL of 3% v/v H_2O_2), thermal (105°C), and photolytic degradations at 231 nm, for determining the steady nature of drugs. Percent drug degradation values during acidic and alkaline degradation were observed to be 26.5% and 12.3%, respectively. In contrast, less than 2% degradation was reported in the case of oxidative, thermal, and photolytic degradations, which indicate the drug's completely resistant behaviors to the above stress conditions. The detailed descriptions of forced degradation activities are listed in Table 7. The representative chromatograms of the sample in various stress conditions by incorporating a mixture of excipients (a), acid (b), alkali (c), oxidative (d), photolytic (e), and thermal degradation (f) studies are depicted in Figure 8a-f, respectively.

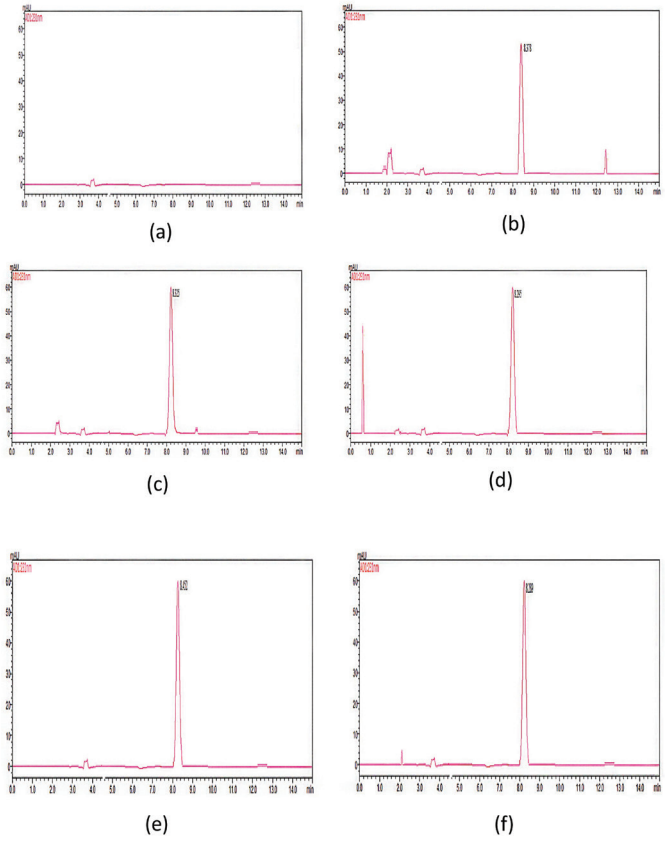

Figure 8. Schematic diagram indicating (a) the mixture of excipients, (b) sample acidic degradation, (c) alkali degradation, (d) peroxide degradation, (e) photolytic degradation, and (f) thermal degradation studies

Stability of analytical solution

The solution stability study was carried out by observance of sample and standard solutions at 25±2°C for 40 h. After analysis, it has been concluded that the drug standard and sample were stable for up to 40 h (Table 7). The anticipated method was effectively validated and met the necessities as per the recommended stability guidelines of ICH.

Assay of formulations

The chromatogram of the marketed sample is depicted in Figure 9. The calculated values of the percentage of the assay of various marketed formulations are exhibited in Table 8. The results show that all the values of the formulations are within the acceptance limit, that is, 98-102%.

CONCLUSION

Chemometrics-assisted method development affords regulatory flexibility, the formation of homogeneous or robust finished products of assured quality characteristics as per Food and Drug Administration (FDA) concerns, and ICH stability stressed conditions. In addition to this, the AQbD method minimizes the process of revalidation, lessens solvent consumption and development time, and enhances optimum robust analytics. By implementing the DoE, the Box-Behnken statistical design can evaluate the independent variables (CMPs) concurrently with adding common interactions among the critical factors to optimize the tentative conditions. It is explicit that the application of this BBD with RSM is an adaptable practice to reduce the

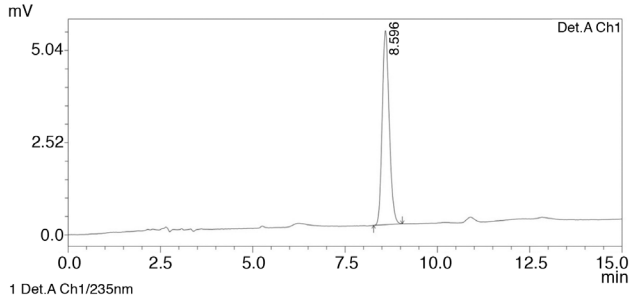

Figure 9. Chromatogram of the formulation (10 µg mL^{-1})

Table 8. Assay data of abiraterone acetate

Assay of marketed formulations			
Brand name	**Label claim (mg)**	**Drug obtained**	**% Recovery**
Brand I (XIBRA)	250	247.96	99.18
Brand II (ZYTIGA)	250	251.02	100.41
Brand III (ABIRAPRO)	250	249.47	99.79

total experimental runs to obtain sustainable, robust analytics, reducing the chance of revalidation, requisite for analytical development. The optimization of the RP-HPLC method for abiraterone acetate can produce the highest intense data and boost efficiency within a short period as per the ICH Q8 (R2) and FDA perspectives. The proposed method was found to be linear, with concentrations of 2-100 ppm having r^2: 0.998. The remarkable % recovery (within 98-102%) of the drug reflects that the excipients existing in the tablet formulation have no impediment in the quantitation of the drug. The optimized conditions by AQbD of the anticipated method established that the proposed study was cost-effective, extremely robust, and stability indicating. Therefore, the developed stability-indicating method was economical, accurate, and precise. It can be successfully implemented for the routine analysis of abiraterone acetate in its bulk and pharmaceutical formulations.

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ORIGINAL ARTICLE

In silico Repurposing of Drugs for pan-HDAC and pan-SIRT Inhibitors: Consensus Structure-based Virtual Screening and Pharmacophore Modeling Investigations

pan-HDAC ve pan-SIRT İnhibitörleri Olarak İlaçların *İn silico* Olarak Yeniden Konumlandırılması: Konsensüs Yapı Tabanlı Sanal Tarama ve Farmakofor Modelleme Araştırmaları

Suat SARI, Ahmet AVCI, Ebru KOÇAK ASLAN*

Hacettepe University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Ankara, Turkey

ABSTRACT

Objectives: Drug repurposing is a highly popular approach to find new indications for drugs, which greatly reduces time and costs for drug design and discovery. Non-selective inhibitors of histone deacetylase (HDAC) isoforms including sirtuins (SIRTs) are effective against conditions like cancer. In this study, we used molecular docking to screen Food and Drug Administration (FDA)-approved drugs to identify a number of drugs with a potential to be repurposed for pan-HDAC and pan-SIRT inhibitor activity.

Materials and Methods: The library of FDA-approved drugs was optimized using MacroModel. The crystal structures of HDAC1-4, 6-8, SIRT1-3, 5, 6 were prepared before the library was docked to each structure using Glide, FRED, and AutoDock Vina/PyRx. Consensus scores were derived from the docking scores obtained from each software. Pharmacophore modeling was performed using Phase.

Results: Based on the consensus scores, belinostat, bexarotene, and cianidanol emerged as top virtual pan-HDAC inhibitors whereas alosetron, cinacalcet, and indacaterol emerged as virtual pan-SIRT inhibitors. Pharmacophore hypotheses for these virtual inhibitors were also suggested through pharmacophore modeling in agreement with the molecular docking models.

Conclusion: The consensus approach enabled selection of the best performing drug molecules according to different software, and good scores against isoforms (virtual pan-HDAC and pan-SIRT inhibitors). The study not only proposes potential drugs to be repurposed for HDAC and SIRT-related diseases but also provides insights for designing potent de novo derivatives.

Key words: Drug repurposing, HDAC, sirtuin, consensus scoring, virtual screening, pharmacophore modeling

ÖZ

Amaç: İlaç yeniden konumlandırma, ilaçlar için yeni endikasyonlar bulmak için oldukça popüler bir yaklaşımdır ve ilaç tasarımı ve keşfi için zaman ve maliyetleri büyük ölçüde azaltır. Sirtuinler (SIRT) dahil olmak üzere histon deasetilaz (HDAC) izoformlarının seçici olmayan inhibitörleri, kanser gibi durumlara karşı etkilidir. Bu çalışmada, pan-HDAC ve pan-SIRT inhibitör aktivitesi için yeniden kullanım potansiyeline sahip bir dizi ilacı belirlemek üzere Gıda ve İlaç Dairesi (FDA) onaylı ilaçları taramak için moleküler docking kullanılmıştır.

Gereç ve Yöntemler: FDA onaylı ilaçlar kütüphanesi MacroModel ile optimize edilmiştir. HDAC1-4, 6-8, SIRT1-3, 5, 6 yapıları hazırlanarak kütüphane her bir protein yapısına Glide, FRED ve AutoDock Vina/PyRx ile kenetlenmiştir. Konsensüs skorları her yazılımdan elde edilen kenetleme skorlarından türetilmiştir. Farmakofor modelleme Phase ile gerçekleştirilmiştir.

*Correspondence: ebrukocak@hacettepe.edu.tr, Phone: +90 312 305 18 72, ORCID-ID: orcid.org/0000-0003-0191-0746

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Bulgular: Konsensüs skorlarına göre belinostat, beksaroten ve siyanidanol en iyi sanal pan-HDAC inhibitörleri, alosetron, sinakalset ve indakaterol ise en iyi sanal pan-SIRT inhibitörleri olarak öne çıkmıştır. Bu sanal inhibitörler için farmakofor hipotezleri, moleküler yerleştirme docking uyumlu olarak farmakofor modellemesi yoluyla da belirlenmiştir.

Sonuç: Konsensüs yaklaşımı, farklı yazılımlara göre en iyi performans gösteren ilaç moleküllerinin seçilmesini ve izoformlara (sanal pan-HDAC ve pan-SIRT inhibitörleri) karşı iyi puanlar alınmasını sağlamıştır. Çalışma, yalnızca HDAC ve SRT ile ilgili hastalıklar için yeniden kullanılabilecek potansiyel ilaçlar önermekle kalmamış, aynı zamanda güçlü de novo türevleri tasarlamak için de yol gösterici olmuştur.

Anahtar kelimeler: İlaç yeniden konumlandırma, HDAC, sirtüin, konsensüs skorlama, sanal tarama, farmakofor modelleme

INTRODUCTION

Histone deacetylases (HDACs) are a class of enzymes that cleaves acetyl groups from ε-*N*-acetylated lysine residues of histone proteins, which wrap DNA molecules. HDAC activity causes DNA molecules to be wrapped more tightly leading to various epigenetic regulations.[1] In the last couple of decades, much effort was put into inhibiting HDAC activity as a strategy to design compounds against a wide range of conditions such as neurodegenerative diseases, inflammatory diseases, cancer, diabetes, cardiovascular diseases, and HIV infections.[1-4]

To date, 18 HDAC isoforms have been identified, which are classified into four classes (class I-IV) according to their sequence homology and tissue distribution (Table 1). Classes I, II, and IV are zinc-dependent classical HDACs, i.e., they require a Zn^{2+} ion in their catalytic site for activity, whereas class III, also known as sirtuins (SIRTs), are a structurally distinct class, which depends on NAD^{+} for catalytic activity.[1] Currently, the crystallographic structures of the human HDAC1-4, 6-8, SIRT1-3, 5, and 6 are available, leading to an increase in high-throughput virtual screening (hVS) for design and identification of novel specific HDAC inhibitors[5,6] as well as drug repurposing efforts.[7] Drug repurposing has become a new approach in drug design as a means to reduce costs and attrition rates in clinical studies and speed up drug development process.[8]

Although isoform-selective HDAC inhibition is required in many HDAC-related treatment strategies[9], pan-HDAC (e.g., trichostatin A, vorinostat, and valproic acid) and pan-SIRT inhibitors (e.g., nicotinamide) attract attention for their clinical effectiveness in diseases like cancer (Figure 1).[10-12] Because most HDAC isoforms are associated with tumorigenesis and tumor progression, such polypharmacological approaches may prove more effective than isoform-specific inhibitors.[13]

Molecular docking is an *in silico* method to predict preferred binding orientation and affinity of a ligand with respect to a receptor. It has been used as a robust tool to identify hit matter as part of virtual screening for a long time. To improve virtual screening performance, consensus scoring is applied by combining scoring functions of multiple software programs, which usually is considered more accurate than single-score methods.[6]

In this study, we identified a number of drug molecules with potential to show pan-HDAC and pan-SIRT inhibitor activities using consensus structure-based hVS of the library of the Food and Drug Administration (FDA)-approved drugs in order to suggest drugs to be repurposed. The study also suggests pharmacophore hypotheses for virtual pan-HDAC and pan-SIRT drugs, through pharmacophore modeling, which are expected to improve our understanding of the design of potent *de novo* derivatives.

Table 1. HDAC classes and isoforms

Class I	HDAC1-3, 8
Class IIa	HDAC4, 5, 7, 9
Class IIb	HDAC6, 10
Class III	SIRT1-7
Class IV	HDAC11

HDAC: Histone deacetylase, SIRT: Sirtuin

MATERIALS AND METHODS

Ligand preparation

The collection of FDA-approved drugs was obtained as 3D-coordinates sdf file from DrugBank (http://www.drugbank.ca) (accession: July 3, 2019).[14] After removing the experimental, investigational, and nutraceutical compounds, the remaining 1502 ligands were prepared by removing salts and counter ions using LigPreP (2019-2, Schrödinger, LLC, New York, NY), and optimized geometrically using MacroModel (2019-2, Schrödinger, LLC, New York, NY) and conjugate gradients method according to OPLS_2005 forcefield parameters.[15] The optimized library was directly used for Glide (2019-2, Schrödinger, LLC, New York, NY)[16-18] and converted to sdf format for FRED (v3.3.1.2, Open Eye Scientific Software; Santa Fe, NM).[19] For AutoDock Vina (v1.1.2, The Scripps Research Institute, San Diego, CA) the library was converted to pdbqt format by Open Babel (v2.4).[20]

Molecular docking protocol

The crystal structures of HDAC1-4, 6-8, and SIRT1-3, 5, 6 were downloaded from the RCSB Protein Data Bank (www.rcsb.org).[21] The protein structures were prepared for docking by removing unwanted chains and residues, adding missing atoms, assigning hydrogen atoms, bond orders, partial charges

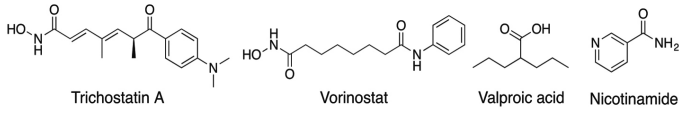

Figure 1. Known pan-HDAC and pan-SIRT inhibitors

(for Glide only), and setting ionization and tautomeric states, as well as the H bonds of the protein residues using the Protein Preparation Wizard of Maestro (Epik, Impact, Prime: 2019-2, Schrödinger, LLC, New York, NY).[22] The prepared protein structures were assigned Gasteiger charges and converted to their pdbqt format using AutoDockTools (v1.5.7, The Scripps Research Institute, San Diego, CA) for AutoDock Vina. Grid maps of the active site of each protein were prepared using the receptor grid generation panel of Maestro (2019-2, Schrödinger, LLC, New York, NY) for Glide, Make Receptor (v3.3.1.2, Open Eye Scientific Software; Santa Fe, NM) for FRED. This procedure is automatically performed by AutoDock Vina. The central coordinates of the catalytic site of each structure were taken for a grid box of $27x10^3$ Å^3 size (see Electronic Supplementary Material for details). Molecular docking on Glide was performed at standard precision of 50 runs per ligand with the following settings: A scaling factor of 0.80 with 0.15 charge cut-off (absolute value) being applied for the ligands, Epik (2019-2, Schrödinger, LLC, New York, NY) state penalties were added to docking scores, nitrogen inversions and ring conformations were sampled, and post docking minimization was enabled. For FRED, docking was performed at high resolution mode with 50 runs per ligand. For AutoDock Vina, the default parameters were used and the virtual screening tool PyRx (v0.8, The Scripps Research Institute, San Diego, CA) was used to run the docking simulations on AutoDock Vina.[23] Each ligand was assigned a docking score of the identified best pose from each software upon visual evaluation. The consensus score of a ligand for a given structure was determined by calculating the average of the three scores from the three software. A pan-HDAC and a pan-SIRT score were determined for each ligand by calculating the average of the consensus scores for all HDAC and SIRT structures, respectively.

Pharmacophore modeling

3D pharmacophore models for pan-HDAC and pan-SIRT inhibitor drugs were created with Phase (2019-2, Schrödinger, LLC, New York, NY)[24] using the top-scoring three ligands according to each pan-HDAC (belinostat, bexarotene, and cianidanol) and pan-SIRT scores (alosetron, cinacalcet, and indacaterol) according to the following settings: Finding best alignment and common features method was applied, 50 conformers were generated for each ligand, three to five features were required in each hypothesis, all the query compounds were required to match each hypothesis, and the hypotheses (6 for pan-HDAC and 20 for pan-SIRT) were ranked according to PhaseHypoScore and the best hypothesis was selected for each group. The FDA-approved drug library was screened against each selected hypothesis using the Phase Ligand Screening panel with the default settings.

Statistical analysis was not performed in this study.

RESULTS AND DISCUSSION

Consensus structure-based VS

A total of 1502 FDA-approved drug molecules was *in silico* screened against HDAC1-4, HDAC6-8, SIRT1-3, 5, and 6 using three different docking software. For each drug molecule, a consensus score was assigned regarding each HDAC and SIRT isoform, which was the mean of the scores from the three software. A pan-HDAC score was then determined for each molecule by calculating the mean of the consensus scores for HDAC1-4 and HDAC6-8 (Table 2). The pan-SIRT scores were similarly calculated using the consensus scores for SIRT1-3, 5, and 6 of each drug molecule (Table 3). This approach assured that the molecules with good scores from all of the software and good consensus scores for all the isoforms ranked higher.

Virtual pan-HDAC drugs

Among the classical HDAC isoforms, classes I and II HDACs share a similar catalytic site topology. A zinc cofactor, chelating with two aspartate and one histidine residues, and a substrate, is at the bottom of a narrow lipophilic gorge that forms the catalytic site (Figure 2). Therefore, compounds with a linear lipophilic moiety with H-bond donor and acceptor groups at the tip such as trichostatin A can effectively occupy this site by chelating with the zinc and interacting with the zinc ligands.

Table 2. Top 10 pan-HDAC scoring drugs and their consensus scores for each isoform

Compound	HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	HDAC7	HDAC8	pan-HDAC
Belinostat	-7.3	-9.4	-5.9	-8.5	-9.6	-7.7	-9.1	-8.2
Bexarotene	-6.8	-8.1	-6.2	-9.1	-9.6	-8.0	-8.0	-8.0
Cianidanol	-6.6	-7.0	-6.8	-10.5	-8.1	-8.3	-8.3	-7.9
Phenacemide	-7.0	-8.9	-6.2	-8.2	-8.6	-7.7	-7.1	-7.7
Frovatriptan	-7.2	-7.0	-6.3	-8.5	-8.6	-8.0	-8.1	-7.7
Levodopa	-7.0	-7.8	-5.9	-9.0	-8.0	-7.4	-8.0	-7.6
Chlorphenesin	-6.6	-8.3	-5.5	-8.5	-8.6	-7.3	-8.1	-7.6
Masoprocol	-7.5	-7.0	-6.1	-9.7	-7.7	-7.3	-7.7	-7.6
Benzylparaben	-6.8	-7.7	-6.2	-9.0	-7.3	-8.2	-7.5	-7.5
Ensulizole	-6.8	-7.2	-7.5	-8.8	-7.6	-6.3	-8.4	-7.5

HDAC: Histone deacetylase

Table 3. Top 10 pan-SIRT scoring drugs and their consensus scores for each isoform

Compound	SIRT1	SIRT2	SIRT3	SIRT5	SIRT6	pan-SIRT
Alosetron	-9.1	-11.4	-7.7	-7.8	-8.5	-8.9
Cinacalcet	-10.1	-11.5	-8.3	-6.7	-7.6	-8.8
Indacaterol	-10.4	-10.1	-8.5	-7.5	-7.6	-8.8
Ziprasidone	-8.8	-12.2	-6.6	-7.8	-8.7	-8.8
Phenprocoumon	-9.6	-10.9	-8.5	-6.7	-8.0	-8.8
Ethinylestradiol	-9.5	-10.4	-7.8	-7.5	-8.6	-8.7
Diflunisal	-9.2	-10.1	-7.5	-8.2	-8.6	-8.7
Bexarotene	-9.7	-11.3	-7.0	-7.2	-8.4	-8.7
Estrone	-9.8	-10.6	-7.4	-7.6	-8.1	-8.7
Tolcapone	-9.3	-10.0	-8.2	-8.0	-7.9	-8.7

HDAC: Histone deacetylase, SIRT: Sirtuin

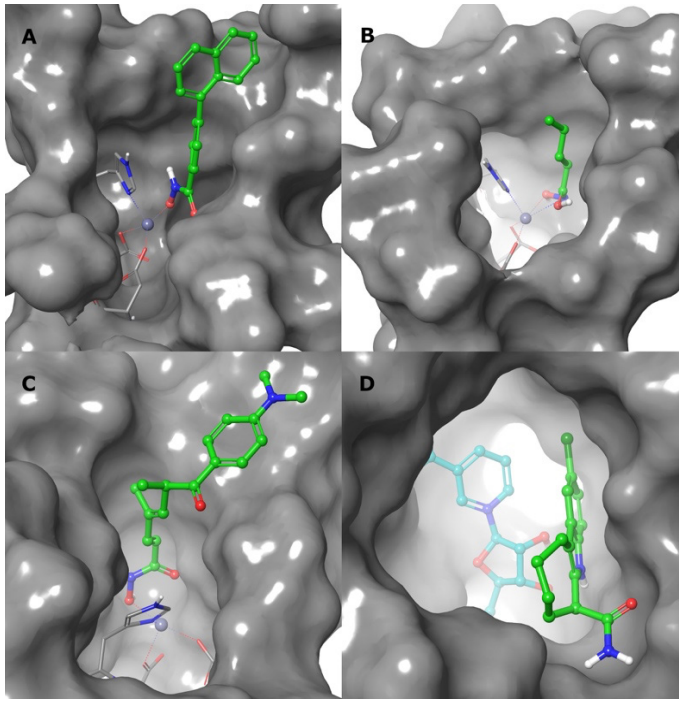

Figure 2. Catalytic site of HDAC8 (A), HDAC7 (B), HDAC6 (C), and SIRT1 (D). Inhibitors are represented as green stick-ball, zinc as gray sphere, zinc ligands as gray stick, NAD+ as teal stick-ball, and protein active sites as solid sphere

HDAC: Histone deacetylase, SIRT: Sirtuin

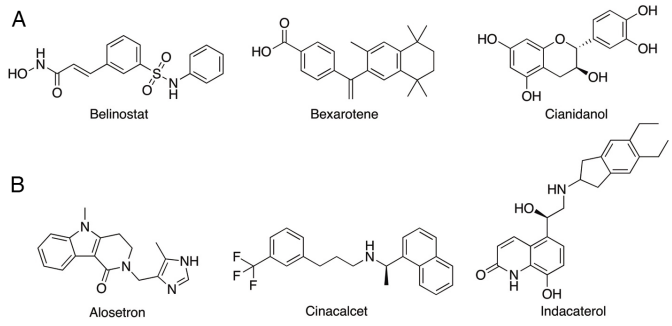

Figure 3. Top-scoring drugs according to pan-HDAC (A) and pan-SIRT (B) scores

The active site of classical HDACs leaves little room for conformational flexibility, thus FRED, Glide, and AutoDock Vina usually produce similar poses for the molecules. Belinostat, bexarotene, and cianidanol were recorded as the top pan-HDAC scoring molecules in our study (Figure 3).

As expected for a pan-HDAC inhibitor anticancer drug, belinostat had the highest pan-HDAC score. Belinostat ranked 6th according to consensus HDAC1 scores and 1st according to consensus HDAC2, 6, and 8 scores (see Electronic Supplementary Material for details). Belinostat's IC_{50} values of 7.20, 7.31, 7.82, and 7.16 nM have been reported against these targets.[25-28] A hydroxamic acid at the end of a cinnamyl moiety is typical of potent classical HDAC inhibitors. The hydroxamic acid moiety of belinostat was in close contact with zinc and its ligands (Figure 4A-C). The cinnamyl benzene stacked with the histidine ligand (e.g., His709 of HDAC7) of zinc, as well as other aromatic sidechains of the nearby residues, e.g., Phe679 and Phe738 of HDAC7, in HDAC active sites (see Electronic Supplementary Material for details), corroborates findings of previous studies.[29]

Bexarotene, the second highest pan-HDAC scoring drug, is also an antineoplastic drug approved for the treatment of cutaneous T-cell lymphoma.[30] The retinoid X receptor activator has not been tested against any HDACs so far, but it obtained the 7th best consensus HDAC2 and the 2nd best consensus HDAC6 score in our study. The benzoic acid moiety of bexarotene was mainly responsible for binding to HDAC active sites, in which the carboxylate group interacted with the zinc, its ligands, and nearby residues like Tyr308 of HDAC2. The benzene stacked with the aromatic side chains of the nearby residues such as

Phe155 and Phe210 of HDAC2 (Figure 4D-F). Bexarotene was also the 8th best pan-SIRT scoring molecule, making it a likely inhibitor of the HDACs of all classes.

Cianidanol [(+)-catechin], a natural flavonol, has been withdrawn as a drug due to hematological toxicity. However, it is still marketed as an over-the-counter product for weight loss.[31] It has been clinically evaluated for several cancer types but has no anti-HDAC activity record. In this study, it was the 2nd, 9th, and 4th best compound according to consensus HDAC4, 7, and 8 scores. Cianidanol is structurally different from the classical HDAC inhibitors regarding the zinc-interacting group, which is an *ortho* phenolic hydroxyl instead of a hydroxamic or carboxylic acid. Whereas these hydroxyl groups engaged with the zinc and its ligands, the benzene bearing the hydroxyls stacked with the aromatic side chains of the nearby residues such as Phe155 and Phe210 of HDAC2, as was recorded for belinostat and bexarotene (Figure 4G-I).

Virtual pan-SIRT drugs

The NAD^+- dependent SIRT active site is composed of NAD^+-binding Rossmann-fold subdomain and a distal zinc-binding pocket, and is more deeply buried and roomier than HDACs (Figure 2).[32] The active pockets of SIRTs show large variations among the isoforms resulting in varied binding modes among the drug molecules and among the software for the same molecule. Since apo (NAD^+ free) and holo (NAD^+ including) states of SIRTs are both inhibited by inhibitors of diverse topology,[32,33] we preferred the apo form of SIRTs in the hVS process to avoid missing out bulky drug molecules. Alosetron, cinacalcet, and indacaterol emerged as the best three drug molecules from the hVS study according to pan-SIRT scores (Figure 3).

Alosetron is a "setron", i.e., 5-HT_3 receptor antagonist, used for the treatment of irritable bowel syndrome.[34] Although the effects of alosetron on SIRTs are yet to be studied, it obtained the best pan-SIRT score and the 7th best consensus SIRT2 score. Alosetron appeared to have two important moieties for interacting with the relevant key SIRT residues: (1) The imidazole ring which donates and accepts H bond (e.g., Arg71 of SIRT5) and makes π-π interactions (e.g., His158 of SIRT5), and (2) the indol-1-one that widely engages in π-π interactions (e.g., Tyr255 of SIRT5) (Figure 5A-C).

Cinacalcet is an allosteric activator of the calcium-sensing receptor and is used for the treatment of hyperthyroidism and hypercalcemia.[35,36] The compound has no SIRT-related record but it obtained the 7th and 4th best consensus scores for SIRT1 and 2, respectively. The binding of Cinacalcet to SIRTs was supported by the π-π stacking via its two aromatic rings (e.g., His 58 and Tyr255 of SIRT5) as well as strong electrostatic contacts via the CF_3 group with the active site residues (Figure 5D-F). For some SIRTs, the secondary amine formed H bonds (see Electronic Supplementary Material for details).

Indacaterol is a β adrenoceptor agonist used for the treatment of chronic obstructive pulmonary disease.[37] The molecule, which ranked the third according to the pan-SIRT scores, has not been tested against SIRTs before. Indacaterol obtained the 2nd and 6th best consensus score for SIRT1 and 3, respectively. The

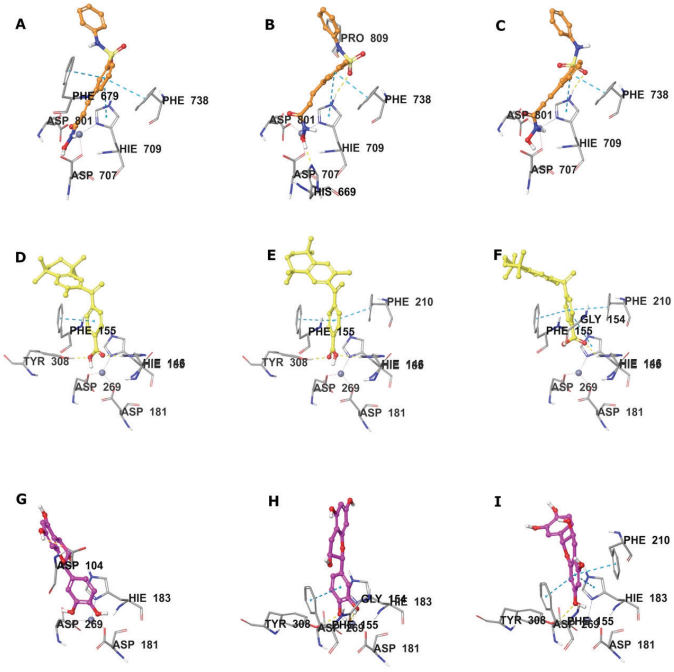

Figure 4. Binding interactions of belinostat with HDAC7 (A-C), bexarotene with HDAC2 (D-F), and cianidanol with HDAC2 (G-I), predicted by FRED (A, D, G), Glide (B, E, H), and AutoDock Vina (C, F, I), respectively. Drug molecules are represented as color stick-ball, protein residues as gray stick, and interactions as color dash

HDAC: Histone deacetylase, SIRT: Sirtuin

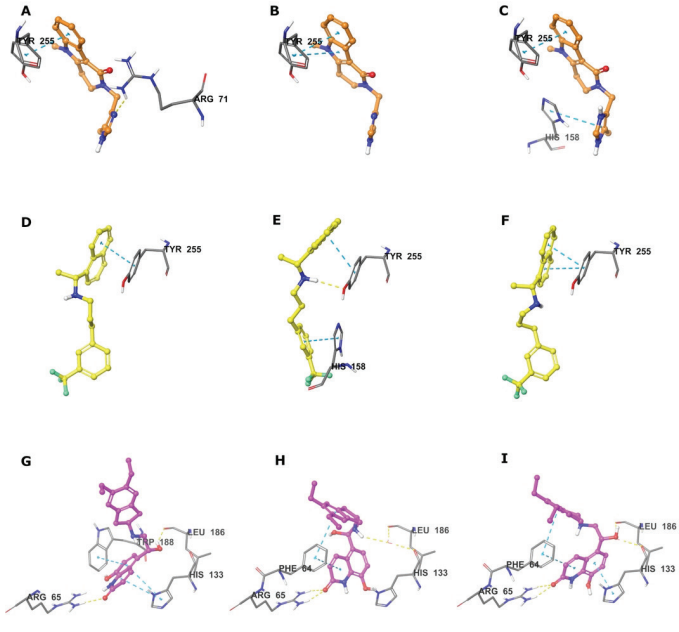

Figure 5. Binding interactions of alosetron with SIRT5 (A-C), cinacalcet with SIRT5 (D-F), and indacaterol with SIRT6 (G-I) predicted by FRED (A, D, G), Glide (B, E, H), and AutoDock Vina (C, F, I), respectively. Drug molecules are represented as color stick-ball, protein residues as gray stick, and interactions as color dash

SIRT: Sirtuin

two condensed ring systems of indacaterol greatly contributed to its theoretical binding affinity to SIRTs (Figure 5G-I). While these rings engaged in π-stacking, with residues like Phe64, His133, and Trp188 of SIRT6, the hydroxyl and amino groups made H bonds in most cases to further stabilize the binding (e.g., His133 and Leu186 of SIRT6).

Pharmacophore models for virtual pan-HDAC and pan-SIRT molecules

Pharmacophore models are hypothetical spatial orientations of the common pharmacophores (functional groups considered important for biological activity) for a set of ligands (or a single ligand) that share a biological property (activity, toxicity, etc.), which are widely exploited in rational drug design applications.[38,39] We created a set of possible pharmacophore hypotheses for virtual pan-HDAC and pan-SIRT inhibitor drug molecules using top three scoring molecules of each group and selected the best hypothesis for each group according to PhaseHypoScore and BEDROC scores (scores showing how much a hypothesis fits to the query ligands in general) (Table 4).

The selected hypothesis for the virtual pan-HDAC inhibitor drugs (hypothesis 1) consists of a closely located H-bond acceptor (A) and donor (D) groups, and a distal ring (R). The alignment of belinostat, bexarotene, and cianidanol with hypothesis 1 suggests that A and D represent the hydroxamic, carboxylic, and phenolic groups interacting with the zinc and its ligands, while R aligns with the aromatic ring of these compounds that stack with the aromatic side chains of active site residues (Figure 6A-C). The best hypothesis for alosetron, cinacalcet, and indacaterol (hypothesis 2) comprises two adjacent rings for the condensed ring of these drugs, a vertical third ring regarding the two for a separate aromatic group, and a hydrophobic group (H) close to the third ring representing a hydrophobic substitution to the third ring, namely methyl, trifluoromethyl, and ethyl (Figure 6D-F). Thus, hypothesis 2 reflects the hydrophobic nature of SIRT catalytic site. Cianidanol and cinacalcet showed the best alignment to hypothesis 1 and 2, respectively (see Fitness score in Table 4).

To test the accordance of these hypotheses with the consensus structure-based hVS campaign, we screened the drug molecules against both hypotheses and compared the results with respect to pan-HDAC and pan-SIRT scores from the molecular docking by calculating the 10% enrichment factor (Table 4). This metric shows how many of the drug molecules that are among the top 150 pan-HDAC scoring molecules (i.e., top 10% drugs) are listed in the top 150 compounds according to PhaseScreenScore (a score that shows how much a screened molecule fits to the pharmacophore hypothesis) for hypothesis 1. The same applies for pan-HDAC scores and hypothesis 2. The 10% enrichment factor was 20% for both hypotheses, showing that both methods predicted somewhat similar drug molecules as top virtual pan-HDAC and pan-SIRT inhibitors.

CONCLUSION

A total of 1502 FDA-approved drugs were screened against a set of classical HDACs and SIRTs with available crystal structures using FRED, Glide, and AutoDock Vina. The drug molecules were ranked according to their average consensus HDAC and SIRT scores to identify the drug molecules that can potentially inhibit as many HDAC or SIRT isoforms, i.e., virtual pan-HDAC and pan-SIRT inhibitors. The consensus approach in this method works in two ways: Consensus among the software used and among the isoforms. Belinostat, bexarotene, and cianidanol were the best scoring virtual pan-HDAC inhibitors. Although bexarotene and cianidanol have not been tested against HDACs, they have potential against HDACs and could be repurposed for HDAC-related indications. Specifically, bexarotene may show potent *in vitro* activity against HDAC2 and 6; and cianidanol against HDAC4, 7, and 8. Among these molecules, belinostat is already a confirmed pan-HDAC inhibitor used as an anticancer agent, which shows the effectiveness of the hVS methodology. On the other hand, other known pan-HDAC inhibitors such

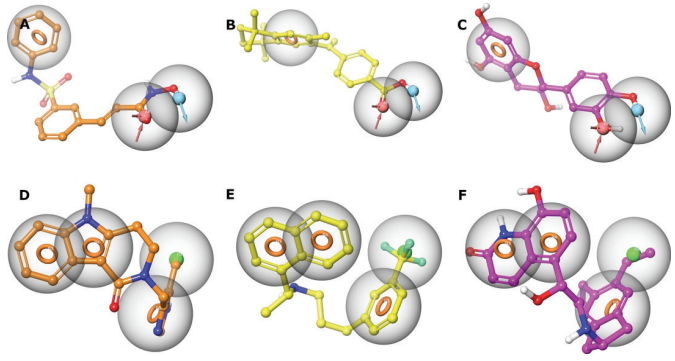

Figure 6. Hypothesis 1 (A-C) and hypothesis 2 (D-F) aligned with belinostat (A), bexarotene (B), cianidanol (C), alosetron (D), cinacalcet (E), and indacaterol (F). Drug molecules are represented as color stick-ball, pharmacophore features as color ring (ring) and sphere (pink for H-bond acceptor, blue for donor, and green for hydrophobic)

Table 4. Pharmacophore hypotheses and their specifications

Hypothesis	Features	PhaseHypoScore	BEDROC score	Fitness score	10% enrichment (%)
1	A, D, R	0.95	0.75	Belinostat: 2.0 Bexarotene: 2.1 Cianidanol: 3.0	20
2	H, R, R, R	1.20	0.97	Alosetron: 1.9 Cinacalcet: 3.0 Indacaterol: 1.8	20

A: H-bond acceptor, D: H-bond donor, H: Hydrophobic, R: Ring

as vorinostat were not listed among the top-scoring drugs suggesting that the hVS method has weaknesses as well. Alosetron, cinacalcet, and indacaterol obtained the best pan-SIRT scores. Although these compounds have no SIRT record, they may be useful in pan-SIRT-related conditions. Alosetron could be a promising inhibitor of SIRT2, cinacalcet of SIRT1 and 2, and indacaterol of SIRT1 and 3. Bexarotene was also listed among the top-ten pan-SIRT scoring drugs, which could be a potent inhibitor of all HDAC classes and repurposed for a unique indication in this regard. Taken together, these drug molecules may find new indications related to pan-HDAC and pan-SIRT inhibition.

Two pharmacophore hypotheses were formulated, one for top pan-HDAC scoring drug molecules (hypothesis 1) and the other for pan-SIRT scoring drug molecules (hypothesis 2). The top three pan-HDAC and pan-SIRT scoring drugs aligned very well with their respective hypothesis. The pharmacophore features in these hypotheses were in compliance with the binding interactions of the drug molecules predicted by docking. Rankings of the drug molecules according to the pharmacophore hypotheses and molecular docking screens bore similarities, i.e., some of the top-scoring molecules in pharmacophore screens were also among the top pan-HDAC and pan-SIRT scoring drugs. Therefore, the ligand- and structure-based hVS methods yielded compatible results.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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ORIGINAL ARTICLE

In silico Modeling and Toxicity Profiling of a Set of Quinoline Derivatives as c-MET Inhibitors in the treatment of Human Tumors

İnsan Tümörlerinin Tedavisinde c-MET İnhibitörü Olarak Kullanılan Bir Dizi Kinolin Türevinin *İn silico* Modellemesi ve Toksisite Profili

Gülçin TUĞCU[1]*, Filiz Esra ÖNEN BAYRAM[2], Hande SİPAHİ[1]

[1]Yeditepe University Faculty of Pharmacy, Department of Toxicology, İstanbul, Turkey
[2]Yeditepe University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, İstanbul, Turkey

ABSTRACT

Objectives: 4-(2-fluorophenoxy) quinoline derivatives constitute one of the chemical classes of hepatocyte growth factor receptor (c-MET) inhibitors, a promising treatment against various human tumors. There are three aims of the present study: (1) To develop a robust and validated quantitative structure-activity relationship model to predict the c-Met kinase inhibition; (2) to examine the toxicity profiles of these compounds; (3) to design new quinoline derivatives and apply the developed model on these compounds to observe its pertinence.

Materials and Methods: A multiple linear regression method was used to develop the model with calculated descriptors. State-of-the-art internal and external validation parameters were calculated. The *in silico* toxicity profiles including structural alerts and the lowest observed adverse effect level (LOAEL) values were evaluated using online tools. New derivatives were designed and tested on the developed model.

Results: A series of 4-(2-fluorophenoxy) quinoline derivatives was linearly modeled and vigorously validated to predict the molecules' c-MET kinase inhibition potential. Statistical metrics of the developed model showed that it was robust and able to make successful predictions for this chemical class. The mass, electronegativity, partial charges, and the structure of the molecules had an effect on the activity. Moreover, the toxicity profiles of the studied compounds were found to be adequate.

Conclusion: Five of the synthesized compounds were observed to be auspicious for the toxicity/activity ratio. The developed model is useful in the virtual screening and in the design of new anti-tumor compounds.

Key words: Toxicity, LOAEL, anti-tumor, c-Met, QSAR

ÖZ

Amaç: 4-(2-florofenoksi) kinolin türevleri, insan tümörlerinin tedavisinde kullanılan hepatosit büyüme faktörü reseptörü (c-MET) inhibitörleri kimyasal sınıflarından biridir. Bu çalışmanın üç amacı vardır: (1) c-MET kinaz inhibisyonunu tahmin etmek için sağlam ve doğrulanmış bir kantitatif yapı-aktivite ilişkisi modeli geliştirmek; (2) üzerinde çalışılan bileşiklerin toksisite profillerini incelemek; (3) yeni kinolin türevleri tasarlamak ve geliştirilen modeli bu bileşikler üzerine uygulayarak uygunluğunu incelemektir.

Gereç ve Yöntemler: Bu çalışmada hesaplanmış tanımlayıcılarla modeli geliştirmek için çoklu doğrusal regresyon yöntemi kullanılmıştır. Güncel iç ve dış doğrulama parametreleri hesaplanmıştır. Böylelikle *in silico* toksisite profilleri yapısal uyarılar ve gözlemlenen en düşük advers etki düzeyi değerleri çevrimiçi araçlar kullanılarak değerlendirilmiştir. Yeni bileşikler tasarlanmış ve geliştirilen model üzerinde test edilmiştir.

Bulgular: Bir dizi 4-(2-florofenoksi) kinolin türevi doğrusal olarak modellenmiş ve moleküllerin c-MET kinaz inhibisyon potansiyelini tahmin etmek için doğrulanmıştır. Geliştirilen modelin istatistiksel metrikleri, sağlam olduğunu ve bu kimyasal sınıf için başarılı tahminler yapabildiğini göstermiştir. Kütle, elektronegatiflik, kısmi yükler ve moleküllerin yapısının aktivite üzerinde etkili olduğu görülmüştür. Ek olarak, incelenen bileşiklerin toksisite profilinin kabul edilebilir olduğu görülmüştür.

Sonuç: Sentezlenen bileşiklerden beşinin risk-yarar profili incelendiğinde, etkisinin risklerinden daha fazla olduğu görülmüştür. Geliştirilen model, sanal tarama ve yeni anti-tümör bileşiklerinin tasarlanmasında yararlı olduğu belirlenmiştir.

Anahtar kelimeler: Toksisite, LOAEL, anti-tümör, c-MET, QSAR

***Correspondence:** gulcin.tugcu@yeditepe.edu.tr, **Phone:** +90 216 578 00 00, **ORCID-ID:** orcid.org/0000-0002-9750-6563

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INTRODUCTION

Cancer is a worldwide health problem.[1] Hepatocyte growth factor receptor (c-MET) kinase inhibition has been a novel treatment for various human cancers.[2] In addition to being effective in treating diseases, the approved drugs are expected to be safe at an acceptable level.[3] In other words, the risk-benefit profile of these drugs was considered to be greater than their risks.[4] Among these compounds, crizotinib and cabozantinib are two c-MET inhibitors that meet these criteria and are used in the treatment of various cancers.[5] There are other molecules that have been added to the list of approved drugs.[6] However, studies on promising c-MET tyrosine kinase inhibitors such as JNJ-38877605 were suspended in phase 1 due to the high risk of toxicity.[7] Therefore, before starting clinical trials, it is greatly important to explore the safety profile of a drug candidate in the preclinical phase as early as possible so that studies for safer drugs continue expeditiously.[8] Quantitative structure-activity-relationship (QSAR) modeling can be utilized to reduce costs, save time, and understand mechanisms of active substances even before a chemical is synthesized.

The safety evaluation of drugs is determined as the ratio of the safe amount of the drug exposed to the amount of drug that is effective. All preclinical data on the efficacy and safety endpoints need to be used as early as possible to understand the preliminary therapeutic index (TI). This index is calculated by dividing the highest amount of the drug that does not cause any toxicity to the amount that yields the aimed effect. The derivation of TI ratios from translational studies helps in proper decision-making for a drug in the next stage of drug development.[3]

This study developed a QSAR model to estimate the c-MET kinase inhibition of a set of compounds. The compounds' toxicity profiles are then explored *in silico*, and several compounds were determined to be both effective and safe. The model, which revealed satisfying internal and external validation statistics, was then applied to a set of newly designed compounds.

MATERIALS AND METHODS

Data set

A data set of 32 4-(2-fluorophenoxy) quinoline derivatives as c-MET kinase inhibitors was obtained from the literature.[9] The negative logarithm of the activity value (p c-MET) was used as the dependent variable in the model equation. Table 1 lists the structures and biological activities (IC_{50}) of the studied compounds.

Molecular descriptors and descriptor selection

Compounds were drawn in Spartan v.18 (Wavefunction Inc., Irvine, CA) and corresponding geometries were optimized with the semi-empirical PM7 using MOPAC 6.[10] Chemopy v.3.2,[11] PaDEL v.2.21,[12] and alvaDesc 1.0.20 (www.alvascience.com/alvadesc) descriptors were calculated using geometry-optimized molecule files.

Descriptor selection was performed via genetic algorithm (GA) using QSARINS v.2.2.4[13,14] following the removal of the constant and near-constant value descriptors. A search with GA within the software follows the tournament selection method.[15]

Model development and validation

The data set was split into training and test sets for an external validation procedure by systematically selecting compounds for the test set from the data set sorted according to activity. In this way, the activities were distributed homogenously and the training set was set as large as possible. Additionally, training and test sets are congruent so that the test set resides in the applicability domain. The training set consisted of 80% of the whole data set.

Selected significant descriptors were used as independent variables in MLR models to predict the biological activity. The delta K limit was set at 0.05 to eliminate models with collinear descriptors.[16] The maximum number of descriptors in the model has been limited to at least five compounds per descriptor (Topliss Ratio)[17] to avoid overfitting in the model. Linear models were developed using the ordinary least square method, which is carried out in QSARINS v.2.2.4[13,14] with the selected descriptors as the independent variables.

Internal and external validation procedures were performed vigorously using widely known parameters in the literature. The coefficient of determination (R^2), leave-one-out cross-validation (Q^2_{LOO}), standard error (SE), root mean squared error (RMSE), and Fischer statistics (F) as model fitting and internal validation criteria were listed. The reliability of the developed model was tested by the randomization procedure (Y-scrambling). Activity values in the training set were rearranged and new correlation coefficients were calculated for the randomized models. The average correlation coefficient of the new models was expected to be significantly low corresponding to the model correlation coefficient, indicating the absence of a chance correlation. External validation parameters, representing the predictive ability of the model, were listed as R^2, RMSE, Q^2_{F1} (0.70), Q^2_{F2} (0.70), Q^2_{F3} (0.70), CCC (0.85), and $r_m^{\,2}$ (0.65) of the test set[18] with the recommended lower limits given in parentheses. Furthermore, conditions described by Golbraikh and Tropsha[19] were applied to the test set predictions:

I. $(R^2 - R_o^2)/R^2 < 0.1$ and $0.85 \le k \le 1.15$

or

$(R^2 - R_o'^2)/R^2 < 0.1$ and $0.85 \le k' \le 1.15$

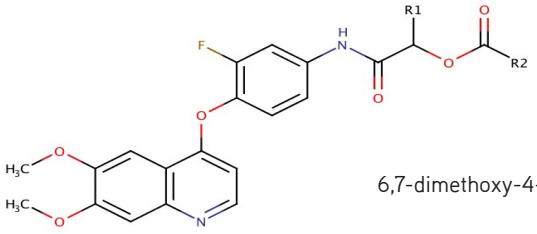
II. $|R_o^2 - R_o'^2| < 0.3$,

where R^2 corresponds to the predicted vs. observed, R'^2 refers to the observed vs. predicted, k and k' are slopes, and R_o^2 and $R_o'^2$ are coefficients of determination passing through the origin.

Applicability domain

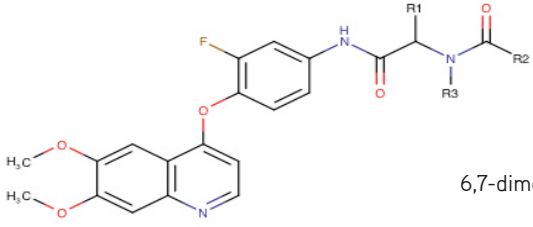
The applicability domain of the model was defined via the Williams plot. Leverage (hat) values lie on the x-axis, while standardized residuals are on the y-axis in the graph. Response outliers were diagnosed by the limit of ±2.5 standardized

Table 1. Molecular structures and activities of studied compounds



6,7-dimethoxy-4-(2-fluorophenoxy)quinoline derivatives

Compound	R1	R2	IC_{50} (nm)
10a	CH_3-	Cyclopentyl	52.86
10b	CH_3-	2-thiophenyl	37.46
10c	CH_3-	Naphtyl-	48.36
10d	CH_3-	Phenyl-	22.74
10e	propyl	Phenyl-	19.45
10f	cyclohexyl	Phenyl-	30.38
10g	phenyl	Phenyl-	54.42
10h	^{t}But	Phenyl-	8.35
10i	^{t}But	4-methylphenyl-	12.35
10j	^{t}But	4-methoxyphenyl-	21.48
10k	^{t}But	3,4,5-trimethoxyphenyl-	35.64
10l	^{t}But	^{t}But-	18.45
10m	^{t}But	4-fluorophenyl-	2.49
10n	^{t}But	3-fluorophenyl-	4.72
10o	^{t}But	2-fluorophenyl-	3.13
10p	^{t}But	4-chlorophenyl-	7.43
10q	^{t}But	3-chlorophenyl-	7.86
10r	^{t}But	2-chlorophenyl-	8.12
10s	^{t}But	4-bromophenyl-	18.44
10t	^{t}But	4-CF3-phenyl-	36.62
10u	^{t}But	2,3-dichlorophenyl-	18.66
10v	^{t}But	2-thiophenyl	32.74
10w	^{t}But	2-furanyl-	42.68
10x	^{t}But	Cyclopentyl-	66.54
10y	^{t}But	Naphtyl-	46.87



6,7-dimethoxy-4-(2-fluorophenoxy)quinoline derivatives

Compound	R1	R2	R3	IC_{50} (nm)
11a	^{t}But	4-fluorophenyl-	Butyl-	10.46
11b	^{t}But	4-fluorophenyl-	^{t}But-	18.46
11c	^{t}But	4-fluorophenyl-	Cyclohexyl-	20.54
11d	^{t}But	4-fluorophenyl-	Phenyl-	30.92
11e	^{t}But	4-fluorophenyl-	3,4-dimethoxyphenyl-	24.45
11f	^{t}But	4-fluorophenyl-	4-fluorophenyl-	36.46
11g	^{t}But	4-fluorophenyl-	H	9.12

residuals, while the structural outlier threshold was set at the critical hat (h*). The critical hat value was calculated as 3p'/n, where p is the number of descriptors plus one and n is the number of molecules in the training set.

Exploring toxicity profile of c-MET inhibitors

The toxicity profile of c-MET inhibitors was predicted using online tools and software. As a first step, structural alerts were sought for pan-assay interference compounds (PAINS)[20] and Brenk et al.[21] using SwissADME.[22] These alerts filter unwanted substructural features and screen compounds for lead-likeness. One of the parameters used in the safety evaluation is the lowest observed adverse effect level (LOAEL), which is defined as the lowest dose in a study at which adverse effects were observed, although it can lead to a significant overestimation of the TI.[3] LOAEL values (obtained from oral rat chronic toxicity tests) were predicted via pkCSM.[23]

The TI can be used as the ratio of the highest amount of the drug that does not cause any toxicity to the amount that yields/creates the desired effect in a translational research environment. This study used in vitro efficacy and *in silico* safety endpoint to compare safety margin interpretations. The greater the difference between the effective dose of a drug candidate molecule and its toxic dose, the safer that drug is. Consequently, a ratio was calculated as LOAEL to IC_{50} to evaluate the toxicity over the activity. This study defined a threshold level of 1 for this ratio.

Design of new c-MET inhibitors

New compounds were designed to explore potential anti-tumor agents. Inspired from papers of Parikh and Ghate[24] and Liu et al.[25] useful molecular fragments were selected. For instance, pyrazol and oxolane functional groups were introduced to the designed molecules. Additionally, the single methoxy group on the quinoline backbone was proved on some compounds. Designed compounds were tested using the developed model, and predicted activities and leverages were plotted on a graph.

RESULTS AND DISCUSSION

The training and test set selection was performed using systematic division. Activity values were sorted in decreasing order. The most and the least active compounds were assigned into the training set to provide a broader applicability domain. Finally, 80% of the data set was allocated into the training set. A total of 5182 descriptors were calculated using Chemopy (633), Padel (1444), and alvaDesc (3105) programs for all compounds. Selected descriptors were used as independent variables in the model developed on the training set. A GA-based search with parameters of 500 iterations, a population of 50 models, and a mutation rate of 20 was employed to find significant descriptors for the model. The four-descriptor MLR model (Eqn. 1) below was found to be predictive with an acceptable goodness-of-fit value. SEs of the coefficients were given in the parentheses ($p<0.05$).

p **c-MET** = 30.428 (±8.794)+0.087 (±0.018) **PEOEVSA2** + 0.034 (±0.016) **AATSC4m** -5.751 (±1.094) **SpMin8_Bh(e)** -25.005 (±8.584) **VR2_RG** (1)

n_{tr} = 26, R^2 = 0.812 R^2_{adj} = 0.776 $RMSE_{tr}$ = 0.163 MAE_{tr} = 0.138

SE = 0.182 F = 22.635 Q^2_{loo} = 0.725 R^2_{Yscr} (average) = 0.159

n_{test} = 6, R^2_{test} = 0.782 $RMSE_{test}$ = 0.130 MAE_{ext} = 0.102,

Q^2_{F1} = 0.800, Q^2_{F2} = 0.756, Q^2_{F3} = 0.881, CCC = 0.877

r^2_m (average) = 0.692, $|R_o^2 - R_o'^2| = 0.018$

$R_o'^2$ = 0.758, k' = 1.019, $(R^2 - R_o'^2)/R^2$ = 0.030

R_o^2 = 0.776, k = 0.974, $(R^2 - R_o^2)/R^2$ = 0.007

The reliability of the model was investigated using a randomization test, which was run for 2000 iterations for the developed model. The average R^2 was 0.159. Additionally, the distinct distribution of R^2 and Q^2 values belong to randomized models, and the developed model (Figure SI) revealed that the generated model is robust and the descriptors are not selected by chance.

The four-descriptor linear model has fulfilled the internal and external validation criteria listed in the materials and methods section. Figure 1 depicts the predicted versus observed activities, which are scattered around the y = x line. Table SI1 gives the predicted values by the model, leverages, and standardized residuals for the studied compounds.

The result indicated that partial charges, surface area, mass, electronegativity, and the structure of the molecules contributed to the activity (Table 2). Table 2 lists the standardized coefficients of the descriptors in the model, representing their relative importance. Partial charges, surface area contributions, and the Sanderson electronegativity were seen to be the most influential properties of the compounds. Table SI2 gives the calculated descriptor values, while Table

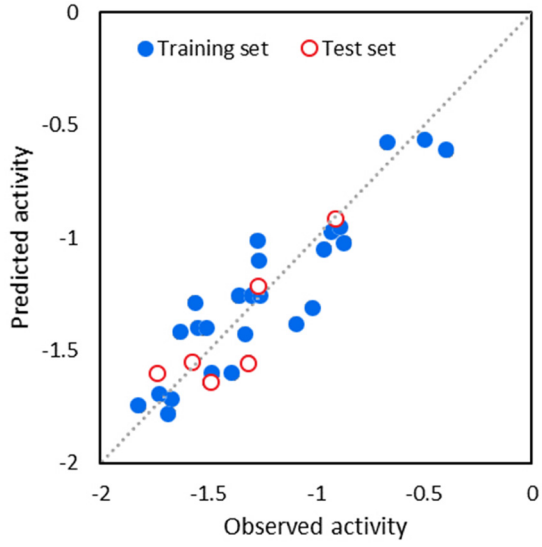


Figure 1. Predicted versus experimental activity values of training and test set compounds

3 lists the correlation matrix of the descriptors of the model, showing that the descriptors were not intercorrelated.

The Williams plot was depicted to show both the structurally distant compounds to the others (leverages) and the standardized residuals that represent the model's prediction accuracy. Horizontal reference lines are at ±2.5 σ and the vertical reference line is at the critical hat value (h*=0.577). The model did not have any outliers in terms of leverages and standardized residuals. Hence, all compounds are located within the AD of the model. Supplementary information Table SI1 lists the leverages and standardized residuals for the studied compounds.

The compounds were inspected *in silico* according to their structural alerts and LOAELs to explore the toxicity profile of the set of c-MET inhibitors as potential anti-tumor agents. No alert was fired for PAINS and Brenk screens in the SwissADME software. LOAEL values that were predicted via pkCSM were used to evaluate the safety of drugs. LOAEL predictions varied between 0.237 and 75.683 mg/kg/day. 10m, 11a, 11b, 11c, and 11g with the highest toxic dose/effective dose ratio were seen to be safe at the effective dose as expected from a drug (Table SI3) (Figure 2).

As described in the materials and methods section in detail, 20 quinoline derivatives were designed. The developed model was deployed on this set of new compounds (Table SI4) to explore possible inhibitors. Among 20 compounds, 16 of them were located in the AD and showed promising results as drug candidates (Figure 3). Compounds D06 and D07 were structurally distant from the modeled compounds. D02 and D13 had extreme prediction values, resulting in falling outside the AD of the model.

CONCLUSION

A QSAR model was developed with four descriptors to predict the c-MET inhibitory activity. The model revealed that the mass, electronegativity, partial charges, and the structure of the molecules have an influence on the c-MET inhibition. The model was validated internally and externally to prove its robustness and predictivity. Compounds were predicted for their availability as drug candidates and toxicity profile. It can be concluded that the compounds are opportune to be drug candidates and five of them ensued promising as both active and relatively safe. Additionally, the developed model was applied to 20 newly designed compounds. The model showed promising results on the new compounds. The model can be used in the early design process of c-MET inhibitors as anti-tumor agents.

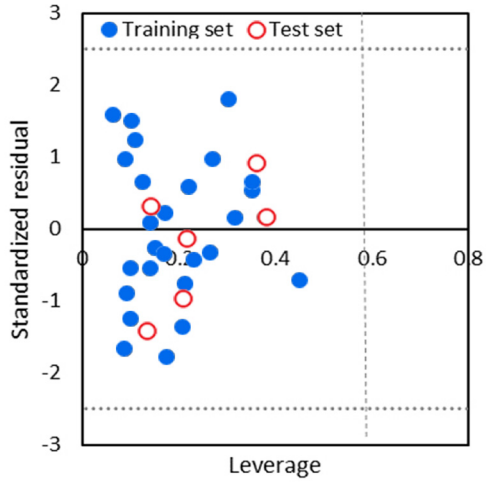


Figure 2. Williams plot for the applicability domain. The critical hat limit is at h*: 0.577

Table 2. Descriptors used in the model along with their standardized coefficients

Descriptor	Software	Meaning	Standardized coefficient
PEOEVSA2	ChemoPy	MOE-type descriptor using partial charges and surface area contributions	0.492
AATSC4m	PaDEL	Autocorrelation descriptor Average-centered Broto-Moreau autocorrelation - lag 4 (weighted by mass)	0.297
SpMin8_Bh(e)	alvaDesc	Burden eigenvalues Smallest eigenvalue n.8 of the Burden matrix weighted by the Sanderson electronegativity	-0.610
VR2_RG	alvaDesc	3D matrix-based descriptor Normalized Randic-like eigenvector-based index from the reciprocal squared geometrical matrix	-0.350

ChemoPy: Chemoinformatics in Python, Padel: PaDEL-Descriptor, alvaDesc: Alvascience Descriptor

Table 3. Correlation matrix of descriptors used in the model

	PEOEVSA2	AATSC4m	SpMin8_Bh(e)	VR2_RG
PEOEVSA2	1	-	-	-
AATSC4m	0.14	1	-	-
SpMin8_Bh(e)	-0.17	0.52	1	-
VR2_RG	0.02	-0.61	-0.25	1

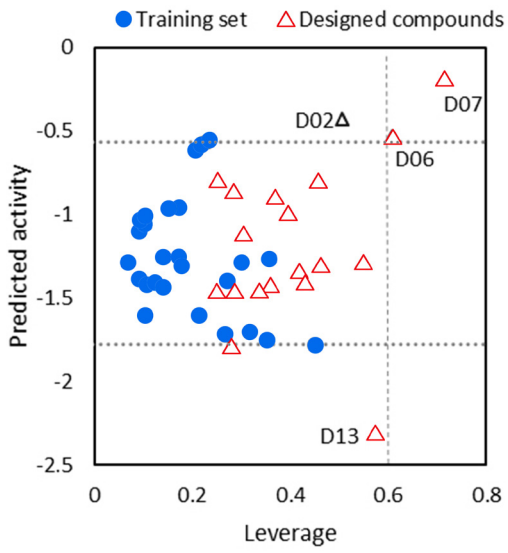


Figure 3. Performance of the designed compounds with the model

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ORIGINAL ARTICLE

The Antibacterial, Insecticidal and Nematocidal Activities and Toxicity Studies of *Tanacetum falconeri* Hook. f.

Tanacetum falconeri Hook. f.'nin Antibakteriyel, İnsektisidal ve Nematosidal Aktiviteleri ve Toksisite Çalışmaları

Muhammad ISMAIL[1]*, Atia KOWSAR[1], Salma JAVED[2], M. Iqbal CHOUDHARY[3], Sher Wali KHAN[4], Qamar ABBAS[4], Yanran TANG[5], Wei WANG[5]

[1]Karakoram International University, Department of Chemistry, Gilgit, Pakistan
[2]University of Karachi, National Nematological Research Centre, Karachi, Pakistan
[3]University of Karachi, International Center for Chemical and Biological Sciences, Karachi, Pakistan
[4]Karakoram International University, Department of Biological Sciences, Gilgit, Pakistan
[5]Hunan University of Chinese Medicine Faculty of Pharmacy, TCM and Ethnomedicine Innovation & Development International Laboratory, Hunan, China

ABSTRACT

Objectives: Secondary bioactive metabolites from plants may be realistic alternatives to conventional synthetic chemicals. The aim of the present research was to test the *Tanacetum falconeri* Hook. f. in different bioassays to evaluate its potential as nematocidal, insecticidal, antibacterial, cytotoxic, and phytotoxic agent. The plant *T. falconeri* was further studied for its chemical constituents.

Materials and Methods: The methanolic extract from *T. falconeri* was fractionated into various solvent fractions. All solvent fractions were further subjected to different bioassays. Antibacterial activity and cytotoxicity were determined by broth micro-dilution method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, respectively. Nematocidal activity was evaluated by using second-stage juvenile of *Meloidogyne incognita*. Insecticidal activity and phytotoxicity were measured with the help of *Rhyzopertha dominica* and *Tribolium castaneum* and fronds of *Lemna minor* L., respectively. Compound-1 was isolated by using liquid chromatography and its structure was determined by spectroscopic data as *cis*-dehydromatricaria ester.

Results: The excellent nematocidal activity (reduction in 100% motility) of the compound-1 against a root-knot nematode was obtained at 1% concentration after 72 h incubation time, whereas the reductions in motilities were 95% and 75% at concentrations 0.5% (w/v) and 0.125% (w/v), respectively. Similarly, the mortalities were 90% and 82% at 1% and 0.5% concentrations, respectively, after 24 h of the treatment. Compound-1 also exhibited the excellent insecticidal activity against *Sitophilus oryzae* at 1% concentration (providing 100% mortality) with effective dose 50 (EC_{50}) of 0.08 mg/L. The EC_{50} of phosphine, which was used as standard, was 0.07 mg/L. Two fractions of *T. falconeri* showed a moderate cytotoxic activity against 3T3 cells lines at the concentration of 30 mg/mL with inhibitory concentration 50 values of 22.4 and 25.8 mg/L corresponding to TfP and TfM, respectively.

Conclusion: This study demonstrates that *cis*-dehydromatricaria ester-1 provides nematocidal activity (100% mortality) against the root-knot nematodes. Its insecticidal activity against *S. oryzae* may enable its use as marketable insecticide.

Key words: Pesticides, *Tanacetum falconeri*, secondary metabolites, root-knot nematodes, toxicity

ÖZ

Amaç: Bitkilerden elde edilen ikincil biyoaktif metabolitler, geleneksel sentetik kimyasallara gerçekçi alternatifler olabilir. Bu araştırmanın amacı farklı biyoyöntemlerle *Tanacetum falconeri* Hook. f.'nin nematosidal, insektisidal, antibakteriyel, sitotoksik ve fitotoksik ajan olarak potansiyelini test etmektir. *T. falconeri* bitkisi, kimyasal bileşenleri için daha ileri incelemelere de tabi tutulmuştur.

*Correspondence: dr.ismail@kiu.edu.pk, Phone: +925811960045, ORCID-ID: orcid.org/0000-0002-7512-5380
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Gereç ve Yöntemler: *T. falconeri*'den elde edilen metanolik ekstre çeşitli çözücü fraksiyonlarına bölünmüştür. Tüm çözücü fraksiyonları ayrıca farklı biyoyöntemlere tabi tutulmuştur. Antibakteriyel aktivite ve sitotoksisite, sırasıyla broth mikro seyreltme yöntemi ve 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür yöntemi ile belirlenmiştir. Nematosit aktivitesi, *Meloidogyne incognita*'nın ikinci evre yavrusu kullanılarak değerlendirilmiştir. İnsektisidal aktivitesi ve fitotoksisitesi, sırasıyla *Rhyzopertha dominica* ve *Tribolium castaneum* ve *Lemna minor* L.'nin yaprakları yardımıyla ölçülmüştür. Bileşik-1, sıvı kromatografi kullanılarak izole edilmiş ve yapısı cis-dehidromatricaria ester olarak spektroskopik verilerle belirlenmiştir.

Bulgular: Bileşik-1'in bir kök-düğüm nematoduna karşı mükemmel nematosidal aktivitesi (%100 motilitede azalma), 72 saatlik inkübasyon süresinden sonra %1 konsantrasyonda elde edilirken, motilitelerdeki azalmalar %0,5 ve %0,125 (a/h) kosantrasyonlarda sırasıyla; ka %95 ve %75 olarak belirlenmiştir. Benzer şekilde, uygulamadan 24 saat sonra ölümler sırasıyla %1 ve %0,5 konsantrasyonlarda %90 ve %82 olarak belirlenmiştir. Bileşik-1 ayrıca, 0,08 mg/L'lik etkin doz 50 (EC_{50}) ile %1 konsantrasyonda (%100 ölüm sağlar). *Sitophilus oryzae*'ye karşı mükemmel insektisidal aktivite sergilemiştir. Standart olarak kullanılan fosfinin EC_{50}'si 0,07 mg/L olarak belirlenmiştir. *T. falconeri*'nin iki fraksiyonu, sırasıyla TfP ve TfM'ye karşılık gelen 22,4 ve 25,8 mg/L'lik inhibitör konsantrasyon 50 değerleri ile 30 mg/mL konsantrasyonda 3T3 hücre hatlarına karşı orta derecede sitotoksik aktivite göstermiştir.

Sonuç: Bu çalışma, cis-dehidromatricaria ester-1'in kök-düğüm nematodlarına karşı nematosidal aktivite (%100 ölüm) sağladığını göstermektedir. *S. oryzae*'ye karşı insektisidal aktivitesi, pazarlanabilir insektisit olarak kullanılmasını sağlayabilir.

Anahtar kelimeler: Pestisitler, *Tanacetum falconeri*, sekonder metabolitler, kök-düğüm nematodları, toksisite

INTRODUCTION

Asteraceae family belongs to the flowering plants having 23,000 species of 12 sub-families and 1,620 genera.[1] The family is quite diverse group of vascular plants containing shrubs, trees, and vines widely grown in sub-tropical and lower temperature latitude regions.[2] Worldwide, this family is used in medicines, cosmetics, and pesticidal products.[2,3] The genus *Tanacetum* commonly known as tansy belongs to the family *Asteraceae* and contains 200 species across the globe and abundantly found in Europe and Western Asia.[4,5] These species contain several biologically active compounds that are extensively used in herbal medication and in cosmetics.[6] *Tanacetum* species have vast medicinal importance and used to cure different diseases for many centuries. Many species of *Tanacetum* are used as an edible vegetable as well as medicinal plants. Different classes of secondary metabolites, including flavonoids, phenolic acids, sesquiterpene lactone, monoterpene, diterpene, glycosides, alkaloid, phytosterol, heterocyclic compounds, and polyacetylenes, have been reported from the different species of *Tanacetum*. Biologically, plants of genus *Tanacetum* have shown a variety of activities including insect anti-feedant and antimicrobial properties.[7] In addition to this, different species of genus *Tanacetum* also have biological activities like antimicrobial, cytotoxicity, growth regulating, phytotoxic, anti-ulcer, anthelmintic, anti-fungal, and antioxidant activities.[2] *Tanacetum chiliophyllum* has shown a promising insecticidal activity against the stored granary products pest *S. granaries*.[8] Similarly, compounds isolated from *T. chiliophyllum* have been reported in the literature to possess cytotoxic, antimicrobial activities, and acetylcholinesterase, butyrylcholinesterase inhibitory effects.[9] Many sesquiterpene lactones and flavonoids as anti-inflammatory agents have been isolated from *Tanacetum sinaicum*.[10] Pyrethroids are commercial insecticidal compounds isolated from *Chrysanthemum cinerariaefolium*.[11]

In this paper, we report *cis*-dehydrometricaria ester (compound-1) for the first time from *T. falconeri*, which has been further tested for its nematocidal and insecticidal activities. In addition to this, different fractions of *T. falconeri* extract have been screened for their potential nematocidal, insecticidal, antibacterial, cytotoxic, and phytotoxic activities.

MATERIALS AND METHODS

General experimental procedure

Analytical and laboratory grade of different chemicals including reagents and solvents were purchased from the trustworthy chemical companies (e.g., E. Merck, Fisher Scientific). For soaking and extraction, commercial grade of different solvents (methanol, *n*-hexane, ethyl acetate, and dichloromethan) were utilized. While chromatography and purification of the isolated compound was carried out by using distilled solvents. Silica gel was used as adsorbents for liquid column chromatography (E. Merck: 100-380 μm mesh). For determining samples purity pre-coated silica gel plates (GF 254, TLC) were used. Bruker AM-400 and AMX-500 and 125 MHz instrument was utilized for ^{1}H-and ^{13}C-NMR (1D and 2D spectra), respectively. By using the reference solvent proton signal of $CDCl_3$, chemical shifts values were represented in ppm and coupling constant (J) were represented in Hz. To determine the exact mass of the pure compounds, at 70 eV Electron-impact mass spectra was noted by using Finnigan MAT-112 instrument.

Collection of plant material

The collection of plant *Tanacetum falconeri* Hook. f. (5.2 kg) was carried out in July 2017 from Astore (Daosai). The identification of the plant was done in Karakoram International University Gilgit Baltistan, by the taxonomist Dr. Sher Wali Khan. The specimen (voucher no. 145/17) has been stored and protected at the herbarium of the department of biological sciences, KIU for future reference.

Preparation of sample extracts

The air-dried aerial parts of *T. falconeri* Hook. f. (2.18 kg) was extracted three times (3×2 L) with 95% methanol at 20°C by soaking for three days each time. Following filtration, the combined methanol extracts were evaporated by using a rotary evaporator at 40°C to dryness, to obtain the *T. falconeri* whole

plant extract (TfP) (105 g, TfP). The combined and concentrated TfP was further dissolved in water (500 mL) and extracted with *n*-hexane (3×500 mL) first, and water was evaporated by using a rotary evaporator to get the concentrated MeOH extract [91 g, *T. falconeri* methanolic (TfM)]. The methanolic extract was further fractionated through the successive solvent-solvent extractions with ethyl acetate (3×300 mL), and *n*-butanol saturated with H_2O (3×250 mL) in a separatory funnel. Each extract, as well as its remaining aqueous phase (R-H_2O) after solvent extractions were evaporated to dryness under a reduced pressure to yield an *n*-hexane fraction [14 g, *T. falconeri* hexane (TfH)], EtOAc fraction [37 g, *T. falconeri* ethyl acetate (TfE)], *n*-BuOH fraction [13 g, *T. falconeri* butanolic (TfB)], and R-H_2O fraction [25 g, *T. falconeri* aqueous (TfA)], respectively.

Isolation of compound-1

The EtOAc extract (37 g) obtained from the methanolic extract of *Tanacetum falconeri* Hook. f. was subjected to silica gel column chromatography. At first, system was eluted with 100% *n*-hexane, and then by using respective solvent system of *n*-hexane: EtOAc (98:2, 95:5, 93:7, 90:10, 88:12, 85:15, 80: 20, 70:30, 50:50); and finally washed with 100% ethyl acetate; and then with 50:50 ethyl acetate; and methanol (1L with each polarity). At the end 64 fractions (TF1-TF64) were obtained from the column. At gradient of *n*-hexane: Ethyl acetate (98:2) pure compound-1 was obtained.

Physical state: Colorless needle crystals. ESI-MS (+Ve) m/z: 173.0589[M+H]$^+$. Molecular formula: $C_{11}H_8O_2$. ^{1}H-NMR: ($CDCl_3$, 600 MHz) δ ppm: 6.25 (1H, d, J=11.4 Hz, H-2), 6.14 (1H, d, J=11.4 Hz, H-3), 3.75 (3H, s, H-1′), 1.99 (3H, s, H-10). ^{13}C-NMR: ($CDCl_3$, 150 MHz) δ ppm: 164.5 (C-1), 132.53 (C-2), 121.55 (C-3), 86.14, 80.82, 72.19, 70.97, 64.82, 58.39 (C-4-C-9, acetylenic carbons), 51.7 (C-1′), 4.80 (C-10).

Biological assays

All the extract fractions and pure compound-1 of *Tanacetum falconeri* were subjected to different biological assays including nematocidal, insecticidal, cytotoxic, antibacterial, and phytotoxic activities.

Nematocidal activity

The impact of six extracts and compound-1 of *T. falconeri* were used to study the larval mortality of root-knot nematode. Inhabitants of second-stage juvenile (J2) of *Meloidogyne incognita* was obtained from culture on tomato plants in microplot of a screen room. From the infected tomato plant, the egg masses were collected and placed in a cavity block with water. The cavity block was placed under conditions that promote the development of egg hatching at an ambient temperature for 72 h. In the next stage, 100 larvae were counted in a chamber for each dose and replicated thrice to introduce in 3×3 glass cavity block. The stock solution was prepared by using 10 mg/mL plant extracts in 5% dimethyl sulfoxide (DMSO). Three concentrations of 1%, 0.5%, and 0.125% were applied at a rate of 1 mL at each cavity block. Furadan was chosen as standard drug, while 5% DMSO as a control treatment. Stereoscopic microscope was used to observe the percentage death rate (mortality) after an interval of 24, 48, and 72 h. Nematodes were considered dead when no movement was detected after a mechanical nudge. Then the nematode bodies were transferred into distilled water for conformation of irreversible mobility.

Insecticidal activity

By using the impregnated filter paper, *Rhyzopertha dominica* and *Tribolium castaneum* (insect species) were subjected to the methanolic extract and all solvent fractions.[12-14] Stock solution was made by mixing the sample (200 mg) in methanol (3 mL). On Petri-plates with the help of micropipette samples (1019.10 µg/cm^2), were applied to filter paper of size 9 cm or 90 mm. The solvent evaporated after 24 h. From each species, ten insects were set in each plate for test and control. For positive and negative control, 239.5 µg/cm^2 of Permethrin and methanol were used, respectively. After maintaining the ambient temperature and 50% humidity in growth chamber, the test plates were incubated in the chamber for 24 h. The next day, from each species the number of survivals was calculated, and percentage mortality *(%M)* was determined by applying the formula given below:

$$\%M = \frac{100 - \text{No. of insects alive in test}}{\text{No. of insects alive in control}} \times 100$$

Insecticidal activity of compound-1

In laboratory conditions, compound was diluted in 5% DMSO to acquire the different concentrations (1%, 0.5%, and 0.125%). Ten active adults of rice weevil were collected from rearing cage and commence in Petri dishes (90 mm diameter) bottomed with filter paper disk (whatman no. 1), applied 1 mL each compound and concentration separately. The Petri dishes were sealed by parafilm (PM-996) and kept at 28±2°C. Pesticide phosphine was chosen as a standard drug. Mortality was recorded after 24, 48, and 72 h of intervals with 5% DMSO as a control treatment. Each treatment was replicated three times.

Statistical analysis

To analyze the treatment differences multifactor analysis of variance was used. By using SPSS statistical software, the obtained data was further submitted to Duncans' multiple range test ($p \leq 0.05$). Probit analysis was done under survival analysis for effective dose 50 (EC_{50}) values by SAS, 2000 software.

Antibacterial activity

With the help of broth microdilution method, minimum inhibition concentration was recoded. The required tests were carried out by application of *Tween-80,* and a final concentration of 0.5% (v/v) in Mueller Hinton Broth. For preparation of serial doubling dilutions of the extract 96-well microtiter plate (200-25 ppm) were used. A concentration of 10 µL of indicator solution and 10 µL of Mueller Hinton Broth were mixed. Then, 10 µL of bacterial suspension (106 CFU/mL) was mixed to each well to attain a concentration of 104 CFU/mL. Each plate was covered with cling film to avoid water loss. The plates were prepared in triplicates to calculate the average of three values. The sample plates were then set in an incubator for 24 h at 37°C. The lowest concentration was obtained by observing the color change with

naked eye. The growth of microorganism was indicated by turbidity.[15,16]

Cytotoxicity (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT)

The cytotoxicity of different extracts of *T. falconeri* was studied by applying the standard MTT colorimetric assay on 96-well micro plates.[17] In this process, mouse fibroblast cells (3T3) were cultured in Dulbecco's modified eagle medium and mixed with 5% of fetal bovine serum, 100 IU/mL of penicillin and 100 µg/mL of streptomycin in 75-cm^2 flasks. The flasks were placed in incubator at 37°C having 5% CO_2. Hemocytometer was used to calculate the growing cells, and the dilution of cells with medium was also done. Cell suspension (5×10^4 cells/mL) was prepared and added (100 µL/well) into 96-well plates. After 12 h of incubation, the medium was removed and 200 µL of fresh medium containing different concentrations of samples (1-30 mg/L) was added. After 48 h, the medium was removed and 200 µL MTT (0.5 mg/mL) was added to each well after 48 h and was kept for further 4 h incubation. Later, 100 µL of DMSO was mixed to each well. The amount of MTT decrease to formazan within cells was obtained with the help of a micro plate reader by determining the absorbance at 540 nm. The cytotoxic activity was noted as inhibitory concentration 50 (IC_{50}) for 3T3 cell. At last, % inhibition of cells was determined by applying the formula which is given below:

$$\%\ \text{inhibition} = \frac{100 - (\text{mean of O. D of test compound - mean of O. D of - ve control})}{(\text{mean of O. D of + ve control - mean of O.D of - ve control})} \times 100$$

Phytotoxicity

The crude methanolic and remaining solvent fractions of *T. falconeri* were subjected to the phytotoxicity assay.[18,19] For this purpose, a medium was prepared at a pH of 6.0-7.0 by adding distilled water (1000 mL) to KOH pellets. The extract (30 mg) was mixed with methanol (1.5 mL) to a prepared stock solution. Three types of concentrations (10, 100, and 1000 µg/mL) were obtained after a dilution of stock solution of the extract. A total of nine flasks were obtained, among which three were prepared for each dilution. Under sterilized conditions, the solvent was kept overnight to evaporate the solvent. In next stage, to each flask, 20 mL medium and 10 plants were added, each one containing a rosette of two fronds of *Lemna minor* L. A flask with a medium used as a positive control and Paraquate (reference plant growth inhibitor) as a negative control. The sample flasks were placed in the growth cabinet of the incubator for one week at 30°C. After incubation, the number of fronds in each sample flask was measured, and by using the following formula, growth regulation as a percent regulation was calculated.

$$\%\ \text{regulation} = \frac{100 - \text{No. of fronds in test}}{\text{No. of fronds in} - \text{ve control}} \times 100$$

RESULTS

From the EtOAc fraction of *T. falconeri, cis*-dehydromatricaria ester-1 has been obtained (Figure 1). Column chromatography was used for the purification of the compound, and the structure was identified with the help of modern spectroscopic techniques. The structure of compound obtained from *T. falconeri* (EtOAc fraction) was confirmed as *cis*-dehydromatricaria ester by the analysis of spectral data of NMR (1D and 2D), whereas the molecular mass was obtained by using EI-MS. The compound's data (^{1}H-NMR and ^{13}C-NMR) were compared and matched with the reported data in the literature.[20]

In the current study, we evaluated the crude methanolic extract (95%) and different fractions of *T. falconeri* for bioassays like nematocidal, cytotoxic, insecticidal, and phytotoxic activities, whereas the methanolic extract and all the solvent fractions of *T. falconeri* were also analyzed for their nematocidal activities.

Nematocidal activity

The nematocidal activity of *T. falconeri* extracts on larval mortality of *Meloidogyne incognita* (root-knot nematode) was studied at various concentrations after 24, 48, 72 hours incubation. The nematocidal activities of TfA, TfB, TfE, TfH, TfM, and TfP of *T.* falconeri were more consistent at the concentration of 1%, 0.5%, and 0.125% with the total larval mortality rate of 50-68% after 72 h incubation time. The results presented in Table 1 indicate that all the fractions of *T. falconeri* showed moderate activities of 60%. The active fractions of *T. falconeri* were comparable to each other showing the activity against root-knot nematodes. We recommend *in vivo* testing of active extracts, which have been never reported yet to promote the green practices for sustainable agriculture and protection of the environment.

In addition to this, compound-1 was also tested for its nematocidal activity against root-knot nematode (*M. incognita*). Compound-1 showed excellent and exceptional activity of 100%, 95%, and 75% mortality at 1%, 0.5%, and 0.125% after 72 h of the treatment, respectively. Whereas the activity at 1%, 0.5%, and 0.125% after 48 h of treatment was recorded as 90%, 82%, and 37% mortality of root-knot nematodes (Table 1).

Insecticidal activity

The insecticidal effect of *T. falconeri* TfP and all the solvent fractions were studied against *R. dominica* and *S. oryzae*. The insecticidal activity of all six fractions of the extract of *T. falconeri* was carried out using 1019.10 µg/cm^2 with a reference to the standard drug permethrin (239.5 µg/cm^2). The results revealed that activity of all fractions remain insignificant at all concentration. Therefore, the data results have not been

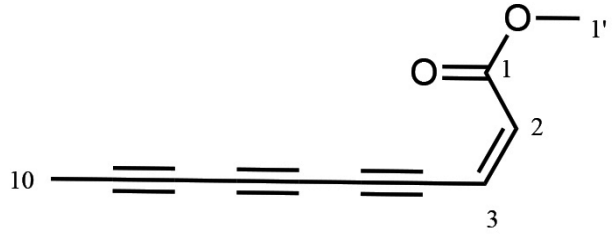

Figure 1. Structure of *cis*-dehydrometicaria ester (1) isolated from *T. falconeri*

presented in the tabular form. The *cis*-dehydrometracaria ester (1) isolated from *T. falconeri* showed the strong insecticidal activity against stored grain pest (*S. oryzae*). In general, the mortality rate was increased with an increase in the concentration of compounds and exposure time. Compound-1 showed 100% mortality at 1% concentration comparable to a standard pesticide (phosphine). The EC_{50} values of compound-1 against *S. oryzae* were 0.0897 mg/L at 1% concentration after 72 h of incubation (Table 2).

Cytotoxicity

The cytotoxicity of *T. falconeri* (extract and all fractions) was examined at concentrations of 30 mg/mL with a reference to the standard drug cyclohexamide. The extracts of *T. falconeri* (methanol and whole plant) showed low inhibition with IC_{50} values of 22.4 and 25.8 mg/L against the 3T3 normal cell lines, respectively (Table 3). The rest of the extracts including *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions were found inactive in MTT assay with inhibition <20%. Therefore, the insignificant data results have been not presented in the tabular form.

Antibacterial activity

The antibacterial susceptibility of all six extracts TfA, TfB, TfE, TfH, TfM, and TfP of *T. falconeri* were tested against *E. coli*, *B. subtilis*, *Staphylococcus aureus*, *P. aeruginosa,* and *Salamonella typhi*. The bacterial isolates were revived on nutrient agar. The bacterial cultures were preserved at 28°C prior to use, and periodic sub-culturing was done to maintain these cultures. The antibacterial activity of all the extract of *T. falconeri* with a standard drug ofloxacine presented in Table 4. All extracts have low inhibitory activity against tested bacterial strains, whereas,

Table 1. Nematocidal activity of different fractions from *T. falconeri* against root-knot nematode

Sample	Time (h)	Concentration in % (w/v)			*EC_{50} (± SE)
		0.125*	0.5*	1*	
Compound-1	24	22±2.0c	35±2.5b	42±1.3a	3.4±06
	48	37±1.2c	82±1.0b	90±1.5a	0.18±0.04
	72	75±1.0c	95±1.4b	100±0.0a	0.04±0.1
TfA	24	10±1.0c	15±1.0b	25±0.5a	2.1±0.1
	48	45±1.5b	45±1.5b	50±2.0a	1.09±0.5
	72	55±1.1Cb	60±1.5Ba	60±0.5a	0.01±0.5
TfB	24	10±2.0c	10±1.0b	15±1.0a	4.5±0.2
	48	35±1.0a	35±1.1a	35±1.5a	4.5±0.2
	72	60±1.0b	60±1.1b	65±1.0a	4.5±0.2
TfE	24	15±1.5b	15±1.0b	25±1.1a	2.6±1.8
	48	40±1.0b	40±1.5b	45±2.0a	1.92±0.5
	72	62±1.0b	62±1.0b	68±1.5a	0.001±1.0
TfH	24	15±0.5bc	18±1.0b	20±1.0a	4.8±0.3
	48	30±1.0c	30±2.0b	35±1.5a	3.4±0.4
	72	60±1.0bc	62±0.2b	65±1.5a	2.9±0.8
TfM	24	12±1.0c	15±2.0b	18±1.0a	4.1±0.2
	48	32±1.0b	32±1.5b	35±1.0a	5.0±0.8
	72	60±1.0c	65±1.1b	68±0.5a	0.01±0.1
TfP	24	10±0.5b	10±0.5b	15±0.2a	4.5 ±0.2
	48	30±2.0b	30±2.0b	32±1.0a	4.5±0.6
	72	50±0.5a	50±2.0a	50±1.0a	4.5±0.6
Carbofuran (furadan)	24	40±1.5b	100±0.0a	100±0.0a	0.13±0.02
	48	80±2.5b	100±0.0a	100±0.0a	0.07±0.3
	72	100±0.0a	100±0.0a	100±0.0a	0.07±0.3

*Values are in mg/L: The concentrations of 1%, 0.5%, and 0.125% were prepared by dissolving extract in 5% DMSO (w/v). Means followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$). TfA: *T. falconeri* aqueous, TfB: *T. falconeri* butanolic, TfE: *T. falconeri* ethyl acetate, TfH: *T. falconeri* hexane, TfM: *T. falconeri* methanolic, TfP: *T. falconeri* whole plant extract, EC_{50}: Effective dose 50, SE: Standard error, DMSO: Dimethyl sulfoxide

TfP was found inactive against *B. subtilis*, *P. aeruginosa*, and *Salmonella typhi*.

Phytotoxicity

The phytotoxic and insecticidal constituents are vital for the development of green herbicides and insecticides that are more eco-friendly than synthetic ones. The phytotoxic effect of the studied samples on *L. minor* was analyzed to have dose-dependent activity because low activity was noticed in TfP, TfM, TfE, TfB, and TfA fractions, with 12.5%, 0%, 20%, 26.56%, and 0% inhibition at 100 µg/mL, respectively, and 0% inhibition at 10 µg/mL for all, in comparison to the Paraquat (0.015 µg/mL) as a standard drug and control. Among the six fractions, *n*-hexane fraction had moderate and good phytotoxic activity (31.25%, 77.08% inhibition) at concentration at 10 and 100 µg/mL, respectively. A very significant phytotoxic effect (100.0% inhibition) was observed at concentration of 1000 µg/mL for all fractions except TfB (68.75%) and TfA (25%) fractions of *T. falconeri*.

DISCUSSION

Meloidogyne incognita belongs to the family of nematodes and commonly called as root-knot nematode. The root-knot nematode found worldwide damages the roots of plants and ultimately decreases both quality and quantity of the plant. The affected plants show relatively slow growth rate and poor performance. To control the population of nematodes usually synthetic nematocides are used but these conventional nematocides are more vulnerable to the non-targeted organisms and ecologies. Hence more risk for environmental pollution problems arises. In these circumstances the green nematicodes (botanical nematicodes) are the best substituent of conventional chemicals to control the nematodes. The present study discussed certain botanical nematocides against *M. incognita*. In the present study, antibacterial, nematocidal, insecticidal, cytotoxic, and phytotoxic activities of *T. falconeri* were evaluated with the help of standard assay protocols. Extraction method is a key factor in obtaining the maximum quantity of active formulations from

Table 2. Insecticidal activity compound-1 isolated from *T. falconeri* against rice weevils

Sample	Time (h)	Concentration in % (w/v)			*EC_{50} (± SE)
		0.125*	0.5*	1*	
Compound-1	24	30±1.5c	52±1.0b	62±1.0a	0.44±0.17
	48	45±1.0c	70±1.2b	80±2.5a	0.163±0.08
	72	62±1.5c	88±1.2b	100±0.0a	0.08±0.03
Phosphine	24	80±0.5c	90±1.0b	100±0.0a	0.05±0.3
	48	90±2.0b	100±0.0a	100±0.0a	0.07±0.3
	72	100±0.0a	100±0.0a	100±0.0a	0.07±0.3

*Values are in mg/L: The concentrations of 1%, 0.5%, and 0.125% were prepared by dissolving extract in 5% DMSO (w/v). Means followed by the same letter are not significantly different according to Tukey's test (p≤0.05). EC_{50}: Effective dose 50, SE: Standard error, DMSO: Dimethyl sulfoxide

Table 3. Cytotoxic activity of two fractions of *T. falconeri* against 3T3 cell lines

Extract	Concentration (mg/mL)	% Inhibition	*IC_{50} ± SD
TfP	30	55	22.4±2.8
TfM	30	67	25.8±2.5
Std: Cyclohexamide	30	70	8±0.2

*Values are in mg/L. TfM: *T. falconeri* methanolic, TfP: *T. falconeri* whole plant extract, IC_{50}: Inhibitory concentration 50, SD: Standard deviation, Std: Standard

Table 4. Antibacterial susceptibility of *T. falconeri* against standard bacteria

Bacteria	% Inhibition of different extracts (conc. 3000 µg/mL)						% Inhibition of drug
	TfP	TfM	TfH	TfE	TfB	TfA	Ofloxacine
E. coli	11.32	18.01	10.84	22.55	27.37	6.80	95.52
B. subtilis	0.00	1.23	16.37	17.65	22.42	29.57	95.19
Staphylococus aureus	2.04	33.64	19.17	6.74	0.19	4.82	90.93
P. aeruginosa	0.00	2.08	5.95	15.29	5.05	2.35	90.99
Salamonella typhi	0.00	0.24	14.67	20.23	1.69	1.73	92.15

Means followed by the same letter are not significantly different according to Tukey's test (p≤0.05). TfA: *T. falconeri* aqueous, TfB: *T. falconeri* butanolic, TfE: *T. falconeri* ethyl acetate, TfH: *T. falconeri* hexane, TfM: *T. falconeri* methanolic, TfP: *T. falconeri* whole plant extract

target plant species. Methanol was used initially to ensure the maximum extraction of secondary metabolites from *T. falconeri* whereas, four different solvents with the variable degrees of polarity along with water were used in this study to divide the main extract into separate fractions, based on ingredients polarity. The methanolic and ethyl acetate fractions were more efficient than solvents with both lower polarity (hexane) and higher polarity (water). These results suggest that nematocidal compounds probably possess an intermediate degree of polarity or that *T. falconeri* contains several nematocidal compounds with different degrees of polarity.

Although the sensitivity of plant parasitic nematodes to nematocides varies among different extractives in general, in present study, the results are closely related to each other except the results for the methanolic extract. The results strongly support the profound ethnobotanical application of *Tanacetum* species and *cis*-dehydrometracaria ester (1). These results also demonstrate its potential for use in botanical pest control strategies.[20] Control treatment fails to kill nematode while standard control carbofuran gave 100% mortality after 72 h of incubation with EC_{50} value 0.07±0.3 mg/L, but it is toxic and extremely lethal to the mammals and the wildlife. Whereas in human beings, it causes reproductive disorders, genotoxic abnormalities, and endocrine disrupting activity.[21] Carbofuran (Furadan®) is used to control broad spectrum of insect is highly soluble white crystalline solid chemical and possess low adsorption properties in soil.[22] The results of extractives and compound-1 showed an excellent activity and may be used as safe alternatives to replace the hazardous chemical products in the market. We recommend *in vivo* testing of active extracts, which have never been reported yet to promote the green practices for sustainable agriculture and the protection of the environment. In line with nematocidal activities, the insecticidal activity of the compound-1 was found to be exceptional, which is consistent with the literature data. It has been reported that *cis*-dehydrometracaria ester isolated from *Artemisia ordosica* was tested against *Tribolium castaneum* at different concentrations (62.91, 12.58, 2.52, and 0.5 nL/cm^2). The pest repellent value has been reported higher than 90% at both 2 and 4 h incubation time.[20] Phosphine pesticide has led to the selection of strong resistance against the major stored grain pest, including *Sitophilus oryzae, Cryptolestes ferrugineus*, *Rhyzopertha dominica*, *Tribolium castaneum*, and *Liposcelis bostrychophila.* It causes lethargy in humans which has been referred to as narcosis or anesthesia in animals.[23] The promotion and development of green practices is in utmost need for the sustainable agriculture and the protection of the environment. The methanolic extract and different fractions of *T. falconeri* were found inactive against cytotoxicity, antibacterial, and phytotoxicity assays. This indicates the safe and effective nature of formulations from *T. falconeri* to the plants and animals for its sustainable application in agriculture, as well as health and well-being.

In summary, the results of the present study indicate that medium polar extracts in general and compound-1 of *T. falconeri* potentially be developed into a commercial nematocide. Although plant-based materials and extractives can be used in sustainable and organic farming, but isolation and identification of the nematocidal compounds is essential for further development of commercial products. Similarly, synthesis of active compounds or their derivatives with a higher nematocidal activity are likely to be a more promising means of developing a nematocide based on *T. falconeri* or related plant species.

Study limitations

Compound-1 could not have been tested for antibacterial, cytotoxic, and phytotoxic activities due to the limited quantity in hand. The nematocidal activity have been carried out on *Meloidogyne incognita* only whereas insecticidal activity against *Rhyzopertha dominica* and *Tribolium castaneum*. The results of *T. falconeri* extracts and compound-1 are limited to the microorganisms stated in this study.

CONCLUSION

The investigation on green pesticides from plant origin is essentially vital for the progress of new botanical pesticides, especially in view of the vast worldwide flora. In summary, the current study revealed the ability of the different fractions of *T. falconeri* for their potential as cytotoxic, antibacterial, phytotoxic, nematocidal, and insecticidal agent. The results indicated that in development of plant-based green pesticides these plant species are a new potential source. These plants species may provide best alternative paths for controlling the different pests than synthetic pesticides with no worse impacts on ecology. Hence, detailed studies are required to find and investigate the functioning compounds, and their path of mechanism of action of these plant formulations, to introduce more safe products to the market, and substitute some of the present toxic chemicals already available and in practice. Opportunities exist to reduce chemical inputs to the environment provided by exploring the potential of pesticides based on plant products.

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ORIGINAL ARTICLE

Minocycline Hydrochloride Controlled-release Microsphere Preparation Process Optimization Based on the Robust Design Method

Sağlam Tasarım Yöntemine Dayalı Minosiklin Hidroklorür Kontrollü Salınımlı Mikroküreleri Hazırlanma Sürecinin Optimizasyonu

Mohammad Karim HAIDAR[1]*, Fumiyoshi YAMASHITA[2], Mitsuru HASHIDA[2]

[1]Erzincan Binali Yıldırım University Faculty of Pharmacy, Department of Pharmaceutical Technology, Erzincan, Turkey
[2]Kyoto University Graduate School of Pharmaceutical Science, Department of Drug Delivery Research, Kyoto, Japan

ABSTRACT

Objectives: The objective of the present study is to establish a robust preparation method that could steadily produce minocycline hydrochloride (MCH) microspheres regardless of used polymer types.

Materials and Methods: Taguchi's Robust Experimental Design methodology was employed to optimize the process parameters for MCH-loaded poly(D,L-lactide-co-glycolide) (PLGA) microspheres. In the experimental design, seven controllable factors, i.e., preparation method, pH of the aqueous phase, volume of the aqueous phase, volume of dichloromethane, rotation speed, temperature, and amount of polyvinyl alcohol, were considered for the optimization of process parameters. PLGA types with different lactide/glycolide ratios were considered the uncontrollable (noise) factor. Based on the L18 orthogonal array, 18 experimental runs were conducted for each type of PLGA. The encapsulation efficiency (EE) and *in vitro* release rate were evaluated for all the prepared formulations.

Results: Regardless of the PLGA type with different lactic/glycolic acid ratios, microspheres prepared via the solid-in-oil-in-water (S/O/W) method, showed a much higher EE and faster drug release than the microspheres prepared via the co-solvent method. Preparation methods, pH of the aqueous phase, and volume of the aqueous phase were the most influencing parameters on the EE. The confirmation experiment results indicated that the signal-to-noise ratio increased by 5.76 db from that of an initial condition. The release of minocycline was fastest with the PLGA (50:50) microspheres, followed by PLGA (75:25) and PLGA (85:15).

Conclusion: Although the interaction between the selected factors in the evaluation was ignored, the orthogonal array design of the experiment based on Taguchi's robust experimental design methodology was sufficient to optimize the process parameters for the PLGA microspheres of MCH. The S/O/W was the main factor affecting the EE. Microspheres prepared via the S/O/W method exhibited a higher EE and faster drug release than the microspheres prepared via co-solvent method. The pH and volume of the aqueous phase were also effective parameters on the EE. A robust experimental design has been successfully applied to the optimization of the process parameters for microsphere preparation.

Key words: Design of experiment, minocycline hydrochloride, PLGA microspheres

ÖZ

Amaç: Bu çalışmanın amacı, kullanılan polimer türlerine bakılmaksızın sürekli olarak minosiklin hidroklorür mikroküreleri sağlayabilen sağlam bir hazırlama yöntemi oluşturmaktır.

Gereç ve Yöntemler: Minosiklin hidroklorürün poli (D, L-laktit-ko-glikolid) (PLGA) mikroküreleri için işlem parametrelerini optimize etmek için Taguchi'nin Güçlü Deneysel Tasarım metodolojisi kullanılmıştır. Deneysel tasarımda yedi faktör yani hazırlama yöntemi, sulu fazın pH'si, sulu fazın hacmi, diklorometan hacmi, dönüş hızı, sıcaklık ve polivinil alkol miktarı kontrol edilebilir faktörler olarak kabul edilmiştir ve laktit/glikolid oranını değiştiren PLGA türleri, işlem parametrelerinin optimizasyonu için kontrol edilemeyen (gürültü) bir faktör varsayılmıştır. L18 ortogonal dizisine (L18 OA) göre, her PLGA tipi için 18 deneysel çalışma gerçekleştirilmiştir.

*Correspondence: mk_haidar@hotmail.com, Phone: +90 553 283 66 54, ORCID-ID: orcid.org/0000-0003-1786-4449
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Bulgular: PLGA tipine bakılmaksızın, yağda-katı-suda (K/Y/S) çözücü buharlaştırma yöntemiyle hazırlanan mikroküreler, yardımcı çözücü yöntemine göre çok daha yüksek kapsülleme verimliliğine ve daha hızlı ilaç salımına sahiptir. Hazırlama yöntemleri, sulu fazın pH'si ve sulu fazın hacmi, kapsülleme verimliliği üzerinde en çok etkileyen parametreler olarak belirlenmiştir. Doğrulama deneyi, oranınınbaşlangıç oranına göre 5,76 db arttığını göstermiştir. Minosiklin salınımı en hızlı PLGA (50:50) mikroküreler, ardından PLGA (75:25) ve PLGA (85:15) ile olmuştur.

Sonuç: Değerlendirmede seçilen faktörler arasındaki etkileşimin göz ardı edilmesine rağmen, Taguchi'nin Güçlü Deneysel Tasarım metodolojisine dayalı deneyin ortogonal dizi tasarımı, minosiklin hidroklorürün PLGA mikroküreleri için proses parametrelerini optimize etmek için yeterli bulunmuştur. Mikroküreler hazırlama yönteminin yağda katı suda (K/Y/S) kapsülleme verimliliğini etkileyen ana faktör olduğu keşfedilmiştir. (K/Y/S) yöntemiyle hazırlanan mikroküreler, yardımcı çözücü yöntemiyle hazırlanan mikrokürelere göre daha yüksek kapsülleme etkinliği ve daha hızlı ilaç salımı göstermiştir. pH ve sulu faz hacmi de kapsülleme verimliliği üzerinde etkili parametreler olarak bulunmuştur. Minosiklin salınımı en hızlı PLGA (50:50) mikroküreler ile olmuştur; ardından bu sıralamayı PLGA (75:25) ve PLGA (85:15) izlenmiştir. Mikrokürelerin hazırlanması için proses parametrelerinin optimizasyonuna sağlam deneysel tasarım başarıyla uygulanmıştır.

Anahtar kelimeler: Deney tasarımı, minosiklin hidroklorür, PLGA mikroküreler

INTRODUCTION

Quality by design (QbD) is the main part of recent approaches to achieve pharmaceutical quality. It refers to designing and developing formulations and manufacturing processes to ensure predefined product quality specifications.[1] According to the ICH Q8 guideline, "product quality cannot be ensured by testing alone and should be built in by design".[2] Multiple variables are involved in the fabrication of pharmaceutical products with consistent quality specifications. Therefore, an important part of QbD implementation is the identification of critical quality attributes and understanding how these parameters affect the product quality and are optimized with respect to the final specifications.[3] The QbD method can be utilized to simultaneously investigate the effect of several variables on the quality of a product by performing a limited number of experiments.[4]

Design of experiment (DOE) is a systematic approach that designates the relation of variables influencing a method and responses generated by the process. Statistical DOE provides an organized and efficacious plan for experimentation to attain specific objectives and allows the simultaneous study of several control variables.[5] Statistically designed experiments consist of several stages: 1) Selection of predefined quality specifications; 2) identification of independent factors, which are influencing the procedure; and 3) estimating the different factor treatments (or levels) for an optimum response.[6]

The separate examination of several variables that affect product quality requires several experiments and is a time-consuming process. Moreover, a quantitative evaluation of these effects requires special statistical techniques.[7] In this regard, several DOE methods have been extensively used to minimize this problem. Particularly, Taguchi's design, developed by Dr. Genichi Taguchi, is a reliable DOE method.[8] This method was designed to improve the quality of final products based on the concepts of factorial/fractional designs and orthogonal arrays.[9]

According to Taguchi's suggestions, the design process contains three stages, i.e., system design, parameter design, and tolerance design. The first two stages are more significant to maximize product reliability and reducing cost, whereas the last stage has minimum effectiveness at reaching desirable products.[10] In Taguchi's method, the parameter design is the main step to achieve a product with high quality without incremental cost. In this method, the arrangement of variables in an orthogonal array can provide simultaneous studies on numerous parameter spaces by performing only a small number of experiments.[11] In an orthogonal array, all parameters and levels are designed equally. Hence, the independent evaluation of each parameter is attainable, and the effect of one parameter does not influence the estimation of other parameters.[12] According to Taguchi's design assertion, all parameters that cause variability are not controllable. This method evaluates and recognizes the controllable parameters that attenuate the effect of noise parameters.[13]

Minocycline hydrochloride (MCH), a semi-synthetic tetracycline derivative, was chosen as a model drug for the preparation of poly(D,L-lactide-co-glycolide) (PLGA) microspheres. MCH has a broad-spectrum antibacterial activity and is the main antibiotic in the treatment of acne vulgaris.[14]

In this study, Taguchi's robust experimental design methodology was applied to optimize the process parameters for MCH-loaded PLGA microspheres. The PLGA type was used as an uncontrollable factor. Seven controllable variables with three levels were selected. The L18 orthogonal array (L18 OA) was used to design the experiments. Eighteen runs were conducted for each type of PLGA to optimize the seven process parameters. The encapsulation efficiency (EE) and *in vitro* release rate were evaluated for all the formulations prepared.

MATERIALS AND METHODS

Materials

MCH, PLGA 50:50 (molecular weight: 38,000-54,000), PLGA 75:25 (molecular weight: 66,000-107,000), PLGA 85:15 (molecular weight: 190,000-240,000), and polyvinyalcohol (PVA) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). All other chemicals, such as dichloromethane (DCM), which were used for microsphere preparation, were of analytical grade and used without further purification.

Preparation of microspheres

Two methods, i.e., solid-in-oil-in-water (S/O/W)[15] and co-solvent method, were utilized to investigate the EE of MCH microsphere. In the S/O/W method, 350 mg PLGA was dissolved

in DCM, and the mixture was further sonicated with occasional vortexing to ensure a complete dissolution of the polymer in DCM. Then, the S/O dispersion was produced by suspending 105 mg (30% w/w) of MCH into the PLGA DCM solution using T25 digital ULTRA-TURRAX® (13500 rpm). Subsequently, the PLGA/MCH mixture was slowly added to the PVA aqueous solution dropwise with continuous stirring. Mechanical stirring was performed for 3 h to completely evaporate the organic solvent. The resulting microspheres were collected via filtration and subsequently rinsed three times with deionized water to remove the non-loaded drug and remaining solvent. The washed microspheres were freeze-dried (FDU-2200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) over night. In the co-solvent method, MCH was dissolved in methanol and then added to a polymer-containing DCM solution. The following procedure was the same as that of the S/O/W method. The investigated process parameters were preparation methods, pH and volume of the PVA solution, volume of DCM, rotation speed, temperature, and amount of PVA (Table 1).

In vitro drug release

The *in vitro* release tests were performed in triplicate in a phosphate-buffered solution (pH 7.4), and the temperature was kept constant (37°C). 10 mg of microspheres were suspended in a 5 mL phosphate-buffered solution containing 0.05 % (w/v) sodium azide and incubated at 37°C with 50 rpm stirring. 1 mL aliquot was withdrawn at each predetermined time point and replaced with fresh and preheated phosphate-buffered solution at each time point to maintain the sink condition. Then, the samples were analyzed via high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) in triplicate.

Encapsulation efficiency

3 mg of loaded microspheres were accurately weighed and completely dissolved in 1 mL acetonitrile, and then the organic solvent was evaporated under a nitrogen gas stream. The resultant was reconstituted with 1 mL distilled water and filtered through a 0.45 µm filter. The samples were analyzed via HPLC (Shimadzu, Kyoto, Japan). The HPLC apparatus equipped with a C8 (4.6×150 mm, Nacalai Tesque) reverse-phase chromatography column was flowed by the mobile phase consisting of acetonitrile and methanol, 0.01 M KH_2PO_4, 0.03 mM Na_2EDTA (5:20:72.1, v/v), and 60% $HClO_4$ (2.9 mL). The pH was adjusted to 2.5.[16] The EE was calculated as the amount of drug per unit weight of the microsphere (equation 1).

$$E.E.\% = \frac{Actual\ loading}{Theoretical\ loading} x100 \quad (1)$$

Experimental design

As represented in Table 1, seven variables, which significantly influence the microsphere EE, were investigated in the optimization study. Parameter A had two levels, and all the other parameters were examined at three levels. PLGA types with different lactide/glycolide ratios, which are an uncontrollable factor (noise), and EE as the response were considered to optimize the process parameters. Based on Taguchi's robust experimental design methodology, an orthogonal array L_{18} (Table 2) was employed to reduce the number of experiments for determining the optimal process parameters for (MCH) microspheres. Based on the layout of the orthogonal array (L_{18}), 18 experimental runs were performed. Under the same conditions, each run needs to be performed in triplicate, thus reducing the experimental error. The 18 experimental runs were accomplished for each type of PLGA.

Analysis of the signal-to-noise ratio

The signal-to-noise (S/N) ratio was calculated as a performance measure in a dynamic system to assess the robustness of a process, which showed the importance of the interaction between controllable parameters and noise factors. Primarily, to accomplish the S/N ratio analysis, the mean squared deviation (MSD) needs to be computed. The value of the MSD indicates the deviation from the target value. For the "larger-the-better" quality characteristic, the MSD and S/N ratio were calculated according to the following equations.[11]

$$MSD = \frac{1}{\mathrm{n}} \sum_{i=1}^{n} \frac{1}{Y_i^2} \quad (2)$$

$$S/N = 10\, Log_{10}(MSD) \quad (3)$$

Table 1. List of control factors and associated levels used in L_{18} design

Parameter	Name	Level-1	Level-2	Level-3
A	Preparation method	S/O/W	Co-solvent method	-
B	pH of PVA solution	3.5	4.5	5.5
C	Volume of PVA solution (mL)	150	250	350
D	Volume of DCM (mL)	2	3	4
E	Rotation speed (rpm)	350	450	550
F	Temperature (°C)	25	37	45
G	Amount of PVA (g)	1.5	2.5	3.5

PVA: Polyvinyalcohol, DCM: Dichloromethane, S/O/W: Solid-in-oil-in-water

Statistical analysis

To investigate and specify the relative significance of the different variables, ANOVA was performed. Statistical analyses were conducted with Prism 7 scientific software by GraphPad.

RESULTS AND DISCUSSION

Effect of the preparation method on the microsphere EE

Seven independent parameters, each having three levels (Table 1), were identified based on some preliminary experiments and related literature review. Figure 1 shows the EE of microspheres, which were made of different PLGA types. Regardless of the PLGA type, microspheres prepared by the S/O/W solvent evaporation method (conditions 1-9) had a much higher EE than that prepared by the co-solvent method (conditions 10-18). When the co-solvent, i.e., methanol, was added, the solution viscosity might be lowered in addition to an increase in water miscibility with the inner oil phase. Therefore, MCH might easily diffuse from the inner oil phase to the outer aqueous phase.[17]

Determination of optimal conditions

In this study, the S/O/W and co-solvent methods were used to encapsulate MCH in polymer matrices. The preparation method involves many parameters that impact the properties and quality of the final product. Optimizing all these parameters using the classic method is time-consuming and costly.[18] The S/N ratio was calculated for each of the formulation run with the larger-the-better EE. Table 3 summarizes the structure of Taguchi's L18 orthogonal array design, EE of the microspheres for each PLGA type, and S/N ratio for the EE. The average S/N ratio of each control factor at each level was also calculated. Figure 2 indicates that the S/N ratio increases as the pH of an aqueous phase (B) becomes higher. It would be due to the decreased solubility of MCH in the outer aqueous phase because MCH is

Table 2. Layout of orthogonal array L_{18} (2^1x3^7)

Run	A: Preparation method	B: pH of PVA solution	C: Volume of PVA solution (mL)	D: Volume of DCM (mL)	E: Rotation speed (rpm)	F: Temperature (°C)	G: PVA concentration (g)
1	S/O/W	3.5	150	2	350	25	1.5
2	S/O/W	3.5	250	3	450	37	2.5
3	S/O/W	3.5	350	4	550	45	3.5
4	S/O/W	4.5	150	2	450	37	3.5
5	S/O/W	4.5	250	3	550	45	1.5
6	S/O/W	4.5	350	4	350	25	2.5
7	S/O/W	5.5	150	3	350	45	2.5
8	S/O/W	5.5	250	4	450	25	3.5
9	S/O/W	5.5	350	2	550	37	1.5
10	Co-solvent method	3.5	150	4	550	37	2.5
11	Co-solvent method	3.5	250	2	350	45	3.5
12	Co-solvent method	3.5	350	3	450	25	1.5
13	Co-solvent method	4.5	150	3	550	25	3.5
14	Co-solvent method	4.5	250	4	350	37	1.5
15	Co-solvent method	4.5	350	2	450	45	2.5
16	Co-solvent method	5.5	150	4	450	45	1.5
17	Co-solvent method	5.5	250	1	550	25	2.5
18	Co-solvent method	5.5	350	3	350	37	3.5

PVA: Polyvinyalcohol, DCM: Dichloromethane, S/O/W: Solid-in-oil-in-water

a weak basic drug that is unionized at a higher pH.[19] Volumes of aqueous phase (C) and organic phase (D) are also important. As the volume of the aqueous phase was increased, the MCH of the aqueous solubility became approximately 2% (v/v), which can more readily distribute into the aqueous phase. Therefore, the drug would be more stably deposited in the polymer matrix. Because DCM reduced the viscosity of the organic phase, an initial state of dispersion would be better. The moderate rotation speed (E) and temperature (F) appeared to be optimal, which might be related to the stability of the S/O/W emulsion and evaporation rate of the organic phase. However, nonlinear interactions between factors might be present in the S/N ratios. The amount of PVA (G), which was used as a surfactant for stabilizing the emulsions, appeared to affect the EE of MCH microspheres, whereas the effect was saturated at an amount of 2.5 g. As shown in Figure 2, a greater S/N value corresponds to a better performance. Therefore, the optimized factor levels are the levels with the highest S/N value, which were predicted to be $A_1/B_3/C_2/D_2/E_2/F_2/G_3$, leading to a maximum EE.

Based on the S/N ratio, the optimal level of the control factors were determined, as shown in Table 4. The predicted mean of the S/N ratio (S_{mp}) was estimated to be 27.25±6.57 dB, using the following equation:[20]

$$S_{mp}=+(A_1-Y)+(B_3-Y)+(C_2-Y)+(D_2-Y)+(E_2-Y)+(F_2-Y)+(G_3-Y), \quad (4)$$

where *Y* is the total average of S/N ratio for the experimental test presented in Table 5. To validate the optimal process parameters, confirmation experiments were conducted. In equation 4, the S/N ratio of the EE for the optimum levels $A_1/B_3/C_2/D_2/E_2/F_2/G_3$ was 23.48 dB, which is within the range of 95% confidence intervals of the prediction. In addition, the S/N ratio increased by 5.76 dB from that of an initial condition. Table 4 shows the response table for the S/N ratio of the "the-bigger-is-better" EE, which was obtained for different parameter levels. The analysis of the S/N ratio of the EE reveals that the main factor that caused the EE increase was the preparation method (S/O/W). Other factors are the pH of the PVA solution, temperature (°C), rotation speed (rpm), volume of the PVA solution, amount of PVA (g), and volume of DCM (mL).

ANOVA

The main objective of ANOVA is to investigate and identify the factors that have statistically significant effects on the quality characteristics.[21] In Taguchi's method, after the analysis of

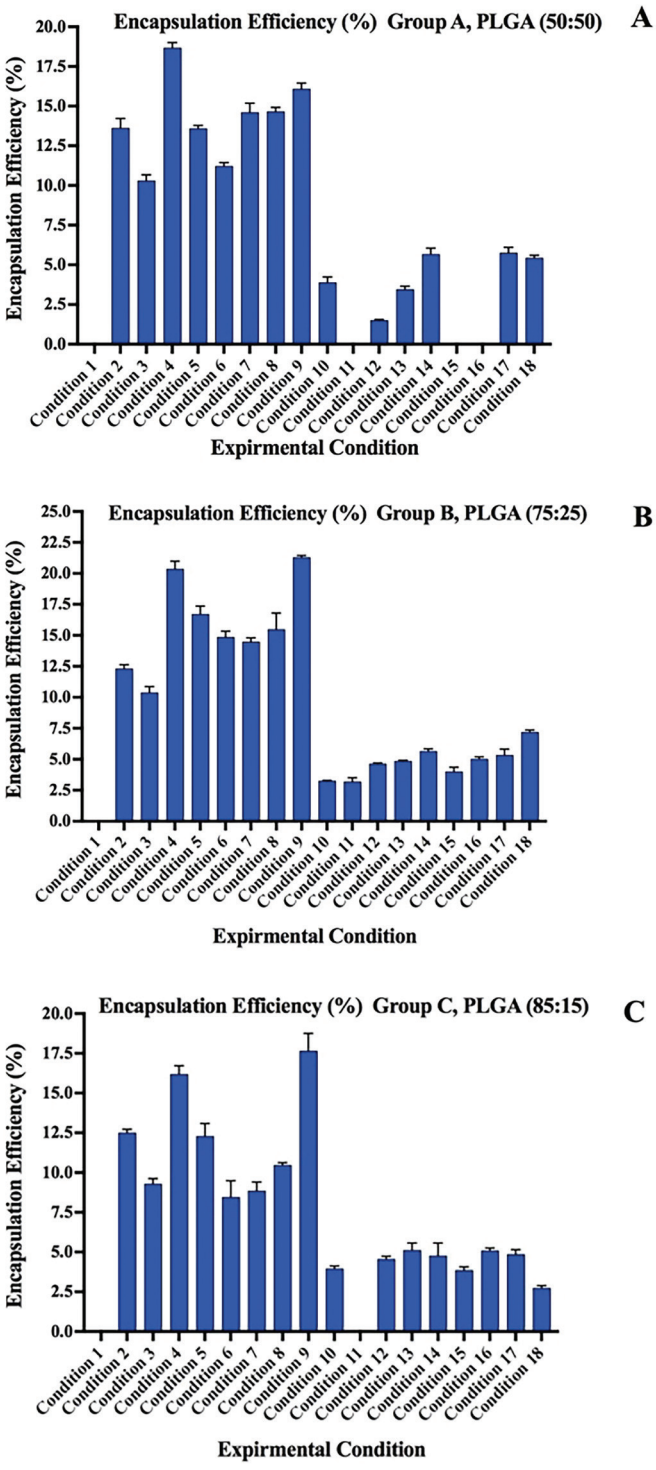

Figure 1. Encapsulation efficiency of minocycline microspheres (MCH) which was made PLGA with different ratio of lactide and glycolide acid (mean ± SD; n=3). A) PLGA (50:50), B) PLGA (75:25), and C) PLGA (85:25). PLGA: Poly(D,L-lactide-co-glycolide), MCH: Minocycline hydrochloride, SD: Standard deviation

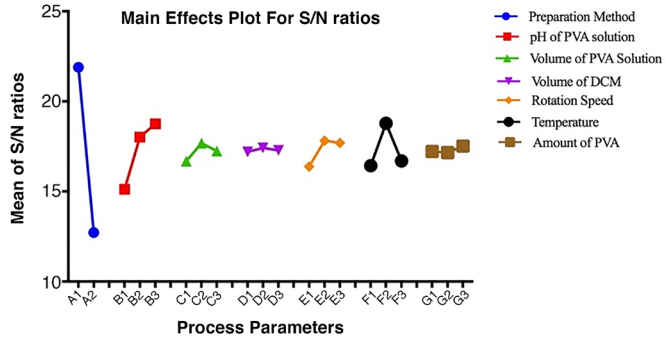

Figure 2. Variation of S/N ratios with factor levels for MCH microspheres encapsulation efficiency. A: Preparation method, B: pH of PVA solution, C: Volume of PVA solution, D: Volume of DCM, E: Rotation speed, F: Temperature, G: Amount of PVA. PVA: Polyvinyalcohol, MCH: Minocycline hydrochloride, DCM: Dichloromethane, S/N: Signal-to-noise

the S/N ratio, an ANOVA should be performed to estimate the variance and specify the relative significance of the different factors. As shown in Table 6, the preparation method and pH of the PVA solution are the most important factors that significantly affect the EE of MCH microspheres.

In vitro release of minocycline from PLGA microspheres

Figures 3A-C depict the release profile of MCH from microspheres, which were made of different PLGA types. The release of MCH from the microspheres was investigated up to 96 h. In the PLGA (50:50) and PLGA (75:25) microspheres, the release rate of MCH was higher in the initial 48 h and became

Table 3. Taguchi's L_{18} orthogonal array design, encapsulation efficiency of microspheres for each type of PLGA and S/N ratio for encapsulation efficiency (mean ± SD; n=3)

Exp. no	Controllable factors								Encapsulation efficiency %				S/N ratio
	A	B	C	D	E	F	G	H	PLGA (50:50)	PLGA (75:25)	PLGA (85:25)	Average	
1	1	1	1	1	1	1	1	1	#	#	#	#	#
2	1	1	2	2	2	2	2	2	13.60±0.62	12.3±0.36	12.5±0.23	12.8±0.70	22.1
3	1	1	3	3	3	3	3	3	10.30±0.39	10.4±0.33	9.27±0.35	9.98±0.61	19.9
4	1	2	1	1	2	2	3	3	18.70±0.35	20.3±0.49	16.2±0.53	18.4±2.09	25.2
5	1	2	2	2	3	3	1	1	13.60±0.22	16.7±0.64	12.27±0.34	14.18±2.27	22.8
6	1	2	3	3	1	1	2	2	11.20±0.24	14.8±0.66	8.98±1.05	11.5±3.31	20.5
7	1	3	1	2	1	3	2	3	14.60±0.58	14.5±0.50	8.84±0.57	12.6±3.28	21.3
8	1	3	2	3	2	1	3	1	14.60±0.29	15.5±0.34	10.5±0.16	13.5±2.68	22.2
9	1	3	3	1	3	2	1	2	16.10±0.37	21.3±1.33	17.6±1.12	18.3±2.67	25.1
10	2	1	1	3	3	2	2	1	3.78±0.35	3.24±0.07	3.93±0.19	3.66±0.38	11.2
11	2	1	2	1	1	3	3	2	#	3.19±0.06	#	3.19±0.06	5.65
12	2	1	3	2	2	1	1	3	1.50±0.05	4.61±0.33	4.54±0.20	3.55±1.77	15
13	2	2	1	2	3	1	3	2	3.44±0.21	4.85±0.10	5.11±0.46	4.47±0.89	12.6
14	2	2	2	3	1	2	1	3	5.65±0.41	5.64±0.06	4.76±0.81	5.35±0.51	14.5
15	2	2	3	1	2	3	2	1	#	3.98±0.20	3.84±0.22	3.91±2.26	11.8
16	2	3	1	3	2	3	1	2	#	5.01±0.38	5.07±0.18	5.04±2.91	14.1
17	2	3	2	1	3	1	2	3	5.74±0.37	5.32±0.18	4.84±0.31	5.3±0.45	14.4
18	2	3	3	2	1	2	3	1	5.42±0.17	7.19±0.50	2.72±0.17	5.11±2.24	12

#: Microsphere does not form, A: Preparation method, B: pH of PVA solution, C: Volume of PVA solution, D: Volume of DCM, E: Rotation speed, F: Temperature, G: Amount of PVA, PLGA: Poly(D,L-lactide-co-glycolide), S/N: Signal-to-noise, PVA: Polyvinyalcohol, DCM: Dichloromethane, SD: Standard deviation

Table 4. S/N ratio of initial and optimized preparation conditions

	Level	S/N ratio (db)
Initial	$A_2/B_2/C_2/D_2/E_2/F_2/G_2$	17.72
Predicted	$A_1/B_3/C_2/D_2/E_2/F_2/G_3$	27±6.57
Experimental	$A_1/B_3/C_2/D_2/E_2/F_2/G_3$	23.42

Improvement of S/N ratio: 5.76 dB, S/N: Signal-to-noise

Table 5. Response table for S/N ratio values for encapsulation efficiency by factor level

	Level	S/N Ratio (db)
Initial	$A_2/B_2/C_2/D_2/E_2/F_2/G_2$	17.72
Predicted	$A_1/B_3/C_2/D_2/E_2/F_2/G_3$	27±6.57
Experimental	$A_1/B_3/C_2/D_2/E_2/F_2/G_3$	23.42

Improvement of S/N ratio: 5.76 dB, S/N: Signal-to-noise

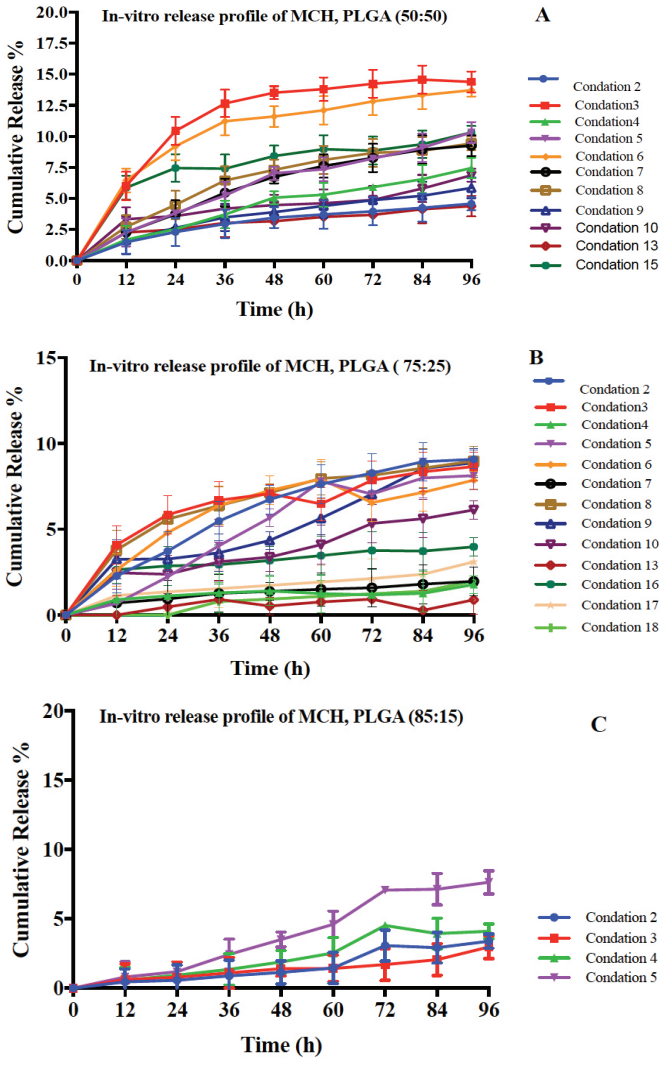

Figure 3. *In vitro* release profile of minocycline hydrochloride in PBS medium pH: 7.4 (mean ± SD; n=3). A: *In vitro* release profile of minocycline hydrochloride form microspheres made of PLGA (50:50), B: *In vitro* release profile of minocycline hydrochloride form microspheres made of PLGA (75:25), and C: *In vitro* release profile of minocycline hydrochloride form microspheres made of PLGA (85:15). MCH: Minocycline hydrochloride, PBS: Phosphate buffered saline, PLGA: Poly(D,L-lactide-co-glycolide), SD: Standard deviation

slower but steady thereafter. Furthermore, many PLGA (85:15) microspheres (Figure 3C) did not show any detectable drug release within 96 h. Release kinetics is well described by the Higuchi's equation, indicating that drug released from the microspheres is diffusion limited.

Optimizing all the parameters of the microsphere preparation methods require to achieve a maximum EE for MCH-loaded PLGA microspheres using the classic method, which is time-consuming and costly.[18] As such, Taguchi's orthogonal array design was employed to investigate the optimal conditions required for the production of microspheres with the highest percentage of EE and to establish a robust preparation method that could steadily provide MCH microspheres regardless of used polymer types. Taguchi's design is meant for investigating the influence of seven independent parameters, each having three-level values.

As shown in Table 3, the mean EE for PLGA (50:50), (75:25), and (85:15) over the 18 experimental runs were in the range of 18.70-1.50%, 21.3-3.1%, and 17.6-2.7%, respectively. Microspheres were not formed in experimental runs 1, 11, 15, and 16 for PLGA (50:50) and in experimental run 11 for PLGA (85:15). Microspheres with the highest EE (18.70%, 21.3%, and 17.6%) were obtained from experimental runs 4 and 9 for PLGA (50:50), (75:25), and (85:15), respectively.

The data obtained from the EE assessment revealed that the microspheres prepared by the S/O/W solvent evaporation method had a much higher EE in comparison to those by the co-solvent method. MCH is a hydrophilic and weak basic drug and shows high solubility in alcohols due to its ability to form hydrogen bonds with solute molecules.[22] Furthermore, the miscibility of methanol with water and increasing solubility of MCH are likely to have resulted in a diffusional loss of MCH in the aqueous phase.[17] Thus, the microspheres prepared via the co-solvent method had a low EE.

Furthermore, the Minitab software was used for the statistical analysis of the L18 OA design results.[23] The response table includes the mean S/N ratio for each level of the parameters and ranks based on the delta value, which shows the relative importance of effects (Table 5), and the parameters are

Table 6. ANOVA results for signal-to-noise ratio for encapsulation efficiency

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	f value	p value
Preparation method	1	383.219	84.66%	342.381	342.381	2116.71	0.001
Volume of PVA solution	2	17.885	3.95%	7.166	3.583	22.15	0.016
Amount of PVA (g)	2	1.541	0.34%	3.852	1.926	11.92	0.037
Temperature	2	17.135	3.79%	16.243	8.121	50.21	0.005
Volume of DCM (mL)	2	2.230	0.49%	0.701	0.351	2.17	0.261
pH of PVA solution	2	20.374	4.50%	18.310	9.155	56.60	0.004
Rotation speed (rpm)	2	9.814	2.17%	9.814	4.907	30.34	0.010
Error	3	0.485	0.11%	0.485	0.162	-	-
Total	16	452.683	100.00%	-	-	-	-

DF: Degrees of freedom, Seq SS: Sequential sums of squares, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares, PVA: Polyvinyalcohol, DCM: Dichloromethane

arranged according to their importance. In the main effect plot (Figure 2), the relatively horizontal lines indicate that the parameter has fewer effects on the response. Therefore, the factors with the highest gradient lines may have the greatest impact. As shown in Figure 2, parameter A (preparation method) is the most significant factor, whereas parameter D (volume of DCM) has no significant effect. The ANOVA results for the selected model outlined in Table 5 show that the preparation method with a p value of 0.001 and maximum contribution of 84.66% has the most significant effect on the EE.

The release profile of MCH-loaded into PLGA 50:50, 75:25, and 85:15 microspheres were investigated for 96 h, and the results are presented in Figure 3. The results indicate that only 14.5%, 8.9%, and 7.6% of the total drug load had been released from the microspheres prepared from PLGA 50:50, 75:25, and 85:15, respectively. This case could be associated with the polymer composition as the most important factor responsible for the hydrophobicity and rate of degradation of a delivery matrix.[24] The hydrolytic degradation of PLGA is related to the lactide/glycolide ratio, end group (ester or free carboxyl group), and molecular weight of the polymer.[25]

CONCLUSION

Although the interaction between the selected factors in the evaluation was ignored, the orthogonal array DOE on the basis of Taguchi's robust experimental design methodology was sufficient to optimize the process parameters for the PLGA microspheres of MCH. The microsphere preparation method (S/O/W) was the main factor affecting the EE. Microspheres prepared via the S/O/W method exhibited higher EEs and faster drug release than microspheres prepared via the co-solvent method. The pH and volume of the aqueous phase were also effective parameters on the EE. The release of minocycline was the fastest with PLGA (50:50) microspheres, followed by PLGA (75:25) and PLGA (85:15) microspheres in this order. A robust experimental design was successfully applied to the optimization of process parameters for MCH-loaded PLGA microsphere preparation.

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ORIGINAL ARTICLE

A Rapid, Precise, and Sensitive LC-MS/MS Method for the Quantitative Determination of Urinary Dopamine Levels *via* a Simple Liquid-liquid Extraction Technique

Basit Sıvı-sıvı Ekstraksiyon Tekniği ile İdrar Dopamin Düzeylerinin Kantitatif Tayini için Hızlı, Kesin ve Hassas Bir LC-MS/MS Yöntemi

Dilek BATTAL[1]*, Ayça AKTAŞ SÜKÜROĞLU[2], Fehmi Burak ALKAŞ[3], İrfan ÜNLÜSAYIN[4]

[1]Mersin University Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Mersin, Turkey
[2]Gazi University Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Ankara, Turkey
[3]Near East University Faculty of Pharmacy, Department of Toxicology, Nicosia, Cyprus
[4]Acıbadem Lab Med Research and Development Laboratory, İstanbul, Turkey

ABSTRACT

Objectives: Dopamine (DA) is a prominent biochemically complex neurotransmitter and immunomodulator. The quantification of DA could contribute to a better understanding of how endocrine system, cardiovascular and renal functions are regulated. The study aims to develop a rapid, precise, and extremely sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for routine clinical quantification of DA in urine.

Materials and Methods: Urine samples were extracted *via* one simple and rapid liquid-liquid extraction technique; then analyzed using a sensitive LC-MS/MS method developed by multiple reaction monitoring mode.

Results: DA and internal standard (IS) retention durations were found to be 2.28 min and 2.24 min, respectively. The mean extraction recovery of DA and DA-IS in urine was above 95.62%. DA calibration curve in urine was linear (r^2≥0.998) ranging from 20 ng/mL to 1000 ng/mL. The maximum intra-day and inter-day precisions were 5.87 and 2.81, respectively and coefficients of variation were 10.55% and 7.57%, respectively.

Conclusion: A rapid, precise, sensitive and quantitative LC-MS/MS detection of DA without the use of derivatization, evaporation, reconstitution and ion-pairing reagents has been developed with a simple and non-invasive sample technique for clinical laboratory applications, basic neuroscience research and drug development studies.

Key words: Dopamine, LC-MS/MS, urine, method validation

ÖZ

Amaç: Dopamin (DA), biyokimyasal olarak kompleks önemli bir nörotransmitter ve immünomodülatördür. DA'nın kantitatif tespiti, endokrin sistem, kardiyovasküler ve renal fonksiyonların düzenlenmesinin daha iyi anlaşılmasını sağlar. Bu çalışmanın amacı, idrarda DA'nın rutin klinik kantitasyonu için hızlı, kesin ve son derece hassas olan sıvı kromatografisi-tandem kütle spektrometresi (LC-MS/MS) yöntemi geliştirmektir.

Gereç ve Yöntemler: İdrar numuneleri, basit ve hızlı bir sıvı-sıvı ekstraksiyon tekniği ve ardından çoklu reaksiyon izleme modu kullanılarak geliştirilen hassas bir LC-MS/MS yöntemi ile hazırlanmıştır.

Bulgular: DA ve internal standardın alıkonma zamanları sırasıyla 2,28 ve 2,24 dakika olarak bulunmuştur. İdrarda DA ve DA-IS'nin ortalama ekstraksiyon geri kazanımı %95,62'nin üzerinde belirlenmiştir. İdrardaki DA kalibrasyon eğrisi, 20 ila 1000 ng/mL arasında doğrusaldır (r^2≥0,998). Maksimum gün içi ve günler arası kesinlik sırasıyla 5,87 ve 2,81 ve varyasyon katsayıları sırasıyla %10,55 ve %7,57'dir.

Sonuç: Türevlendirme, buharlaştırma, yeniden yapılandırma veya iyon eşleştirme reaktifleri kullanılmadan, DA'nın hızlı, hassas ve kantitatif LC-MS/MS yöntemi ile tespiti, invazif olmayan numune ve temel nörobilim ve ilaç geliştirme çalışmaları gibi klinik laboratuvar uygulamaları için kolay hazırlama tekniği ile geliştirilmiştir.

Anahtar kelimeler: Dopamin, LC-MS/MS, idrar, yöntem validasyonu

***Correspondence:** dilekbattal@mersin.edu.tr, **Phone:** +90 505 475 56 11, **ORCID-ID:** orcid.org/0000-0003-1935-5330

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INTRODUCTION

Dopamine (DA) is a basic chemical neurotransmitter with many neurological functions, particularly in the brain (Figure 1). The reward response is intricately linked to DA pathways, otherwise known as dopaminergic pathways.[1] Dopaminergic pathways are commonly activated in response to or in anticipation of reward. DA is assumed to significantly influence motivation and reward-associated satisfaction.[2] The relationship between dopaminergic signaling and reward response has considerable crosstalk, if not a direct influence, on dopaminergic signaling in a vast range of psychotropic pharmaceuticals and illicit drug substances. Therefore, dopaminergic system abnormalities may cause a wide variety of neurological disorders. The neurological and non-neurological syntheses of DA are considered to be largely independent of each other because it is a poor penetrator of the blood-brain barrier.[3] The majority of blood DA is considered to be synthesized in the mesentery or obtained from food digestion, with about 95% of it circulating as the biologically inactive DA sulfate.[4] Thus, unconjugated "(free)" DA is primarily responsible for the biological activity of blood DA. Such activities include vasodilation and noradrenaline release inhibition. There are indications, however not well illuminated, that DA may be released into the bloodstream in response to hypoxic conditions.[5] The immune system is also responsive to DA, with lymphocytes being the most affected. Some of these cells may synthesize and release DA themselves. Although the function of DA in these cells is unclear, it is considered to be an integral component of immunogenetics through lymphocyte activation modulation.[6,7] The possibility for interactions between the nervous and immune systems through DA has been touted as a potential route of interaction between the two systems, with malfunctions of this interaction linked to autoimmune disorders.[7,8]

In the detection and continued monitoring of relatively recent xenobiotic exposure, urine retains its position as the primary matrix of choice owing to several advantages, including its relatively wide temporal envelope of detection, which can last for several days, an increased xenobiotic concentration due to its nature as a concentrating waste carrier, and the ease and non-invasiveness of sample procurement; the patient urinating into a sterile container. Using the aforementioned non-invasive techniques, urine can be used to detect DA in the diagnosis and continued surveillance of several diseases, including stress-induced diseases and sympathoadrenal system dysfunction. Urine is chemically simpler than comparable detection media, obviating the need for tedious, effort-intensive, and complicated preparatory steps. Thus, the sample pretreatment was as simple as dilution in a micellar solution, followed by filtration, and then direct injection.

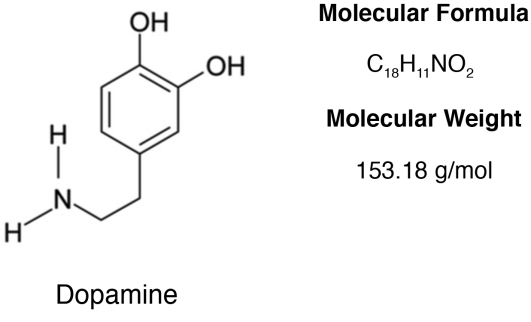

Figure 1. Chemical structure of dopamine

There are many published studies on the analysis of DA and associated species in biological fluids. This creates a landscape containing a variety of methods, including spectrophotometry,[9,10] liquid chromatography (LC)-fluorometry,[11,12] enzyme immunoassays, and LC-electrochemical detection for detecting and quantifying DA.[13-16] LC- tandem mass spectrometry (MS)-based methods are considered the frontrunner because tandem LC-MS methodologies can provide increased selectivity while relying on chromatographic separation only minimally.[17-26] While these methods provide a good combination of selectivity, sensitivity, and ease of use, they are ill-equipped to provide the same quality of output for multitarget analysis, which forms the basis of the present study, thus necessitating the development of novel methods. The simultaneous generation of optimal results for all relevant analytes necessitates substantial method modification and optimization. As a result of these modifications, the method discussed herein has achieved optimal quality in terms of sensitivity, selectivity, and robustness as it applies to DA detection.[27]

The simplicity, robustness, sensitivity, and specificity aspects were the major consideration criteria for this study, with the main objective being to develop an LC-MS/MS method that incorporates and meets all of these conditions. A cost-effective solution was also developed, with a simplified and highly effective liquid-liquid extraction step using a small sample volume, which will be invaluable for routine clinical testing.

Despite its superiority in both selectivity and specificity over other alternatives, an LC-MS/MS platform has not been extensively developed in the past to the degree discussed herein, to the best of the authors' knowledge.[13,14] Thus, this study also serves as a testbed for demonstrating and establishing the method discussed herein in terms of applicability and reproducibility to a larger sample population, as well as the scarcely-discussed clinical applications.[28]

MATERIALS AND METHODS

Chemicals and materials

Analytical grade chemicals were used in all steps of the process. Artificial urine, DA, and creatinine were procured from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_{12}$-DA (99%) and creatinine-d3 standards (both isotopically labeled) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). High performance liquid chromatography (HPLC)-grade methanol, formic acid, and hydrochloric acid fuming 37% were obtained from Merck (KGaA, Darmstadt, Germany). All aqueous solutions were made with deionized water (18.2 MΩ) treated with a Millipore (Simplicity, 185) Milli-Q water purification system (Elga Labwater Veolia, Anthony, France).

Preparation of calibration standards and quality control (QC) samples

Internal standard (IS) solution

To begin, a dilution solution (DS) was prepared using 50 mL of 1N hydrochloric acid and 1 L of water. After that, a stock solution (500 µg/L) was prepared by diluting 5 mg of DA-*d4* IS with the DS. Next, 0.1 Ml of stock solution was diluted with 10 Ml of the DS for a dilute stock solution (5 ng/mL). Finally, a 200 µL of dilute stock solution (100 ng/mL) diluted with 10 Ml of the DS was employed as an IS solution to be used in the analyses.

Standard solutions

50 mg of DA was diluted with 10 mL DS (4000 ng/mL). To prepare the stock standard solution (100 ng/mL), 200 µL of 5000 ng/mL solution was diluted with 10 Ml of the DS. For the intermediate standard stock solution (2000 µg/L), 100 µL of 100 ng/mL solution was diluted with 10 mL of the DS. Preparation of stock standard solution levels are presented in Figure 2.

Sample pretreatment

The sample was vortex for 3-4 minutes after mixing 200 µL of the IS solution (100 µg/L), 800 µL of the DS, and 100 µL of the artificial urine sample.

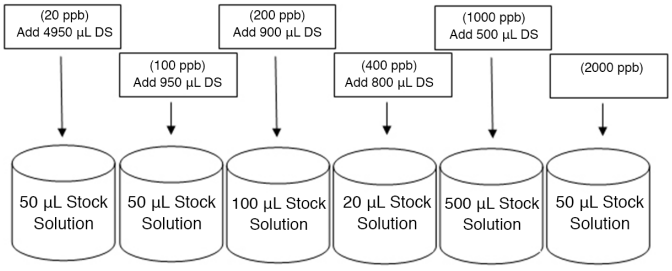

Figure 2. Preparation for stock standard solution levels

LC-MS/MS conditions

Agilent 1200 Series 6460 triple quadrupole mass spectrometry with Jet-Stream atmospheric pressure electrospray ionization source and MassHunter data acquisition/Quantitation software (Santa Clara, USA) were used in the LC system. Chromatographic separation was performed on a Zorbax SB-C18 3.0x50 mm 3.5-micron 600 BAL. The mobile phases comprised 50% formic acid aqueous and methanol (HPLC gradient grade). The flow rate was set to 5 mL/min. The injection volume was set at 40 µL. MS was conducted on an Agilent triple quadrupole mass spectrometer operated in selected reaction monitoring (SRM) mode. The parameters for chromatographic conditions were set up as follows: Capillary voltage: P (1750 V) N (3000V); desolvation gas: 325 L/h; desolvation gas temperature: 375˚C; cone gas: 12 L/min; Nebulizer: 40 psi; Nozzle voltage: 0-0; Chamber current: 0.24 µA; LC stop time: 5.50 min; SRM transitions were monitored at m/z 158.10→141.10 for DA IS (positive) and at m/z 154.0→137.00 for DA (positive). For each analyte, dwell time was set at 150 *ms*.

Method validation

The validation of the LC-MS/MS method was based on the Food and Drug Administration Guidance for Industry on bioanalytical method validation[27] (selectivity, carryover, linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ), accuracy, precision, matrix effect, extraction recovery, and stability). Artificial urine was used throughout the validation process owing to a scarcity of genuine blank urine samples without all targeted analytes.

Preparation of calibration curve

The linearity of the DA method was quantified with a calibration curve constructed in the range of 20-2000 ng/mL (Figure 3), which included the LLOQ. The acceptance criterium for recalculated standard concentrations was not more than 15% of the nominal values and 20% in the LLOQ. Each validation run

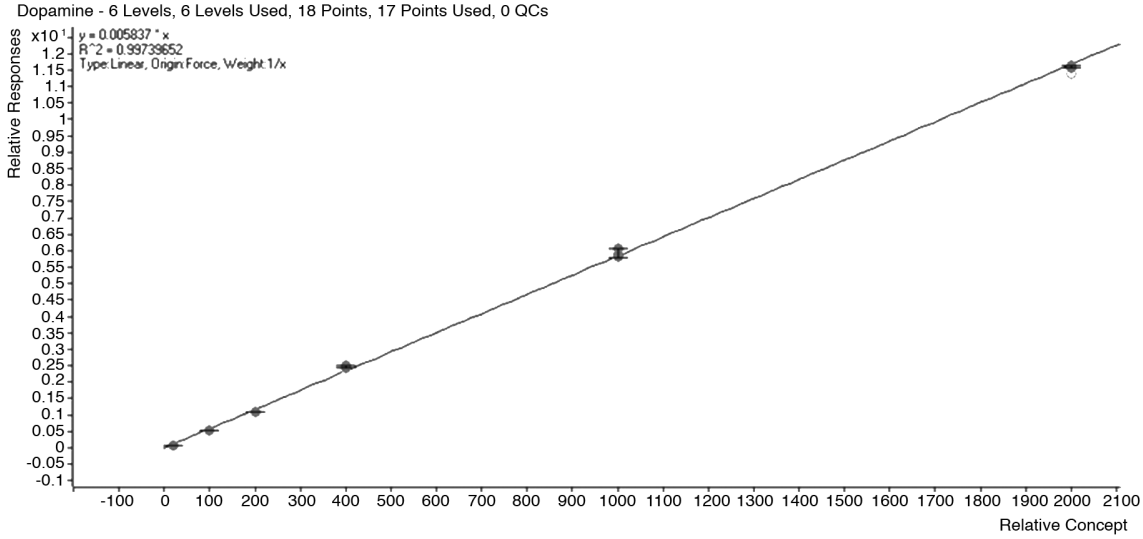

Figure 3. Calibration curve of dopamine

comprised QC samples at three concentrations (n=6, at each concentration). Such validation studies should be completed in three consecutive days.

Accuracy and precision

The accuracy and precision studies should be evaluated and reported as intra-day/within-run accuracy and between-run accuracy with a single injection. Intra-day and inter-day accuracy and precision should be determined using quality control samples at each level of at least five samples and at least four different concentration levels of DA at low-quality control (LQC: 200 ng/mL), medium-quality control (MQC: 400 ng/mL), and high-quality control (HQC: 100 ng/mL) samples, including LLOQ (100 ng/mL). The mean concentration value did not exceed 15%, except at LLOQ of the nominal concentration (20%), and the coefficient of variation (CV %) values was <15% above the calibration range.

Selectivity

The analysis matrix was examined as 6 different lots, the CV % did not exceed 20% of the LLOQ in terms of the substance to be analyzed, and the IS was not affected as it did not exceed 5% of the IS response.

Recovery

By equating the peak area of each analyte, the recovery of DA, six concentration levels, and IS in artificial urine was determined

Stability

The stability of DA was determined using all six replicate samples (LLQC, LQC, MQC, and HQC) stored at +4˚C for one week. The acceptance stability criterion did not exceed 15% of the nominal concentration.

Matrix effect

The matrix effect for DA and its IS is the ratio of the response of the metabolite to be analyzed, added at certain concentrations to six independent blank matrices, to the response of the pure standard solution of the same concentration in the analysis after extraction. The CV % of the matrix factors obtained for 6 different matrices did not exceed 15%.

Statistical analysis

The data were processed using "SPSS v.22". For accuracy, precision, stability, and matrix effect, the results were calculated as mean ± standard deviation (SD), and the relative SD (CV %). The coefficient of regression was also calculated for the linearity parameter.

RESULTS

Selectivity and optimization of chromatographic conditions

To obtain and evaluate the selectivity of the method, six different artificial urine matrices were employed and tested. Subsequently, the interference at the analyte and the IS retention times were also quantified. In the aforementioned chromatographic parameters, DA and IS separation from the artificial urine were both found to be adequate, with retention times of ~2.09 min and ~1.08 min, respectively (Figure 4). The retention time corresponding to DA and the IS showed no significant interference peaks (Figure 5). The results showed that the method developed in this study is highly specific and selective for DA in urine samples.

Linearity, LOQ, and LOD

For DA, the method was validated over the nominal concentration range of 20 ng/mL-2000 ng/mL (Figure 3). For the batch, the correlation coefficient (r) value was 0.998 and the equation was y=0.005938* x-0.053491. With adequate sensitivity and accuracy, LOQ and LLOD were chosen as a 100 µg/L (level 2) subcalibration point for DA. The LOQ and LOD of DA were 1.215 ng/mL and 0.36 ng/mL, respectively.

Precision and accuracy

The maximum values of intra-day and inter-day precisions (RSD %) were 5.87% and 2.81%, respectively (Table 1). In addition, the intra-day and inter-day CV % maximum levels were 10.55% to 7.57% (Table 2) respectively, indicating that this method was accurate and precise for DA quantification in urine.

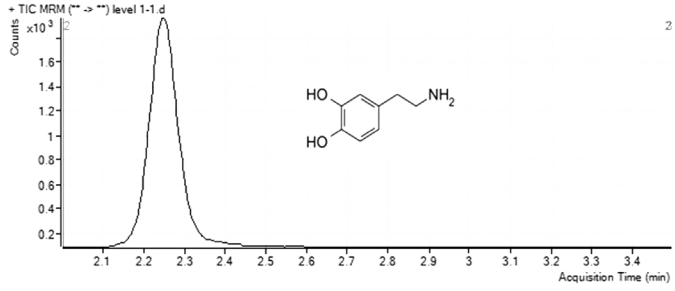

Figure 4. Representative chromatogram of dopamine in urine

Table 1. The intra-day and inter-day precision values of the dopamine in artificial urine (n=5)

		Intra-day precision		Inter-day precision	
	Nominal concentrations (ng/mL)	Concentration found (mean ± SD, ng/mL)	*RSD %	Concentration found (mean ± SD, ng/mL)	*RSD %
LLQC	100	96.45±2.92	3.03	106.519±2.42	2.27
LQC	200	196.29±11.53	5.87	216.943±5.01	2.31
MQC	400	403.63±14.04	3.47	440.063±10.61	2.41
HQC	1000	954.36±42.89	4.48	1058.594±29.75	2.81

*RSD %: The intra-day and inter-day precisions, SD: Standard deviation, LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

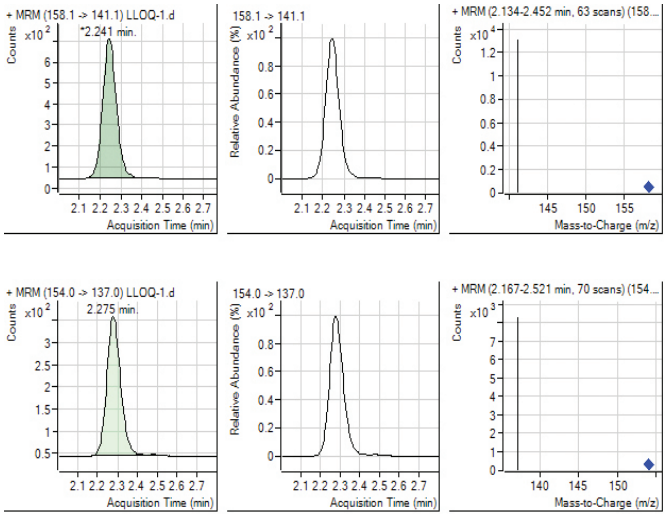

Figure 5. Dopamine and the internal standard peaks

Extraction recovery and matrix effects

The extraction recovery results were listed in Table 3 and found to be within the range of 95.622-106.147%.

Matrix effects of DA are shown in Table 4. The matrix effect values obtained were matrix 1 (CV %, 1.5%), matrix 2 (CV %, 5.5%), matrix 3 (CV %, 2.7%), matrix 4 (CV %, 7.2%), matrix 5 (CV %, 4.3%), and matrix 6 (CV %, 14.3%). It has been determined that there was no effect of urinary matrix variability in the presence of IS in DA quantification and the CV% value was less than the acceptance criterion for matrix effect (15%) as shown in Table 4. DA and IS did not show a matrix effect in urine.

Stability

Six replicates of DA samples were measured for stability using freeze-thaw cycles (frozen 7 days at -20˚C). The CV % did not exceed 15%, proving the freeze–thaw stability of DA in artificial urine (Table 5).

Table 2. The intra-day and inter-day accuracy values of the dopamine in artificial urine (n=5)

		Intra-day		Inter-day	
	Nominal concentrations (ng/mL)	Concentration found (mean ± SD, ng/mL)	*CV %	Concentration found (mean ± SD, ng/mL)	*CV %
LLQC	100	91.42±2.69	8.58	106.39±3.09	6.38
LQC	200	186.14±5.18	6.92	215.16±4.76	7.57
MQC	400	379.10±5.81	5.22	427.51±2.57	6.87
HQC	1000	894.49±19.10	10.55	1064.594±18.10	6.40

*CV %: Accuracy, coefficient of variation, SD: Standard deviation, LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

Table 3. Extraction recoveries of spiked artificial urine samples

Spiked urine standards (n=6)	*Extraction recoveries			Recoveries (mean ± SD)	CV %
1	101.38	98.39	103.83	101.20±2.22	2.17
2	96.84	95.24	94.79	95.62±0.87	0.90
3	97.91	96.91	97.37	97.39±0.40	0.40
4	105.54	105.13	107.78	106.15±1.16	1.09
5	99.99	103.29	99.11	100.80±1.79	1.77
6	97.94	96.66	98.57	97.73±0.79	0,80

*Extraction recovery (%): [(Response of extracted sample/response of post extracted spiked sample) x 100]. CV: Coefficient of variation, SD: Standard deviation

Table 4. The matrix effect of dopamine

Number of spiked samples	Matrix response results (n=6, %)	Correlation coefficient %
Matrix-1	107.00	5.63
Matrix-2	101.80	
Matrix-3	106.46	
Matrix-4	103.94	
Matrix-5	98.15	
Matrix-6	115.69	

Table 5. Stability of dopamine in artificial urine

	Nominal concentration (ug/L)	Concentration found (mean ± SD, ng/mL)	Correlation coefficient %
LLQC	100	88.05±5.51	7.01
LQC	200	188.13±5.89	3.51
MQC	400	395.86±4.87	1.38
HQC	500	987.40±17.76	2.01

SD: Standard deviation, LQC: Low-quality control, MQC: Medium-quality control, HQC: High-quality control

DISCUSSION

With further research clarifying the specific role of DA in the development and maintenance of these systems, urine DA testing could become a mainstay regular test in clinical settings, in addition to neurological analyses. Such testing may also provide unique insight into the biochemical processes behind the disorders in such systems, providing for a better understanding of how such systems develop, maintain, and sustain themselves, as well as their pathological situations.

By measuring DA concentration in other bodily fluids, such as the cerebrospinal fluid (for which this study will be of pioneering significance in terms of method development), neurological disorders related to DA biosynthesis or utilization can also be characterized. Theoretically, such novel techniques may allow for a more effective evaluation of illnesses treatment, as well as early-stage diagnosis of these illnesses.

The method achieved functional sensitivity requirements and quantified analytes over a wide dynamic range. Precise quantification of DA concentrations in urine could help researchers better understand the pathophysiology and pathogenesis of many neuropsychiatric disorders [e.g., drug addiction, schizophrenia, Parkinson's and Alzheimer's disease, and attention deficit hyperactivity/hyperkinetic disorder (ADHD)] and pharmaceutical research on novel drugs. The method developed in this study demonstrates its viability for determining DA levels in urine. The complexity of methodology is greatly reduced by simple precipitation and dilution directly leading to direct injection without intervening steps, a feat that can be considered the primary strength of this procedure, giving it an advantage over its alternatives. Previously published methodologies all have long and tedious sample preparation and derivatization steps, making them unsuitable for high-throughput applications (for example; a busy clinical laboratory serving many patients at the same time). These drawbacks are eliminated in the method described herein, with its simple and straightforward sample preparation and a short-duration chromatographic run, which makes it more applicable and desirable in such high-volume operations (Table 6).

In terms of its speed, reliability, quantitative potency, and cost-effectiveness, the method developed and validated in this study is an improvement over the microextraction methodology developed and employed by El-Beqqali et al.[19] Such advantages are particularly prevalent in a clinical setting, where fast and reliable detection of low DA levels in biological matrices may be crucial.[28] Moriarty et al.[8] developed an SPE/LC-MS/MS method for urinary DA detection in the diagnosis of ADHD. Li et al.[28] discovered that using solid-phase extraction (SPE) can have unforeseen and detrimental effects on the results obtained through LC-MS/MS analyses. The proposed method, by utilizing liquid-liquid extraction, demonstrably prevents the aforementioned problems. The selectivity and specificity attained in the method discussed herein significantly exceed those reported by Woo et al.[32], who utilized SPE as the extraction technique.[32,38,39] Significantly improved analytical reliabilities can be achieved by eliminating variance related to the extraction and analysis methodologies, allowing for widespread implementation of such methods in relevant fields. To prove our point, Zhanga et al.[38] and van de Merbel et al.[39] used SPE to isolate DA from blood plasma. Catecholamines, such as DA, are unstable in alkaline conditions, such as blood, which is a cause of concern. Thus, acidification and antioxidant addition were used to treat the extracts to provide a more amenable and permissive environment for inhibiting DA degradation in such matrices. Our method, which makes use of the naturally acidic urine, prevents these pH-associated effects and allows DA concentrations to be preserved considerably more effectively. When urine is used as the biological matrix of analysis, pH-related effects and their mitigation become a more manageable, if not outright ignorable, inconvenience.

This study aims at demonstrating that a high-speed, low-complexity, robust, sensitive, and specific LC-MS/MS method for urinary DA analysis and quantification can be developed at an affordable rate. To ensure extensive and repeatable reliability, this method was validated following standard laboratory protocols and guidelines. During the validation steps, artificial urine was used. The development of the assay was made possible by the robustification of the method discussed herein, which allowed for the emergence of novel features, such as easy sample preparation, rapid LC-MS/MS detection of DA without the use of derivatization, evaporation, and reconstitution, as well as the use of ion-pairing reagents.

Table 6. Comparison of the proposed method with previous studies

Analyte	Linearity (ng/mL)	LLOQ (ng/mL)	Sample preparation	Equipment	Reference
NE	10-210	6.0	LLE	LC-MS/MS	Diniz et al.[29]
E	3.0-53	1.0	-	-	-
DA	15-1015	11	-	-	-
NMN	30-2130	8.0	-	-	-
MN	20-1420	4.4	-	-	-
NE	10-10000	10	LLE	ESI-MS/MS	Kushnir et al.[24]
E	2.5-10000	2.5	-	-	-
DA	2.5-10000	2.5	-	-	-
NE	1.69-203	1.69[a]	SPE (Bond Elut Plexa)	LC-MS/MS	Whiting[30]
E	1.83-110	1.83[a]	-	-	-
DA	0.77-306	0.77[a]	-	-	-
NMN	0.92-769	0.92[a]	-	-	-
NE	0.39-345	0.39	SPE (WCX µElution)	LC-MS/MS	Peitzsch et al.[31]
E	0.24-500	0.24	-	-	-
DA	0.49-100	0.49	-	-	-
NMN	0.24-125	0.24	-	-	-
MN	0.24-250	0.24	-	-	-
NE	7.4-2359	7.4	SPE (Strata-X-CW)	LC-MS/MS	Woo et al.[32]
E	3.8-2163	3.8	-	-	-
DA	5.4-2825	5.4	-	-	-
NMN	3.7-2569	3.7	-	-	-
MN	3.5-2466	3.5	-	-	-
NE	2.5-500	2.5	SPE (PBA-HLB plate)	LC-MS/MS	Li et al.[33]
E	0.25-250	0.25	-	-	-
DA	2.5-1000	2.5	-	-	-
NE	2.5-500	2.5	SPE (PBA-HLB µElution)	LC-MS/MS	Li et al.[34]
E	0.5-500	0.5	-	-	-
DA	2.5-1250	2.5	-	-	-
NMN	2.5-1250	2.5	-	-	-
NE	5-500	15	SPE (CAT-PBA)	HPLC	Rozet et al.[35]
E	5-500	5	-	-	-
DA	5-500	50	-	-	-
NE	1-150	1[a]	Bio-Rex	HPLC	Manickum[36]
E	3-50	3[a]	-	-	-
DA	3-625	3[a]	-	-	-
A	0.5-20	0.16	SPME	HPLC	Kossakowska et al.[37]
NA	0.25-20	0.08	-	-	-
DA	0.5-20	0.16	-	-	-
L-Tryp	0.25-20	0.09	-	-	-
L-Tyr	0.5-20	0.16	-	-	-
DA	50-4000	1.0	MEPS	LC-MS/MS	El-Beqqali et al.[19]
5-HT	50-4000	1.0	-	-	-
DA	20-2000	0.36[a]	LLE	LC-MS/MS	This study

[a]LOD value was used since LLOQ was not reported. NE: Norepinephrine, E: Epinephrine, DA: Dopamine, NMN: Normetanephrine, MN: Metanephrine, L-Tryp: L-Tryptophan, L-Tyr: L-Tyrosine, 5-HT: Serotonine, MEPS: Microextraction in the packed syringe, LC-MS/MS: Liquid chromatography-tandem mass spectrometry

Urine sampling, as a non-invasive and less expensive sample procurement approach, surpasses preexisting methods, in situations like clinical laboratories conducting neuroscience research or pharmaceutical companies conducting drug development since it is non-invasive and less expensive.

CONCLUSION

This study presents an LC-MS/MS-based methodology that eliminates derivatization, evaporation, reconstitution, and the use of ion-pairing reagents while having considerable speed and simplicity advantages. The proposed method also has the advantages of non-invasive sample procurement and a simplified preparation technique, which are useful in clinical laboratory applications, neuroscience research, and drug development studies.

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ORIGINAL ARTICLE

Determination of Pharmacists' Opinions about Collegial Solidarity

Meslektaş Dayanışması Konusunda Eczacıların Görüşlerinin Belirlenmesi

Bilge SÖZEN ŞAHNE[1]*, Elif ULUTAŞ DENİZ[2], Onur KABAŞ[3], Nilay TARHAN[4], Miray ARSLAN[5], Özlem AKBAL DAĞISTAN[6]

[1]Hacettepe University Faculty of Pharmacy, Department of Pharmacy Management, Ankara, Turkey
[2]Atatürk University Facult of Pharmacy, Department of Pharmacy Management, Erzurum, Turkey
[3]Yumuk Pharmacy, Sinop, Turkey
[4]İzmir Katip Çelebi University Faculty of Pharmacy, Department of Pharmacy Management, İzmir, Turkey
[5]Van Yüzüncü Yıl University Faculty of Pharmacy, Department of Pharmacy Management, Van, Turkey
[6]İstanbul University Faculty of Pharmacy, Department of Pharmaceutical Technology, İstanbul, Turkey

ABSTRACT

Objectives: Colleague solidarity, which emphasizes trust, independent thinking skills, and sharing, enables the problems encountered in the health service delivery to be dealt with effectively. This study aims to identify the current situation regarding colleague solidarity among pharmacists, which is also included in Turkey's pharmacy legislation.

Materials and Methods: "Colleague Solidarity Scale Among Nurses" was used in this study conducted with the questionnaire technique. The scale comprises of 23 items and was scored using a 5-point Likert scale. In addition, there were three demographic questions and six questions to get information from participants related to collegial solidarity in the questionnaire.

Results: As a result of the exploratory factor analysis (Kaiser-Meyer-Olkin: 0.837), three factors reported 51.029% of the total variance. The t-test indicated a significant difference between gender groups only in the negative opinions about solidarity (NOS) factor (p=0.000). Females exhibited more negative thoughts about solidarity. The ANOVA showed a significant difference in the academic solidarity (AS) factor (p=0.007) among the participants' works in community pharmacies and universities. Pharmacists working in universities had higher means in the AS factor. Moreover, the number of working years made significant differences in the emotional solidarity factor (p=0.000) and NOS factor (p=0.002). Additionally, it was found that the average responses in all factors of the participants who thought that they supported their colleagues in need and that solidarity with their colleagues increased significantly during the COVID-19 pandemic period ($p<0.05$).

Conclusion: The findings suggest that colleague solidarity among pharmacists should be addressed profoundly as an element specified in legislation and education processes. It is crucial to determine the level of colleague solidarity and improve it using this scale for different practice areas in pharmacy.

Key words: Colleague, solidarity, pharmacist, communication, health services

ÖZ

Amaç: Güven, bağımsız düşünme becerileri ve paylaşmayı ön plana çıkaran meslektaş dayanışmasının sağlık hizmet sunumunda karşılaşılan sorunların etkin bir şekilde ele alınmasını sağladığı belirtilmektedir. Bu çalışmanın amacı, Türkiye'de eczacılık mevzuatında da yer alan eczacılar arası meslektaş dayanışmasına ilişkin mevcut durumu ortaya çıkarmaktır.

Gereç ve Yöntemler: "Hemşirelerde Meslektaş Dayanışması Ölçeği"ni içeren bu çalışmada veri toplamak için anket tekniği kullanılmıştır. Ölçek, beşli Likert tipi ölçekle hazırlanan 23 maddeden oluşmaktadır. Ayrıca, ankette üç demografik soru ve katılımcılardan meslektaş dayanışması ile ilgili bilgi almak için altı soru yer almıştır.

Bulgular: Açımlayıcı faktör analizi sonucunda (Kaiser-Meyer-Olkin: 0,837), üç faktör toplam varyansın %51,029'unu bildirmiştir. T-testi, yalnızca dayanışma hakkında olumsuz görüşler (NOS) faktöründe cinsiyet grupları arasında anlamlı bir farklılık olduğunu göstermiştir (p=0,000). Kadınlar dayanışma konusunda daha olumsuz düşünceler sergilemişlerdir. ANOVA, katılımcıların serbest eczanelerde ve üniversitelerde yaptıkları çalışmalar arasında akademik dayanışma (AS) faktöründe (p=0,007) anlamlı bir farklılık olduğunu göstermiştir. Üniversitelerde çalışan eczacıların AS faktöründe

*Correspondence: bilge.sozen@hacettepe.edu.tr, Phone: +90 312 305 42 26, ORCID-ID: orcid.org/0000-0003-0746-8303
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ortalamaları daha yüksek olarak belirlenmiştir. Ayrıca, çalışma yılı sayısı duygusal dayanışma faktörü (p=0,000) ve NOS faktörü (p=0,002) arasında anlamlı farklılıklar yaratmıştır. Ayrıca koronavirüs hastalığı-2019 (COVID-19) pandemisi döneminde ihtiyaç sahibi meslektaşlarına destek olduklarını ve meslektaşlarıyla dayanışma içinde olduklarını düşünen katılımcıların tüm faktörlerdeki ortalama tepkilerinin COVID-19 pandemisi döneminde önemli ölçüde arttığı tespit edilmiştir (p<0,05).

Sonuç: Bulgular, eczacılar arasındaki meslektaş dayanışmasının mevzuat ve eğitim süreçlerinde belirtilen bir unsur olarak derinlemesine ele alınması gerektiğini göstermektedir. Eczacılıkta farklı uygulama alanları için bu ölçeği kullanarak meslektaş dayanışmasının düzeyini belirlemek ve geliştirmek çok önemlidir.

Anahtar kelimeler: Meslektaş, dayanışma, eczacı, iletişim, sağlık hizmeti

INTRODUCTION

Solidarity is reflected in a wide range of integrated interests from policy to health and is defined comprehensively as "Networks of social relationships that involve mutual dependencies, responsibilities, and entitlements within a defined group of people or a community".[1]

The word solidarity has been known since the early times. Historically, there have been many uses of the concept of solidarity, from Roman law to the French revolution.[2] According to İbni Haldun, individuals develop strong solidarity by uniting for certain reasons. Solidarity helps individuals in overcoming several difficulties and achieving their desired goals. On account of this solidarity, individuals tend to protect and defend each other, take a common stand on economic, social, and political issues, and take joint actions. It is the society wherein they live, which has intense solidarity, that makes people who are considered superior and successful, not their personal talents.[3]

Particular concepts, such as intergenerational solidarity,[4] social solidarity,[5] and gender solidarity,[6] can be found in the literature. Colleague solidarity is another important aspect related to solidarity. It includes supporting colleagues and sharing professional knowledge, technique, and skills. Additionally, it is related to developing professional knowledge and increasing professional skills.[7]

One of the important benefits of collegiality listed by Benshoff and Paisley[8] is the effect of enhancing the skills that help each other professionally. Similarly, Carroll[9] mentioned "the support and assistance of group members to each other helps group members to integrate with each other" in terms of solidarity.

It is said that colleague solidarity, which emphasizes trust, independent thinking skills, and sharing, enables the problems encountered in the health service delivery to be dealt with effectively. Furthermore, it is known to support a sense of belonging, open communication, cooperation, and support.[10]

Particularly in times of crisis, it can be seen that collaboration among colleagues is required, which makes it easier to overcome problems. Studies have shown that colleague solidarity affects factors, such as job satisfaction, job stress, and professional self-esteem. In addition, it is emphasized that colleague solidarity can be improved with applications in vocational education processes.[11] In healthcare sector, colleague solidarity is as important as the solidarity among different occupational groups owing to its close link with colleague relations. It is known that colleague interactions increase opportunities to speak and reflect, ensuring that everyone involved is aware of their knowledge and experience. Besides colleague relations, it also works when issues, such as patient health, are considered more important than one's personal ambitions.[12] In this context, there are some studies with healthcare professionals, especially with nurses, about colleague solidarity.

Gül and Bahçecik[13] performed a descriptive study with 297 nurses working in hospitals in İstanbul. An introductory information form, a colleague solidarity scale, and a job stress scale were used as data collection tools. This study reported high levels of collegiality and work stress among nurses.[13]

Furthermore, another study with nurses indicated that the more professional solidarity there is among nurses, the higher the job satisfaction. In addition, it was mentioned that generational differences should be considered to increase colleague solidarity and job satisfaction among working nurses.[14]

The professional solidarity of pharmacists in Turkey is based on the Turkish Pharmacists Deontology Regulation, as well as their professional practices.[10] The statement, which is specifically related to solidarity, reads, "Pharmacists establish good relations with their colleagues; they help each other materially and spiritually".[15] Collaboration with colleagues is also included in the National Pharmacy Core Education Program.[16]

Apart from these, there are various studies related to colleague solidarity in the fields of nursing and teaching.[17,18] However, collegiality among pharmacists has not yet been explored in both national and international literature. Therefore, within the scope of the planned study, the current situation regarding colleague solidarity among pharmacists, which is also included in the relevant legislation in our country, will be revealed.

MATERIALS AND METHODS

Measurement tool

In this study, the data were collected questionnaire. The questionnaire included the "Colleague solidarity scale among nurses," developed by Uslusoy and Alpay.[19] This scale comprises three factors, namely: (i) emotional solidarity (ES), (ii) academic solidarity (AS), and (iii) negative opinions about solidarity (NOS). The questionnaire contained 23 items and was scored using a 5-point Likert scale, ranging from (1) never to (5) always.

In addition, there were three demographic questions and six questions to obtain information from participants related to colleague solidarity in the questionnaire.

Sample size and data collection

The minimum sample size was calculated as 385; on 0.05 significance level, z: 1.96, d (sensitivity): 0.05, and p and q values being 0.5. To increase the reliability of the study results, we tried to reach the maximum number of individuals that could be reached. We were able to obtain data from 774 pharmacists working in community pharmacies, hospital pharmacies, public institutions, universities, and in the pharmaceutical industry in Turkey.

The current investigation was conducted between July 17, 2020 and September 12, 2020, after ethical approval was obtained from the Hacettepe University Ethical Committee, and Permit Number 35853172-050.06 was issued.

Statistical analysis

The first eight questions were analyzed using the descriptive statistics. Following this, the negative items on the scale were inverted. Subsequently, exploratory factor analysis (EFA) was performed to extract factors using IBM SPSS® Software version 22. After determining the factor structures, independent sample t-test and ANOVA tests were applied.

RESULTS

Table 1 presents the demographic characteristics of the participants (n=774). Table 2 displays the findings related to the importance of colleague solidarity to the participants.

Table 2 indicates that participants generally give positive answers to the questions about the importance they attach to colleague solidarity. When the participants were asked about the importance of colleague solidarity in problem solving, it was revealed that 40.2% of the participants found it important in improving the professional image, 29.2% in solving ethical problems, and 21.6% in improving the service quality. The participants were then asked about the subjects they communicate on, with their colleagues more frequently. It was found that 47.8% of the participants contacted their colleagues to learn about innovations in professional practices and 43.5% to find solutions to professional problems. Lastly, the participants were asked what could be effective in increasing colleague solidarity. Different answers were received. Upon evaluating the answers, it was found that approximately 70% of the participants emphasized that the scientific and social activities offered by the professional organizations could be effective. This was followed by the effective use of social media (25.6%).

As a result of EFA, a three-factor solution was obtained (Table 3) with the Kaiser-Meyer-Olkin measure 0.837. These three factors explained 51.029% of the total variance. This value proved the adequacy of the variance ratio.

The factors obtained from EFA were found to be the same as the factors in the scale developed by Uslusoy and Alpay.[19] Unfortunately, eight items were removed owing to low factor loading (less than 0.50). According to the calculated Cronbach's alpha internal consistency coefficients (Table 3), the factors of the scale demonstrated high reliability in the pharmacist population with this current form. Table 3 presents the mean values of the scale items. It can be seen that mean values are generally higher than 3.5. It was found that "Q5: I respect the personalities of my colleagues" had the highest mean response (4.726), and "Q20: I warn my colleagues regarding their lack of professional knowledge when I recognize it" had the lowest mean response (3.291).

Using the participants' factor loadings, t-test and ANOVA were conducted to investigate participant differences basis gender, place of duty, and number of working years. The results of the t-test showed that there was a significant difference between gender groups only in the NOS factor (p=0.000). Females displayed more negative thoughts about solidarity. According to

Table 1. Characteristics of the participants

	Frequency (%)
Gender	
Male	50.6
Female	49.4
Place of duty	
Community pharmacy	75.2
Hospital pharmacy	11.8
Public institution	3.5
University	5.3
Pharmaceutical industry	3.1
Others	1.1
Working years	
5 years and less	26.2
6-10 years	22.0
11-15 years	14.6
16-20 years	11.9
21-25 years	8.1
More than 25 years	17.2

Table 2. Participants' views on the importance of colleague solidarity

Questions	Frequency (%)	
	Yes	No
Do you get support from your colleagues in your professional practice?	85.3	14.7
Do you think you are supporting colleagues in need?	93.2	6.8
Do you think your solidarity with your colleagues increased during the COVID-19 pandemic period?	62.8	37.2

COVID-19: Coronavirus disease-2019

the ANOVA, the place of duty only made a significant difference in the AS factor (p=0.007). Since the group variances were homogeneous, the Tukey test was performed. Consequently, a statistically significant difference was found between the participants working in community pharmacies and universities. Pharmacists working in universities had higher means in the AS factor. Furthermore, the number of years they had been working showed significant differences in the ES factor (p=0.000) and the NOS factor (p=0.002). The Tukey test results revealed that these differences came from participants who had worked less than 5 years and others in the ES factors. Unlike this, in the NOS factor, differences came from participants who worked less than 5 years and 6-10 years, and 6-10 years and 16-20 years. In these two cases, it was determined that those with less working years had less negative views.

Moreover, the result of the t-test indicated that the participants who received support from their colleagues in their professional practices had statistically significantly higher mean answers in the ES (p=0.000) and the NOS (p=0.000) factors than those who did not. Additionally, it was found that the average responses in all factors of the participants who thought that they supported their colleagues in need and that solidarity with their colleagues increased during the coronavirus disease-2019 pandemic period were statistically significantly higher ($p<0.05$).

DISCUSSION

Solidarity is a concept emphasized in many international documents and is important in terms of sustainability.[20,21] This concept, which is also crucial in the health sector,[22,23] has been examined within the framework of colleague solidarity of pharmacists in the scope of this study.

Pharmacists are an essential part of the Turkish healthcare system. According to the latest reports of the Turkish Pharmacists' Association (TPA), approximately 57% of 39,377 pharmacists are female, and 43% are male.[24] This rate is approximately half of the pharmacists participating in this study. In addition, the same report noted that approximately 73.79% of pharmacists in Turkey work as community pharmacists.[24] Similarly, 75.2% of the pharmacists who took the questionnaire reported that they were working in community pharmacies.

When it comes to collegiality, helping colleagues in need is a key element.[19] In the study, 93.2% of pharmacists who participated in the survey thought that they supported colleagues in need, and 40.2% found colleague solidarity beneficial in improving their professional image. Nearly 30% of the participants found it helpful in solving ethical problems. Similarly, Arslan et al.[25] identified that support of colleagues in solving unethical issues is valuable for pharmacists.

Table 3. Means of items and exploratory factor analysis rotated factor structure

Items	Mean values	Factor loadings	Cronbach's alpha value
Emotional solidarity			
Q12. I am tolerant of my colleagues	4.469	0.771	0.754
Q5. I respect the personalities of my colleagues	4.726	0.758	
Q15. I feel a spiritual relief when I help my colleagues	4.526	0.645	
Q7. I always treat my colleagues honestly	4.628	0.642	
Q11. I trust my colleagues	3.702	0.619	
Academic solidarity			
Q21. I encourage my colleagues to participate in professional conferences and symposiums	3.605	0.741	0.734
Q20. I warn my colleagues regarding their lack of professional knowledge when I recognize it	3.291	0.687	
Q17. I help my colleagues carry out individual scientific studies	4.163	0.660	
Q22. I share my job experiences with my colleagues so that they will not make the same mistakes	4.296	0.620	
Q4. I willingly take part in the scientific studies of my colleagues	4.094	0.600	
Negative opinions about solidarity			
Q3. I do not try to solve the problems of my colleagues that they share with me	4.438	0.729	0.725
Q2. I do not care about the health problems of my colleagues	4.133	0.714	
Q19. I cannot help my colleagues because of my workload.	3.756	0.688	
Q6. I am not sensitive to the studies of professional organizations	3.926	0.659	
Q23. I never help my colleagues if they do not ask for my help	3.364	0.629	

Providing pharmacists with colleague solidarity is one of the aims of the TPA in Turkey.[24] The pharmacists who participated in the study stated that the activities of the professional organization increased colleague solidarity. In this context, 70% of pharmacists indicated that scientific and social activities offered by the TPA could be effective in terms of collegial solidarity. Concordantly, in one of the latest publications of the TPA, it highlighted that communication channels needed to be strengthened between the pharmacists and the Association.[26]

When the responses of the pharmacists to the scale were examined, it was seen that the highest average was in the expression of respect for the personality of colleagues. This is essential in terms of supportive cooperation as well as positive communication with colleagues, which is also important in health service delivery.[27,28]

In this study, it was revealed that women had more NOS in terms of the NOS factor. Women's views on solidarity have been studied since the end of the 90's.[6] Webber and Giuffre[29] identified that women face various obstacles when it comes to solidarity. In this context, the current results are consistent with the extant literature.

Moreover, solidarity is important in terms of public health and essential to ensure sustainability.[30] The World Health Organization and the United Nations[20] emphasized the importance of solidarity in this period in the documents they published. Garros et al.[31] aver its importance for physicians. In addition, it is suggested that global solidarity is important in terms of pharmaceutical services during the pandemic period.[32] Supporting this, when the scores of the pharmacists participating in the study of the colleague solidarity scale were examined, it was found that the scale scores of pharmacists who thought that solidarity increased during the pandemic process were statistically higher.

Study limitations

There were numerous limitations in this study. The survey was conducted via the online platform and only pharmacists who received the survey participation link became aware of the study. Further studies may be done by conducting the survey on more specific pharmacist groups, making it possible to determine the level of colleague solidarity in different areas.

CONCLUSION

As a result of this study, it was revealed that the "Colleague Solidarity Scale Among Nurses" with three factors and 15 statements is appropriate to a pharmacists' sample. Colleague solidarity among pharmacists should be addressed profoundly as an element specified in legislation and education processes. It is crucial to determine the level of colleague solidarity and improve it by applying this scale for different practice areas in pharmacy. In this regard, it will be possible to make positive contributions to professional satisfaction and motivation. The increase in satisfaction and motivation levels will also increase pharmacists' contributions to the improvement of public health.

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ORIGINAL ARTICLE

Effects of Thermal Treatment, Ultrasonication, and Sunlight Exposure on Antioxidant Properties of Honey

Isıl İşlem, Ultrasonikasyon ve Güneş Işığına Maruz Kalmanın Balın Antioksidan Özelliklerine Etkileri

Görkem YALÇIN*

Isparta University of Applied Sciences, Gelendost Vocational School, Department of Pharmacy Services, Isparta, Turkey

ABSTRACT

Objectives: This study aimed to determine effects of controlled heating, ultrasonication, and sunlight on antioxidant capacity, total phenolic content (TPC), and total flavonoid content (TFC) of honey.

Materials and Methods: Honey was subjected to thermal treatment (for 5-20 min at 30-80˚C), ultrasonication (for 5-20 min at 37 kHz frequency), and sunlight (for 1-10 days), and the impact of these treatments on antioxidant capacity, TPC, and flavonoid contents was evaluated. One-Way ANOVA, followed by Tukey's post-hoc test, was performed to compare the differences between experimental results.

Results: Antioxidant quality of samples heated at 60˚C and 80˚C were negatively affected when compared to untreated samples ($p<0.05$); however, there were no statistically significant differences between untreated samples and samples heated at 30˚C and 45˚C. On the other hand, ultrasonication of honey samples for 60 min enhanced the antioxidant properties when compared to untreated samples ($p<0.05$). In addition, while exposure to sunlight for 10 days decreased the TPC, the TFC and antioxidant capacity began to decrease after 6 days ($p<0.05$).

Conclusion: The results suggest that producers and consumers should consider the adverse effects of sunlight and temperature on antioxidative quality of honey. Additionally, ultrasonication technique has the advantage of preserving the antioxidant properties of honey.

Key words: Honey, temperature, ultrasonication, sunlight, antioxidative quality

ÖZ

Amaç: Bu çalışma, kontrollü ısıtma, ultrasonikasyon ve güneş ışığının balın antioksidan kapasitesi, toplam fenolik içeriği (TPC) ve toplam flavonoid içeriği (TFC) üzerindeki etkilerini belirlemeyi amaçlamıştır.

Gereç ve Yöntemler: Bal, ısıl işleme (30-80˚C'de 5-20 dakika), ultrasonikasyona (37 kHz frekansında 5-20 dakika) ve güneş ışığına (1-10 gün) tabi tutulmuş ve bu uygulamaların antioksidan kapasite, TPC ve flavonoid içerikleri değerlendirilmiştir. Deneysel sonuçlar arasındaki farkları karşılaştırmak için tek yönlü ANOVA ve ardından Tukey'nin post-hoc testi yapılmıştır.

Bulgular: 60˚C ve 80˚C'de ısıtılan numunelerin antioksidan kalitesi, işlem görmemiş numunelere göre olumsuz etkilenmiştir ($p<0,05$); bununla birlikte, işlenmemiş numuneler ile 30˚C ve 45˚C'de ısıtılan numuneler arasında istatistiksel olarak anlamlı bir fark belirlenmemiştir. Diğer taraftan, bal örneklerinin 60 dakika ultrasonikasyonu, işlem görmemiş örneklere kıyasla antioksidan özelliklerini arttırmıştır ($p<0,05$). Ayrıca 10 gün güneş ışığına maruz kalma TPC'yi azaltırken, 6 gün sonra TFC ve antioksidan kapasite azalmaya başlamıştır ($p<0,05$).

Sonuç: Sonuçlar, üreticilerin ve tüketicilerin, güneş ışığının ve sıcaklığın balın antioksidan kalitesi üzerindeki olumsuz etkilerini göz önünde bulundurmaları gerektiğini göstermektedir. Ek olarak, ultrasonikasyon tekniği balın antioksidan özelliklerini koruma avantajına sahiptir.

Anahtar kelimeler: Bal, sıcaklık, ultrasonikasyon, güneş ışığı, antioksidan kalite

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***Correspondence:** gorkemy1@gmail.com, **Phone:** +90 246 381 4280 , **ORCID-ID:** orcid.org/0000-0002-8705-0320

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INTRODUCTION

Honey is a natural product generated by honeybees; it has great market potential due to its health benefits in humans. Honey is a well-known source of enzymatic and non-enzymatic antioxidants, such as glucose oxidase, catalase, phenolics, flavonoids, vitamins, proteins, and Maillard reaction products.[1] Phenolics are the main compounds in honey, which contribute significantly to the antioxidant properties of honey.[2] However, the antioxidant activity varies, depending on the floral source, season, and environmental factors.[3]

Honey has a unique combination of components, a characteristic that makes it a valuable diet for consumers. However, raw honey is not normally commercialized, as further treatment is needed for large scale marketing.[4,5] Controlled heating is one of the important steps in processing honey. It can demolish the bacteria yeast that cause undesirable fermentation during storage of the product; it also facilitates liquefaction, so as to obtain a fluidy and non-crystallized product.[4-6] Besides thermal treatments, ultrasonication has been also used as an alternative to promote the marketability of honey.[7]

Honey is inevitably exposed to sunlight from the period of production to consumption. Sunlight exposure increases ultraviolet (UV) radiation. It is well-known that UV radiation adversely affects the quality of foods,[8] but there is no information on the effects of natural UV radiation on the antioxidant properties of honey.

Given the health benefits and demand for high quality honey, the preservation and enhancement its antioxidant properties during processing and storage are considerably important. Therefore, this study aimed to determine the antioxidant capacity, total phenolic content (TPC), and total flavonoid content (TFC) of honey, following its exposure to controlled heating, ultrasonication, and sunlight.

MATERIALS AND METHODS

Materials

Three bottles of same brands of honey were purchased from a common chain market in Turkey. The honey brand chosen for this study is well-known in Turkey. The brand officially declared that they own British Retail Consortium certificate and that all chemical and physical analysis were performed to assure the authenticity of the honey.

Methods

Thermal treatment

Samples were separately subjected to thermal processing in a water bath for 5, 10, 15, and 20 min at 30°C, 45°C, 60°C, and 80°C. Afterward, antioxidant capacity, TPC, and TFC of the samples were determined at room temperature.

Ultrasonication

Sonication of the samples was performed at 37 kHz frequency for 5, 15, 30, and 60 min using an ultrasonic cleaning bath.

Exposure to sunlight

Samples were placed outdoor during daytime (average maximum temperature: 26.0°C) and night time (average minimum temperature: 15.5°C) in May for 1, 3, 6, and 10 days.

Analysis of antioxidant capacity

Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC was determined according to the method described by Apak et al.[9] In brief, 1 g of processed honey sample was dissolved in 2.5 mL of distilled water. Then, 0.1 mL of the solution was mixed with 0.75 mL of $CuCl_2$ (10 mM), 0.75 mL of neocuproine (7.5 mM), 0.75 mL of CH_3COONH_4 buffer (1M, pH: 7.0), and 0.75 mL of distilled water. Absorbance was measured at 450 nm after 30 min. Trolox was used as a reference standard. Results were expressed as µmol Trolox equivalent (TE) per one gram (µmol TE/g).

Trolox equivalent antioxidant capacity (TEAC)

TEAC was determined according to the method described by on Re et al.[10] In brief, 0.1 mL of honey solution (1 g/2.5 mL) was mixed with 2 mL of $ABTS^{\cdot+}$ solution. After 15 min, absorbance was measured at 734 nm. A standard curve was constructed using Trolox and the results were expressed as µmol TE/g.

TPC and TFC

TPC was determined according to the method described by Fu et al.[11] In brief, 0.1 mL of honey solution (1 g/2.5 mL) was mixed with 1.0 mL of 1:10 diluted Folin-Ciocalteu reagent. 1.0 mL of saturated sodium carbonate solution was added after 4 min. This mixture was incubated for 2 h at room temperature. The absorbance of mixture was measured at 760 nm after incubation. Gallic acid (GA) was used as standard to produce the calibration curve. The results were expressed as mg of gallic acid equivalent (GAE) per 100 g.

TFC was determined according to the method described by Meda et al.[12] In brief, 1.5 mL of 2% aluminum trichloride in methanol was mixed with the same volume of honey solution (1 g/2.5 mL). After 10 min, absorbance was measured at 415 nm. A standard curve was constructed using quercetin and the results were expressed as mg of quercetin equivalent (QE) per 100 g (mg QE/100g).

Statistical analysis

Statistical analysis was done using GraphPad Prism 5 (Prism 5 for Windows Version 5.03, GraphPad Software, Inc) and Microsoft Excel. All experiments were conducted in triplicate. One-Way ANOVA was performed and significant differences between means were determined by Tukey's post-hoc test at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Effect of thermal treatment

Table 1 shows the antioxidant capacity, TPC, and TFC of honey before and after heat treatment. For untreated samples, CUPRAC, TEAC, TPC, and TFC were found to be 2.75, 1.14 (µmol TE/g),

27.75 (mg GAE/100g), and 6.76 (mg QE/100g), respectively. For samples heated at 30°C and 45°C for 5 min, the highest TPC and TFC values, respectively, as compared to the rest, were obtained. In the cases of CUPRAC and TEAC, the highest results were obtained for untreated samples. On the contrary, antioxidant capacity, TPC, and TFC of samples decreased with the increase of treatment temperature. To ascertain whether these differences are statistically significant, One-Way ANOVA, followed by Tukey's post-hoc test, was applied to the data. As seen in Table 1, statistical differences between the untreated samples and samples subjected to 60°C (in CUPRAC and TFC assays) and 80°C heating (in all assays) were significant. Also, findings revealed that the process time and the treatment temperature affected the antioxidant capacity, TPC, and TFC of the samples.

Honey is rich in natural antioxidants, such as enzymes, vitamins, phenolic acids, and flavonoids.[13] However, these compounds may undergo several irreversible changes during thermal treatments.[1] Escriche et al.[4] evaluated the effect of industrial heat treatment on the phenolic compounds of Spanish honeys. According to their results, a significant decrease in the concentration of some phenolic compounds in these honeys was observed after the thermal treatment. Kowalski[14] investigated the impact of heating at 90°C for 60 min on antioxidant properties of honey through TPC and $ABTS^{\cdot+}$ assays. It was observed that there was a significant decrease in the antioxidant properties of honeydew honey after processing. Chaikham and Prangthip[15] reported that TPC, TFC, and antioxidant capacity (measured by FRAP and DPPH assays) of longan-flower honey diminished after heating at 100°C for 5 min.

Finally, heating honey at high temperatures could degrade their antioxidant compounds content,[16] and this could explain why the antioxidant capacity, TPC, and TFC of the treated honey samples decreased when compared to those of the untreated samples.

Effect of ultrasonication

Results of the impact of ultrasonication on antioxidant properties of honey are shown in Table 2. The values of treated samples increased with increase of the treatment time as compared to the values of the untreated samples. However, statistical

Table 1. Effects of thermal treatment on antioxidant capacity, TPC, and TFC of honey

	Method			
	CUPRAC*	**TEAC***	**TPC****	**TFC*****
Untreated sample	2.75±0.10	1.14±0.02	27.75±0.57	6.76±0.06
30°C temperature				
5 min	2.66±0.05	1.04±0.03	28.21±0.07	6.70±0.07
10 min	2.62±0.08	1.04±0.02	27.77±1.25	6.78±0.14
15 min	2.67±0.15	1.07±0.07	27.98±0.69	6.75±0.08
20 min	2.69±0.11	1.08±0.02	26.59±0.79	6.72±0.14
45°C temperature				
5 min	2.71±0.07	1.10±0.02	27.73±0.38	6.79±0.24
10 min	2.68±0.10	1.08±0.01	27.94±0.85	6.69±0.18
15 min	2.69±0.12	1.07±0.06	27.59±1.08	6.57±0.11
20 min	2.73±0.13	1.08±0.03	27.82±0.78	6.50±0.05
60°C temperature				
5 min	2.47±0.07	1.06±0.01	27.60±0.45	6.50±0.04
10 min	2.41±0.11[a]	1.03±0.02	27.12±0.31	6.17±0.12[d]
15 min	2.38±0.15[a]	1.02±0.04	26.86±0.28	5.79±0.15[d]
20 min	2.30±0.01[a]	1.03±0.06	26.23±0.66	5.62±0.11[d]
80°C temperature				
5 min	2.32±0.10[a]	1.02±0.01	27.12±0.18	6.30±0.14[d]
10 min	2.32±0.12[a]	0.96±0.01[b]	27.05±0.74	6.01±0.16[d]
15 min	2.22±0.07[a]	0.96±0.01[b]	24.90±1.03[c]	5.69±0.18[d]
20 min	2.19±0.02[a]	0.96±0.04[b]	24.45±0.70[c]	5.74±0.12[d]

[a, b, c, d]The rows that do not share the same superscripts are significantly different from each other in Tukey's post-hoc test ($p<0.05$). *µmol TE/g, **mg GAE/100g, ***mg QE/100g, CUPRAC: Cupric reducing antioxidant capacity, TEAC: Trolox equivalent antioxidant capacity, TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, QE: Quercetin equivalent

differences between the samples subjected to ultrasonication for 60 min, and the untreated samples in terms of TPC, TFC, and CUPRAC were observed. In the case of TEAC assay, there were no significant differences between the treated and untreated samples. The differences between the antioxidant capacity assays CUPRAC and TEAC were due to the difference in the two assays.[17,18]

Ultrasonication is an alternative and innovative technology to obtain fluidy and non-crystallized products. It is more effective to preserve the nutritional l values of honey by this method rather than thermal treatments.[7,15] However, there are limited data on the impact of ultrasonication on antioxidant properties of honey. Similar to the current assay, Chaikham and Prangthip[15] reported that the TPC, TFC, and antioxidant capacity of honey increased after processing for 20 min. Pollen is one of the important contents of honey;[13] it has multiple essential components, such as proteins, vitamins, and phenolic compounds.[19] Ultrasonication has the capability to increase the permeability of plant tissues caused by cell disruption, thereby resulting in the liberation of all the compounds present in the cell.[20] In view of the fact that a pollen is produced by plants as a male cell, existing antioxidant compounds in pollens could be released after ultrasonication, thereby causing an increase in the TPC, TFC, and antioxidant capacity of honey.

Apart from the limited studies relevant to the impact of ultrasonication on antioxidant properties of honey, many studies have been conducted to examine the influence of ultrasonication in preserving the nutrional qualities of fruit juices, although its positive effect in terms of antioxidant properties have been demonstrated.[21-23]

Effect of sunlight exposure

Table 3 shows the effects of sunlight exposure on the TPC, TFC, and antioxidant capacity of honey. TPC of the samples exposed to sunlight began to change after 10 days, whereas exposure to sunlight caused changes in the TFC and TEAC of the samples after 6 days ($p<0.05$). However, CUPRAC did not change significantly in any of the samples when compared with the untreated sample. Direct sunlight exposure initiates the generation of free radicals that accelerate the degradation reactions that adversely affect the quality of foods and beverages.[8] This could explain the decrease of TPC, TFC, and antioxidant capacity of the honey. Until now, there is no study examining the influence of direct sunlight exposure on the antioxidant capacity, TPC, and TFC of honey. However, several authors report that sunlight induced quality loss of fruit products, such as pummelo (*Citrus maxima*) essential oil[24] and strawberry juice.[25]

CONCLUSION

The treatments significantly affected the antioxidant properties of honey, depending on the processing time. Thermal treatment and sunlight exposure had a negative influence on the antioxidant quality of honey. However, ultrasonication significantly increased the values of these parameters in all assays, except TEAC assay, in which the increment was statistically significant. Therefore,

Table 2. Effects of ultrasonication on antioxidant capacity, TPC, and TFC of honey

Method	Untreated sample	Ultrasonication			
		Process time (min)			
		5	15	30	60
CUPRAC*	2.75±0.10	2.76±0.04	2.86±0.25	2.99±0.14	3.22±0.06[a]
TEAC*	1.14±0.02	1.19±0.03	1.19±0.02	1.22±0.06	1.24±0.08
TPC**	27.75±0.57	28.16±0.41	29.13±0.80	29.18±1.22	30.85±0.56[c]
TFC***	6.76±0.06	6.85±0.03	6.97±0.05	7.10±0.07	7.30±0.09[d]

[a, c, d]Shows significant differences between the treated and untreated samples according to Tukey's post-hoc test ($p<0.05$). *µmol TE/g, **mg GAE/100g, ***mg QE/100g, TPC: Total phenolic content, TFC: Total flavonoid content, CUPRAC: Cupric reducing antioxidant capacity, TEAC: Trolox equivalent antioxidant capacity, GAE: Gallic acid equivalent, QE: Quercetin equivalent

Table 3. Effects of sunlight exposure on antioxidant capacity, TPC, and TFC of honey

Method	Untreated sample	Sunlight exposure			
		Process time (day)			
		1	3	6	10
CUPRAC*	2.75±0.10	2.72±0.04	2.58±0.04	2.55±0.09	2.50±0.11
TEAC*	1.14±0.02	1.11±0.04	1.11±0.05	1.01±0.02[b]	1.01±0.06[b]
TPC**	27.75±0.57	27.88±0.42	26.98±1.66	25.83±1.15	24.49±0.96[c]
TFC***	6.76±0.06	6.63±0.12	6.53±0.10	6.27±0.08[d]	6.26±0.18[d]

[b, c, d]Shows significant differences between the treated and untreated samples according to Tukey's post-hoc test ($p<0.05$). *µmol TE/g, **mg GAE/100g, ***mg QE/100g, CUPRAC: Cupric reducing antioxidant capacity, TEAC: Trolox equivalent antioxidant capacity, TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, QE: Quercetin equivalent

ultrasonication could be an alternative technique for preserving the antioxidant properties of honey instead of industrial thermal treatment. On the other hand, it is suggested that producers and consumers should consider the negative effects of sunlight on the antioxidants properties of honey during storage, since exposure of the honey samples to sunlight resulted in a decrease in the antioxidant capacity, TPC, and TFC.

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ORIGINAL ARTICLE

Investigation of the Genotoxic, Cytotoxic, Apoptotic, and Oxidant Effects of Olive Leaf Extracts on Liver Cancer Cell Lines

Zeytin Yaprağı Ekstrelerinin Karaciğer Kanseri Hücre Hatları Üzerine Genotoksik, Sitotoksik, Apoptotik ve Oksidan Etkilerinin Araştırılması

Muhammed Yunus BEKTAY[1]*, Eray Metin GÜLER[2], Mustafa GÖKÇE[3], Mustafa Volkan KIZILTAŞ[4]

[1]Bezmialem Vakıf University Faculty of Pharmacy, Department of Clinical Pharmacy; Marmara University Institute of Health Sciences, Department of Clinical Pharmacy, İstanbul, Turkey
[2]University of Health Sciences, Hamidiye Faculty of Medicine, Department of Medical Biochemistry; Haydarpaşa Numune Health Application and Research Center, Department of Medical Biochemistry, İstanbul, Turkey
[3]Bezmialem Vakıf University Faculty of Pharmacy, Department of Parhmacology, İstanbul, Turkey
[4]İstanbul University Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul, Turkey

ABSTRACT

Objectives: Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third leading cause of tumor-related deaths worldwide. Mechanisms underlying tumor onset, progression, and metastasis in the case of HCC have not been adequately studied. In this study, we aimed to investigate the genotoxic, cytotoxic, apoptotic and oxidant effects of olive leaf extract (OLE) on HCC cells.

Materials and Methods: H4IIE *Rattus norvegicus* hepatoma cells and *Rattus norvegicus* healthy liver clone-9 cells were treated with the increasing concentrations of OLEs (250-2000 ppm) in ethanol, acetone, dichloromethane, and methanol. ATP cell viability, intracellular reactive oxygen species generation levels, double staining test with acridine orange/ethidium bromide, comet assay, levels of interleukin 1-beta (IL-1β), IL-6, and tumor necrosis factor alpha were measured. Significance was determined using ANOVA test.

Results: Apoptotic, genotoxic, cytotoxic, and oxidative effects of OLEs increased with the increasing concentrations as compared to controls in H4IIE cells ($p<0.001$).

Conclusion: This is the first study to show a significant and selective cytotoxic activity of OLEs in the selected H4IIE cancer cell lines. OLEs could selectively increase the apoptotic damage and show anti-proliferative and pro-apoptotic properties against the H4IIE cells. They could be recommended as potential nutraceuticals in the prevention of cancer.

Key words: Olive leaf, hepatocellular carcinoma, oleuropein, oxidative stress, genotoxicity, apoptosis

ÖZ

Amaç: Hepatoselüler karsinoma (HSK), en yaygın yedinci kanser türüdür ve kanserle ilişkili ölümlerde üçüncü sırada yer almaktadır. HSK'de tümör başlangıcına, ilerlemesine ve metastazlara neden olan mekanizmalar tam olarak bilinmemektedir. Bu çalışmada zeytin yaprağı ekstresinin (OLE) HSK hücreleri üzerine genotoksik, sitotoksik, apoptotik ve oksidan etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: H4IIE *Rattus norvegicus* hepatoma hücrelerine ve *Rattus norvegicus* sağlıklı karaciğer klon-9 hücrelerine etanol, aseton, diklorometan ve metanol içinde artan konsantrasyonlarda OLE'leri (250-2000 ppm) uygulanmıştır. Ekstrelerin sitotoksisitesi ATP testiyle, hücre içi reaktif oksijen türlerinin oluşumu florometrik yöntemlerle, genotoksik etkileri alkalen tekli hücre jel elektroforez (comet assay) yöntemiyle, apoptotik etkileri akridin turuncusu/etidyum bromür yöntemiyle ölçülmüştür. İnterlökin 1 beta (IL-1β), IL-6 ve tümör nekroz faktörü alfa düzeyleri ELISA yöntemi ile belirlenmiştir. İstatistiksel test olarak ANOVA testi kullanılmıştır.

Bulgular: OLE'nin apoptotik, genotoksik, sitotoksik ve oksidatif etkileri H4IIE hücrelerinde kontrole göre artan konsantrasyonlarla istatistiksel olarak anlamlı şekilde yükselmiştir ($p<0,001$).

*Correspondence: yunusbektay@gmail.com, Phone: +90 212 523 22 88, ORCID-ID: orcid.org/0000-0003-2032-9957
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Sonuç: Bu çalışma, H4IIE kanser hücre hatlarında OLE'nin seçici sitotoksik aktivitesini gösteren ilk çalışmadır. OLE apoptozu indüklemiş ve H4IIE hücrelerine karşı anti-proliferatif ve pro-apoptotik özellikler göstermiştir. Kanserin önlenmesinde potansiyel nutrasötikler olarak önerilebilirler.
Anahtar kelimeler: Zeytin yaprağı, hepatosellüler kanser, oleuropein, oksidatif stres, genotoksite, apopitoz

INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third leading cause of cancer-related mortality worldwide. Despite significant advances in the diagnosis and treatment, HCC remains a terminal disease. The most common cause for mortality in HCC is progression and the metastasis. However, the molecular mechanisms underlying tumor onset, progression, and metastasis in the case of HCC have not been adequately studied.[1] The neoplastic development of HCC is related to many histological incidents. Hyperplastic nodules formed in the hepatocyte regeneration observed in the liver in response to the cell deaths due to risk factors are cytologically of normal findings, which will be the first step in HCC formation. These lesions may transform into premalignant dysplastic nodules and cause nuclear aggregation with cytologically well-detected cell changes. As a result of molecular analysis in the HCCs, various genetic and epigenetic changes have been detected.[1]

Epidemiological studies and animal experiments have been revealed that some nutritional compounds play a role in the incidence rates of various cancers, including HCC. One-third of all the known human cancers may be related to specific components of nutrients.[1-3] Many research groups are investigating the effects of nutritional components on cancer processes and state that dietary fats increase the risk of brain, colon, breast, and prostate cancer. In addition, the incidence of the brain, cardiovascular diseases, and colon and breast cancers are lower in the Mediterranean Region than in Europe.[4,5] Around the Mediterranean and European territories olive tree leaf (*Olea europaea* L.) is commonly used as traditional medicine. Olive leaf extract (OLE) has been reported to show anti-aging, immune system enhancing, and antimicrobial effects.[6] In studies, olive leaf antioxidants have been shown to be effective in the treatment of cancers like liver, prostate, and breast.[7,8] Olive leaf contains substantial secoiridoids, flavonoids, phenolic acids, and the lignans. Of these, oleuropein is an important phenolic compound in the secoiridoid group.[7] Oleuropein and its metabolite hydroxytrizole (HT) have antioxidant activity, showed in both *in vivo* and *in vitro* experimental models.[6,7] In addition to *in vivo* and *in vitro* activities, the antioxidant potential of oleuropein and HT has also been investigated on experimental cell line models and animal models.[6-8] In recent epidemiological studies, it was shown that OLEs rich in phenolic compounds correlated with a decreased cardiovascular risk, neurodegenerative disease, and cancer.[6-9] Besides the different phenolics and flavonoids olive leaves contain the most oleuropein content when compared with other parts of the plan such as root, bark, fruit.[9] In this study, it was aimed to investigate the *in vitro* genotoxic, cytotoxic, apoptotic, and oxidant effects of OLE on the HCC cell lines.

MATERIALS AND METHODS

Collection of plant samples

The plants have been purchased commercially as medical drugs with a batch number of 26/0/2019. Plant leaves of *Olea europaea* L. were dried at room temperature, then shredded with a plant shredder and stored at room temperature until they were used in the study.

Preparation of methanol, acetone, dichloromethane, and aqueous-ethanol extracts

The preparation of OLEs was performed for the methanol extract (O-MeOH), acetone (O-ACE), dichloromethane (O-DCM), and aqueous-ethanol extract (O-EtOH), a total of 70 g of the plant sample was ground with a laboratory blender, extracted in the solvent, and after the extraction, the extract has been filtered, followed by the evaporation of the solvent. The amount of oleuropein was expressed as milligram per gram of OLE. The OLE was dissolved in dimethyl sulfoxide (0.1%) prior to the analysis.

Determination of total phenolic and flavonoid content

The total amount of phenolic compounds found in the extracts was determined with the Folin-Ciocalteu Reagent.[10] Gallic acid (GA) was used as a standard phenolic compound. The total amount of phenolic compounds found in all OLEs was evaluated according to the study conducted by Gülçin et al.[11] The findings were given in GA equivalent (GAE) and micrograms.

The total amount of flavonoids found in all the OLEs was determined by the method of Park et al.[12] Quercetin was used as the standard. Total flavonoid concentration was calculated as quercetin equivalent.

Determination of free radical activity

The free radical scavenging activities of the extracts were determined using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical.[13] The absorbance was measured at 517 nm, values of the samples were evaluated against the control. As standard, butylated hydroxy anisole (BHA), BH toluene (BHT), and α-tocopherol (α-Toc) were used.

Determination of ABTS cation radical scavenging activity

This method was used to assess the potential of the extracts to sweep the ABTS cation radical. The absorbance of each solution was detected at 734 nm, and the inhibition % was calculated.[14]

Determination of reduction force

The cupric ion (Cu^{2+}) reduction capacities of the extracts were measured with the CUPRAC method.[15] As standard, BHA, BHT, and α-Toc were used.

High-performance liquid chromatography (HPLC) analysis

The amount of oleuropein in leaf extracts was measured by HPLC according to the method developed by Al-Rimawi.[16] HPLC analysis was performed using the Shimadzu (Japan) LC-20A model, and the peak areas of automatic injections of 20 µL with ultraviolet (UV) detector were calculated using the LC solution computer software. Chromatographic separation was attempted to be optimized using C_{18} (5 µm, 150×4.6 mm) and C_8 (5 µm, 150×4.6 mm) columns.

The calibration coefficient was calculated from the calibration curve created by the concentration-dependent linear mathematical equation of the field data, and linearity was calculated (Figure 1G). The UV detection of the separation was performed using the mobile phase of the water (pH: 3): acetonitrile (70:30) at 1 mL min^{-1} flow over the C_{18} ODS-3 (150 mm, 4.6×5 mm) column at 280 nm. The linear coefficient of the linear calibration curve created in the range of 100-1000 µg/mL was found to be 0.99.

Cell culture

H4IIE [American Cell Cultures Collection (ATCC)® CRL-1548™] *Rattus norvegicus* hepatoma cells were obtained from the (ATCC, Middlesex, UK). Cells were grown in the Eagle's minimum essential medium containing 10% FBS and 1% P/S in a humidified incubator with 5% CO_2 at 37˚C.

Healthy rat liver cell clone-9 cells (ATCC® CRL-1439™) were obtained from ATCC (Middlesex, UK). Cells were grown in an F12K medium containing 10% FBS, and 1% P/S in a humidified incubator with 5% CO_2 at 37˚C. Samples were prepared at different doses and left for 24-hour incubation in cells. Each dose was analyzed with a minimum of four replicates.

Cytotoxicity assay

To measure cytotoxicity in cancer and healthy cells, 1.5×10^4 cells per well were added to 96 opaque white plates. Samples of different concentrations were added and incubated for 24 hours. After incubation, the ATP solution was added to the wells and measured luminometrically using the Cell Titer-Glo® Luminescent Cell Viability Test Kit.

Intracellular reactive oxygen generation

The intracellular reactive oxygen species (ROS) levels were measured using the H_2DCF fluorescence probe. Cancer and healthy cells seeded on the opaque black plates, and samples of different concentrations (250-2000 ppm) were incubated for 24 hours. After the incubation, cells were washed three times with dPBS and incubated at 37˚C in the dark with 5 µM H_2DCFH-DA. After the cells were washed, fluorometric measurements were taken at Ex: 482 nm/Em: 512 nm. Results were normalized according to the ATP levels.

Apoptosis

Acridine orange (AO)/ethidium bromide (EB) dye was used to measure the apoptosis.[17] After mixing AO/EB and cell pellet in a 1:1 ratio, images were taken under a fluorescence microscope (Leica DM 1000, Solms, Germany). The separation of apoptotic cells performed according to the morphological characteristics of the nuclei. Apoptotic cell nuclei take up only EB. On the other hand, AO is taken up by both living and dead cells. Green fluorescence at 480-490 nm was flared by living cells. Healthy cells flared green fluorescence while apoptotic cells luminating yellow-orange and necrotic cells were seen red. Briefly, 2×10^5 H4IIE and clone-9 cells/well were seeded in 6-well plates and incubated for 24 hours. Cells seeded in 6-well plates under IC_{50} doses were washed for 24 hours after incubation with the samples. The preferred solution for collection of the cells and washing was PBSR, then cells were stained with 1:1 mixture of AO/EB (100 µg/mL). Triplicate samples of 100 cells each were counted and scored for the incidence of apoptotic chromatin condensation using a fluorescent microscope (Leica DM 1000, Solms, Germany).[17,18] Apoptotic cells were calculated relative to the total number of cells.[18]

Genotoxicity

Genotoxicity analysis was performed according to the comet assay method in our previous study. DNA damage in cells was given as tail intensity percentage.[19] Comet assay (alkaline single cell gel electrophoresis assay) with a slight modification of Singh et al.'s[19] methods was carried out to assess the genotoxic effects of OLEs on H4IIE and clone-9 cells. The 6-well cell culture containing the cell culture medium plates were used. For the evaluation of genotoxicity approximately 2×10^5 cells per well plated on wells at 37˚C. Then, the OLE samples < IC_{50} concentrations were added and incubated for 24 hours. Trypsin-EDTA (0.25%) used for collection of the cells and, phenol red for 2-3 min in the incubator. The cells were centrifuged at 1500x rpm for 5 min at 4˚C. The supernatant was withdrawn, and the cell density of 2×10^5 cells/mL was adjusted with cold dPBS. Total of 15 µL cell suspension and 85 µL of 0.6% low melting point agarose were mixed and placed on 1% normal melting point agarose precoated slides. The gel mixture was solidified on a cold tray for 2 mins, and the slides were treated for overnight with lysis buffer, pH 10.0 (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, Sigma-Aldrich). In an alkaline solution, the incubation of the slides performed (0.3 M NaOH, 1 mM EDTA, Sigma-Aldrich) for 40 min in the dark in the presence of cooling blocks to unwind the DNA. At 0.72 V/cm (26 V, 300 mA) for 25 min in an electrophoresis tank at 4˚C, 0.72 V/cm (26 V, 300 mA) electrophoresis was performed for 25 min. The slide was again treated with tris buffer (0.4 M Tris, pH: 7.5) for 5 mins. Before staining the slides, ethanol was used for dehydrating the slides. A staining procedure with EB (2 µg/mL in distilled H_2O, 70 µL/slide) applied to the slides and coated with a coverslip and scored under fluorescence microscope (Leica DM 1000, Solms, Germany) using the Comet assay IV software (Perceptive Instruments, Suffolk, UK).

Inflammation assays

Cancer and healthy cells were incubated at concentrations < IC_{50} for 24 hours. The levels of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) in the medium were measured by commercially available ELISA kits (Elabscience, Texas, USA) by photometric methods. Levels of IL-1β, IL-6, and TNF-α in a cell medium after 24-hour incubation were measured by the ELISA

method according to the protocol provided by the manufacturer. All ELISA kits were purchased from Elabscience (Texas, USA). IL1β, IL6, and TNF-α levels were read at 450 nm using an ELISA plate reader (Thermo Scientific, Massachusetts- USA), and the concentrations were calculated according to the standard curves obtained from the external standards.

Statistical analysis

All experiments were repeated for the minimum number of 4 times. Results are given as mean ± standard deviation. Variance analysis was done with One-Way ANOVA. $p<0.05$ level was considered as statistically significant. Regression analysis was done for IC_{50} values. All analyzes were done with the Statistical Package Program for Social Sciences (IBM, Inc version 25).

RESULTS

Flavonoid and phenol content, free radical activity, ABTS cation radical scavenging activity, reduction force, and oleuropein level analysis

Polyphenolic compounds, namely flavonoids, phenolic acids, and their derivatives, are typical phytochemicals for olive leaves. Total flavonoid levels in O-DCM, O-ACE, O-MeOH, and O-EtOH were found 8.52, 7.18, 3.29, and 1.73 mg QUE/mL, respectively (Figure 1A), while total phenolic levels in O-DCM, O-ACE, O-MeOH, and O-EtOH were 11.24, 6.92, 9.96, and 9.81 mg GAE/mL, respectively (Figure 1B).

The free radical scavenging activities of the extracts are determined using the DPPH free radical (Figure 1C). O-DCM showed inhibitions of 0.13%, 0.13%, 18.72%, and 18.85% with different concentrations. O-ACE showed inhibitions of 9.49%, 18.72%, 41.54%, and 81.85% with different concentrations. O-MeOH showed inhibitions of 6.63%, 19.76%, 42.78%, and 81.85% with different concentrations. BHA was used as a standard.

ABTS cation radical scavenging activity of the extracts was showed in Figure 1D. O-DCM showed inhibitions of 9.96%, 0.13%, 18.72%, and 18.85% with different concentrations. O-ACE showed inhibitions of 30.32%, 65.82%, 70.54%, and 87.14% with different concentrations. O-MeOH showed inhibitions of 18.13%, 50.43%, 85.49%, and 85.93% with different concentrations. O-EtOH showed inhibitions of 42.30%, 69.89%, 87.36%, and 86.92% with different concentrations. The determination of reducing ability was showed as absorbance levels of extracts (Figure 1E). O-DCM showed absorbance of 0.14, 0.24, 0.31, and 0.52 with different concentrations. O-ACE showed absorbance of 0.20, 0.43, 0.70, and 1.24 with different concentrations. O-MeOH showed absorbance of 0.17, 0.39, 0.60, and 1.04 with different concentrations. BHA is used as a standard. Oleuropein level determination was conducted with HPLC as three parallels (Figure 1E). Mean values of these three were 19.56 mg/mL (O-DCM), 97.97 mg/mL (O-ACE), and 72.20 mg/mL (O-MeOH).

Inhibition of cell viability

Cytotoxicity results of OLEs to liver hepatoma (H4IIE) and healthy liver clone-9 cells are presented in Figure 2A. These results showed a robust concentration dependent response relationship with cytotoxicity of OLEs. In Figure 2A, a gradual decrease was seen in the viability of H4IIE cells, a statistically significant difference obtained $p<0.001$ with increased concentrations.

Intracellular iROS generation levels

The results of the iROS of OLEs to liver carcinoma (H4IIE) and healthy liver clone-9 cells are presented in Figure 2B. There is a gradual increase in the iROS level of H4IIE cells, with all concentrations of O-MeOH and O-EtOH of 250, 500, 1000, and 2000 ppm showed increased iROS compared to control cells ($p<0.001$) (Figure 2B).

Induction of apoptosis

OLEs induce Apoptosis of H4IIE cells in a concentration-dependent manner, showing a gradual increase of EB-positive cells in OLEs-treated cells compared to the control, and a statistically significant difference was obtained $p<0.001$ (Figure 2C, D).

Induction of DNA damage

Extracts at all concentrations lead to the breaking of DNA single-strand in H4IIE cells, and there is a gradual concentration-response relationship and a statistically significant difference of $p<0.001$. Comet assay images of control and OLEs-treated H4IIE cells are presented in Figures 3A, B.

Anti-inflammatory activity of OLEs and its phenolic compounds

Results showed that all extracts at the concentrations of 250, 500, 1000, and 2000 ppm causes elevated levels of IL-1β, IL-6, and TNF-α in H4IIE and clone-9 cells (Figure 4A-C). The OLEs had a significant activity on IL-1β, IL-6, and TNF-α production in a concentration-dependent manner.

DISCUSSION

Studies on *Olea europaea* L. generally includes data on the chemical and physical properties of the oil extracted from the fruits and their samples. There are few studies on the antioxidant activities of leaf extracts in the literature since the oil of the fruits is very valuable. Ethanol and acetone have been found to be highly effective solvents in extracting the phenolic compounds. Phenolic compounds are one of the main secondary metabolites of olive leaves.[20-23] It has also been found that water/aqueous-ethanol/acetone mixtures are generally more effective solvents to extract the polyphenolic compounds of olive leaves.[20-23]

The antioxidant activity of OLEs may directly be related to the polyphenol content.[21,23] Oleuropein, is the major secondary metabolite and has been extensively studied, but available information is limited for different OLEs.[21-23] Oleuropein is an important metabolite for the antioxidant ability of OLEs.[9] For the OLEs, acetone and ethanol extracts exhibit higher antioxidant capacity in both methods (DPPH and ABTS). Our results were consistent with the literature, and acetone and ethanol extracts with high oleuropein content were effective as antioxidant scavengers.[23-26] In a study using solid-liquid extraction technique and three different extraction solvents

[petroleum ether, water, methanol (80%)], suggested that flavonoids (from 3.33-17.64 mg catechin equivalents/g), phenolics (from 3.64-21.47 mg GAE/g) were abundant in the content of OLEs, and their analysis correlated with antioxidant capacity.[24] These data are consistent with our results presented in this study.

OLEs caused cytotoxicity in many different cancer cells such as human breast cancer cells (MCF7) and colorectal cancer cells (HT29).[25,26] Kimura and Sumiyoshi[27] investigated the effects of OLE and oleuropein on the skin cancer animal model. According to their investigations, oleuropein and OLE were superior to the control group in skin thickness and tumor incidence. Barbaro et al.[28] suggested that olive leaves and oleuropein antitumor activity may be related with ROS scavenging effect, antiproliferative effect, apoptosis induction, angiogenesis inhibition, and antimigration effect. Our study is coherent with the previous studies, and we found that OLEs at different concentrations inhibits H4IIE cancer cells more

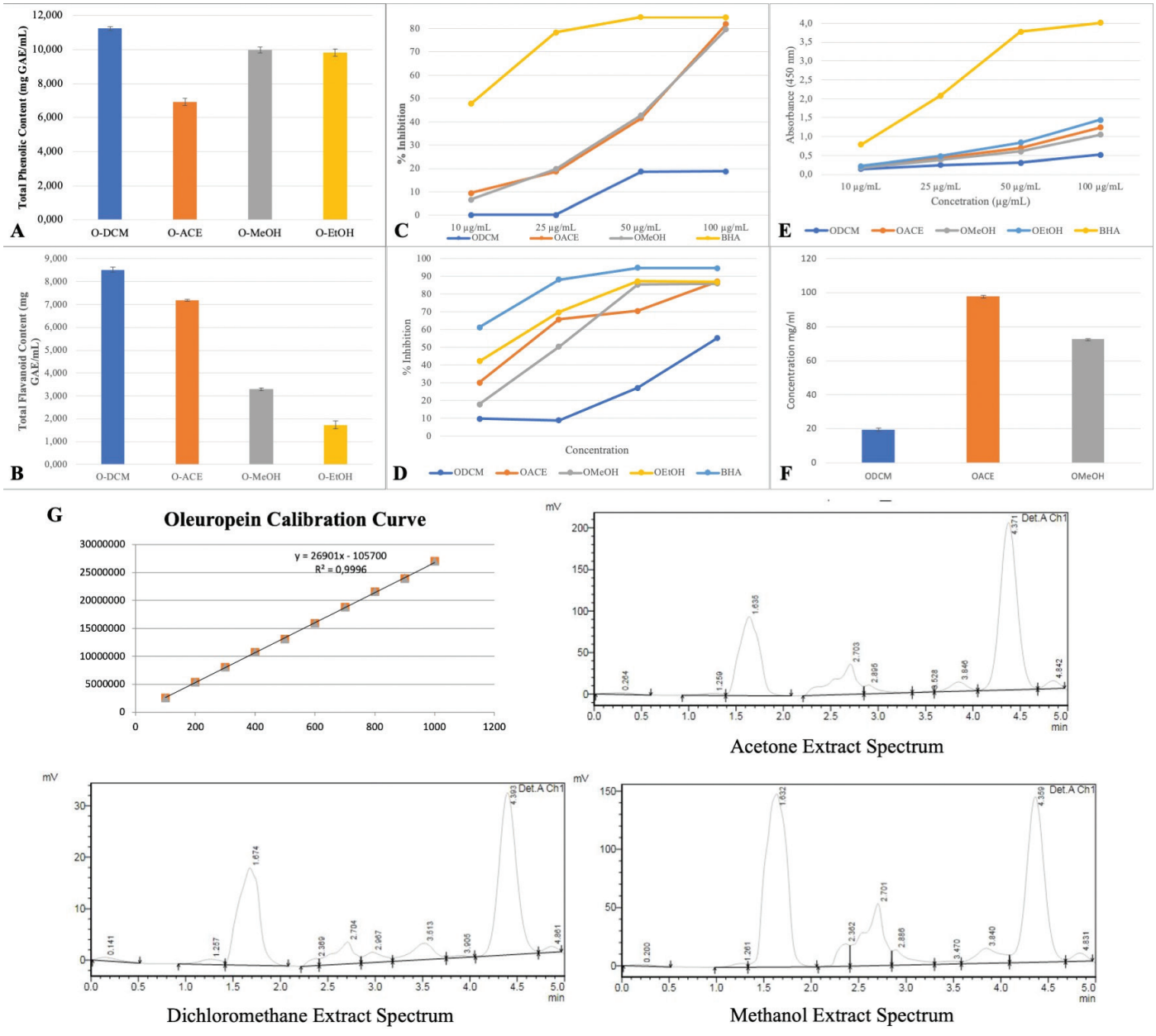

Figure 1. Evaluation of the flavonoid and phenol content, free radical activity, ABTS cation radical scavenging activity, reduction force, and oleuropein level in Olea leaf extracts. (A) Total phenolic compounds of extracts; (B) total flavonoid compounds of extracts; (C) free radical activity of extracts for different concentrations; (D) ABTS cation radical scavenging activity of extracts for different concentrations; (E) reduction force of extracts; (F) oleuropein concentration in extracts. (G) Oleuropein calibration curve and extract spectrums of acetone, dichloromethane, and methanol. GAE: Gallic acid, QUE: Quercetin, O-DCM: OLE with dichloromethane, O-ACE: OLE with acetone, O-MeOH: OLE with methanol, O-EtOH: OLE with ethanol, BHA: Butylated hydroxy anisole

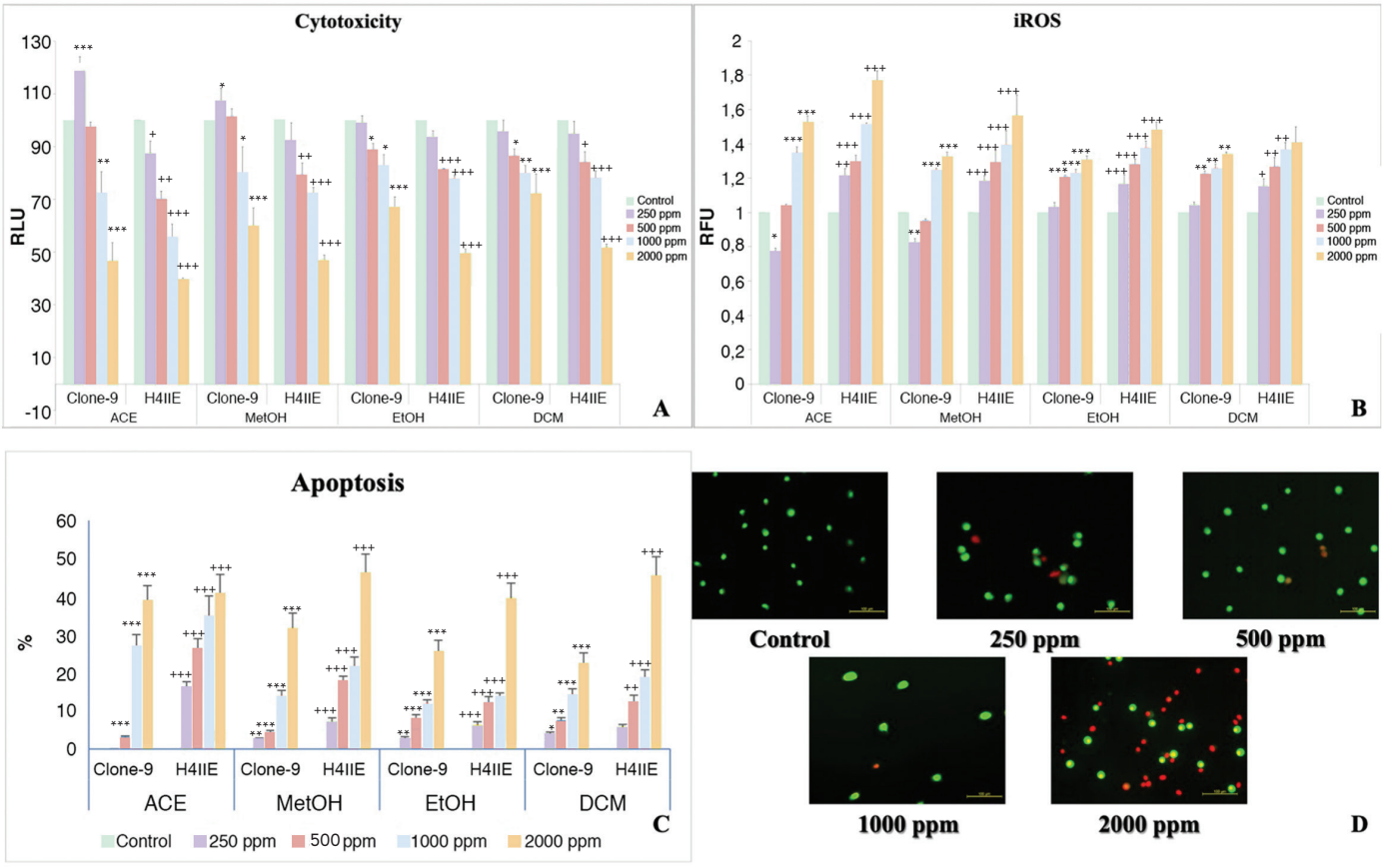

Figure 2. Cytotoxicity, iROS, and apoptosis assays with increasing concentrations (250, 500, 1000, and 2000 ppm) of *Olea europaea* L. extract on clone-9 and H4IIE cells. (A) Cytotoxic effect of *Olea europaea* L. extracts. (B) iROS generation levels of *Olea europaea* L. extracts. (C) Determination of apoptotic and living cells using acridine orange/ethidium bromide double staining test. (D) Representative immunofluorescence images in H4IIE cells. The level of apoptosis increased with increasing concentrations of O-MeOH. O-MeOH showed the highest apoptotic damage among all extracts. Differences between the groups were tested for significance using One-Way ANOVA (*p<0.05, **p<0.01, ***p<0.001, $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001). RLU: Relative Luminescence Units, RFU: Relative Fluorescent Units, O-DCM: OLE with dichloromethane, O-ACE: OLE with acetone, O-MeOH: OLE with methanol, O-EtOH: OLE with ethanol, iROS: Intracellular reactive oxygen species

than clone-9 healthy cells (Figure 2). In fact, at 0.25 mg/mL concentrations, acetone and methanolic extracts positively affected cell viability in terms of Relative Luminescence Units in the healthy clone-9 cell line. This indicates that OLE causes selective cytotoxicity in rat hepatoma cell lines, but not in healthy hepatocytes. This selectivity could be a result of the susceptibility of cancer cells to intracellular ROS formation. Cancer cells have increased ROS producing capacity, and therefore the selective activity of OLE on H4IIE may be due to their increased susceptibility to the iROS. The effects of OLE on H4IIE cells may offer opportunities for new chemoprevention studies for liver cancer.

On the other hand, the higher concentration of OLE was induced cytotoxicity and iROS for both healthy and cancer cell lines. These results indicate that the dose of the extracts have a crucial role in the pharmacological effect. According to concentration, OLE may induce or inhibit cytotoxicity and apoptosis. According to Park et al.[29] mice fed with an oleuropein-supplemented diet attenuated hepatic steatosis in microscopic and macroscopic scale. According to the literature and our results, OLE or oleuropein-supplemented diet has protective effects on liver diseases.

In literature, via various mechanisms, olive leaves may induce apoptosis and inhibit cell proliferation. Oleuropein stops cell growth and promotes the apoptosis in HT29 colorectal cancer cells via a p53-dependent pathway.[25,26] This apoptotic effect with a similar mechanism was also reported in breast cancer cells.[30] In addition, on doxorubicin-induced cardiomyopathy, oleuropein, major phenolic of olive leave showed a protective effect through AMPK activation and iNOS suppression.[27] This study revealed that OLE, which contains oleuropein as major secondary metabolites has antitumor activity in the hepatoma cell lines. These results are partly consistent with the apoptosis mechanism described in MCF-7 cells treated with oleuropein.[30] iROS is thought to be related the activation of AKT.[31,32] Oleuropein may suppress the phosphorylation of AKT. Apoptotic effects of OLE may be related PI3K/AKT pathway, which is a critical pathway for H4IIE cells.

Antigenotoxic agents often show expected therapeutic effects that may be effective to control the cancer. Our results were

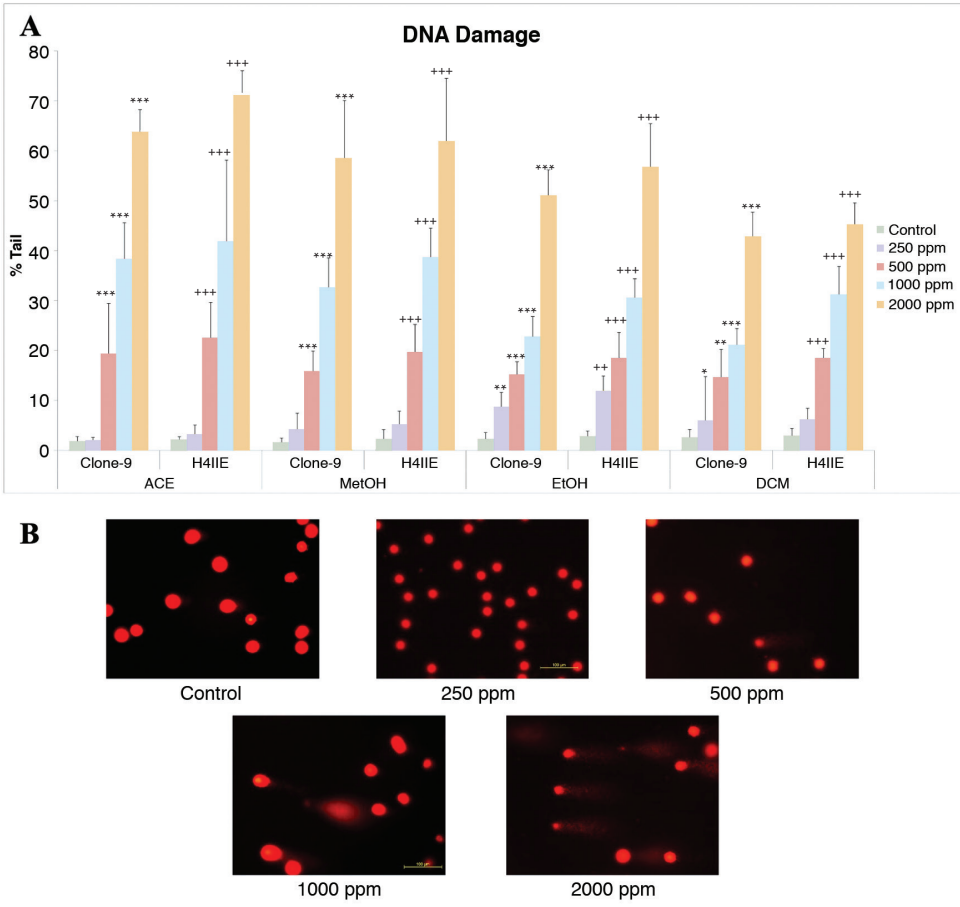

Figure 3. Olive leave extracts cause DNA damage with increasing concentrations (250, 500, 1000, and 2000 ppm) on H4IIE cells. (A) Genotoxic effects of olive leaf extract with increasing concentrations (250, 500, 1000, and 2000 ppm) using comet assay after 24-hour incubation. (B) Representative immunofluorescence images in H4IIE cells. The level of DNA damage increased with increasing concentrations of O-ACE. O-ACE showed the highest genotoxic damage among all extracts. Differences between the groups were tested for significance using One-Way ANOVA (*p<0.05, **p<0.01, ***p<0.001, +p<0.05, ++p<0.01, +++p<0.001). O-DCM: OLE with dichloromethane, O-ACE: OLE with acetone, O-MeOH: OLE with methanol, O-EtOH: OLE with ethanol

promising that OLE reduced cell viability in H4IIE cells and were consistent with the literature showing that OLE may inhibit the proliferation of malignant cells *in vivo*.[29-34] This feature of OLE may be explained by its capacity to act as a strong free radical scavenger, as shown in Figure 1.

Our results pointed out, OLE have induced the IL-1β, IL-6, and TNF-α production in a concentration-dependent manner. A significant decrease in the proinflammatory cytokine levels was found in cells treated with different OLE. Our results were also consistent with the literature in terms of anti-inflammatory effects.[9] In comparison with the healthy and cancer cell lines, anti-inflammatory effects were significantly higher in healthy cell lines. On the other hand, the increased inflammatory response of cancer cell lines is desirable, which starts apoptosis cascades. The possible anti-inflammatory mechanism may be related with arachidonic acid pathway, mitogen-activated protein kinase (MAPK), and MAPK enzymes such as p38, extracellular signal-related kinase, and c-Jun N terminal kinase induce transcriptional and post-transcriptional, activation of COX enzymes. Anti-inflammatory effects of *O. europaea* L. may be accomplished through NFκB activation. Inhibition of this proinflammatory cytokines production could be related either with the oleuropein or other secondary metabolites.[35] Oleuropein is the fundamental OLE component that exhibits anti-inflammatory effects at a concentration of 20 μg/mL.[9] In another study, OLE reduced mRNA expression of E-selectin in human coronary artery endothelial cells stimulated with serum amyloid-A, as well as decreased IL-6 and IL-8 protein levels and reduced matrix metalloproteinase 2 levels in non-stimulated cells.[34] In both studies, the amount of OLE used was 20 μg/mL and 0.5-1 μg/mL, respectively. Compared with our OLE (0.25-2 mg/mL) concentrations, these results show that high amounts of OLE show pro-oxidant activity.

CONCLUSION

To the best of our knowledge, this is the first study in the literature investigating the cytotoxic, genotoxic, and apoptotic activity of OLEs on liver cancer cell lines. OLEs activated cytotoxic and apoptotic mechanisms on cancer cells. For this reason, OLEs may have a potential for the cancer treatment. More detailed *in vivo* studies are needed in the future.

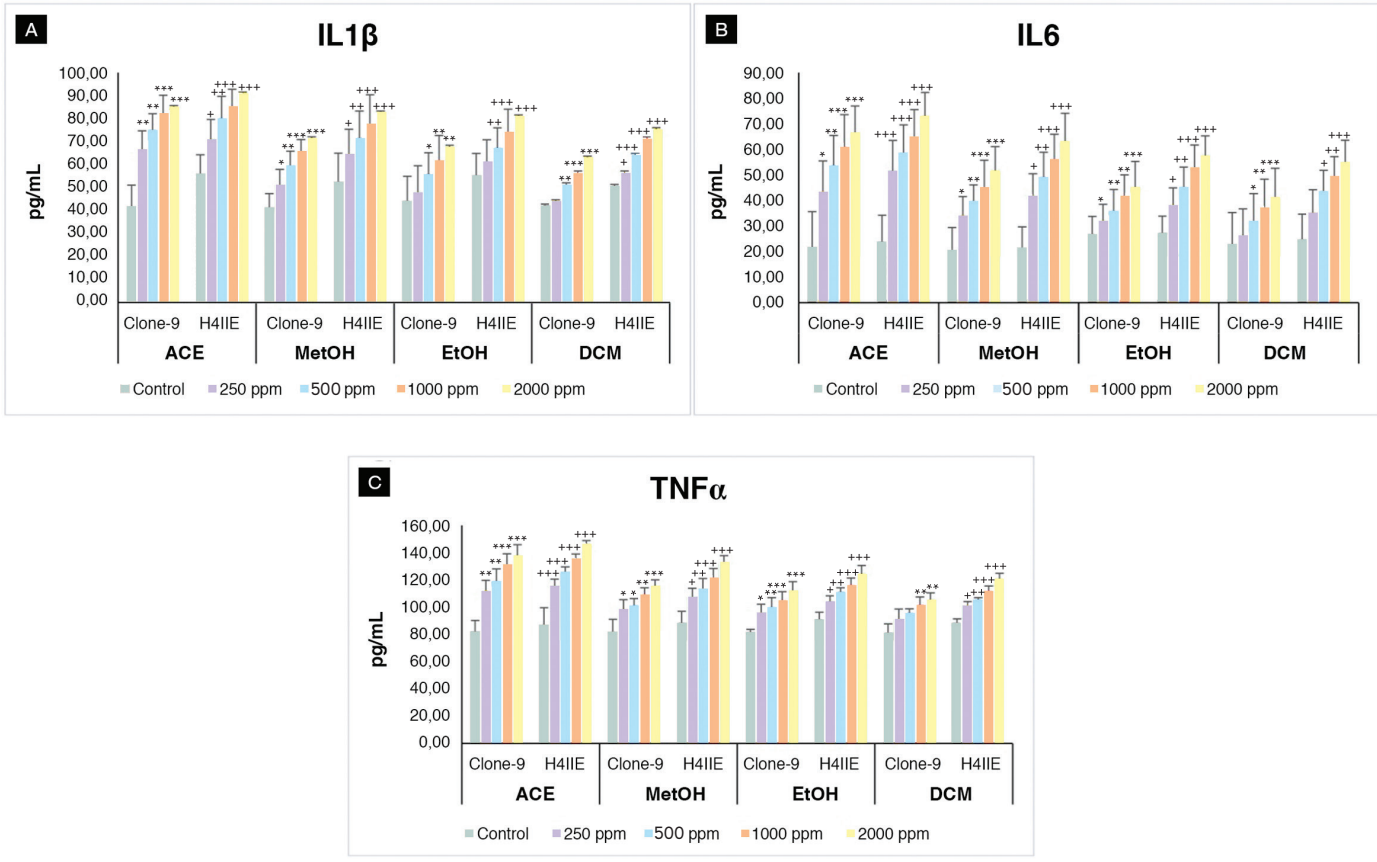

Figure 4. Levels of IL-1β, IL-6, and TNF-α with the increasing concentrations (250, 500, 1000, and 2000 ppm) of OLE on clone-9 and H4IIE cells after 24-hour incubation. (A) IL-1β levels (B) IL-6 levels (C) TNF-α levels. Differences between the groups were tested for sign+ificance using One-Way ANOVA (*p<0.05, **p<0.01, ***p<0.001, $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001). O-DCM: OLE with dichloromethane, O-ACE: OLE with acetone, O-MeOH: OLE with methanol, O-EtOH: OLE with ethanol, IL: Interleukin

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ORIGINAL ARTICLE

Cocrystal Construction Between Rosuvastatin Calcium and L-asparagine with Enhanced Solubility and Dissolution Rate

Gelişmiş Çözünürlük ve Çözünme Hızına Sahip Rosuvastatin Kalsiyum ve L-asparajin Arasındaki Kokristal Konstriksüyon

Venkata Deepthi VEMURİ[1]*, Srinivas LANKALAPALLI[2]

[1]Maharajah's College of Pharmacy, Department of Pharmaceutical Technology, Vizianagaram, India
[2]GITAM (Deemed to be University), Department of Pharmacy, Andhra Pradesh, India

ABSTRACT

Objectives: Rosuvastatin calcium (RSC) is a synthetic biopharmaceutical classification system class-II drug with a low solubility but high permeability. The drug is used in hyperlipidemia management. In this study, the physicochemical properties of RSC were modified via crystal engineering to produce a cocrystal form. The solvent evaporation method was used to fabricate RSC cocrystals with the generally recognized as safe status coformer, L-asparagine.

Materials and Methods: The obtained cocrystals were evaluated using powder X-ray diffraction (PXRD), scanning electron microscopy, fourier-transform infrared spectroscopy, differential scanning calorimetry (DSC), and fourier-transform nuclear magnetic resonance (FT-NMR).

Results: The PXRD analysis revealed the presence of unique crystalline peaks, which provide details of interactions between the active pharmaceutical ingredient and coformer. The changes in the thermal behavior of the cocrystals were confirmed by DSC studies. The formation of a hydrogen bond between the drug and conformer was confirmed by a change in the chemical shift values of the FT-NMR spectra at the O-H group. Comparative studies of the solubility and dissolution rate revealed that the solubility and dissolution rate of the obtained cocrystals were almost two times higher than those of the parent drug.

Conclusion: A new cocrystal form of RSC was obtained with a higher solubility and dissolution rate than those of the parent drug, implying new applications for these cocrystals.

Key words: Cocrystals, rosuvastatin calcium, solubility, dissolution, solvent evaporation cocrystallization

ÖZ

Amaç: Rosuvastatin kalsiyum (RSC), çözünürlüğü düşük ancak geçirgenliği yüksek, sentetik bir biyofarmasötik sınıflandırma sistemi sınıf-II ilacıdır. İlaç hiperlipidemi tedavisinde kullanılır. Bu çalışmada, RSC'nin fizikokimyasal özellikleri, bir kristal formu üretmek için kristal mühendisliği yoluyla modifiye edilmiştir. Genel olarak güvenli durum koformeri olarak kabul edilen L-asparagin ile RSC kristallerini imal etmek için çözücü buharlaştırma yöntemi kullanılmıştır.

Gereç ve Yöntemler: Elde edilen kokristaller, toz X-ışını kırınımı (PXRD), taramalı elektron mikroskobu, fourier-dönüşümlü kızılötesi spektroskopisi, diferansiyel taramalı kalorimetri (DSC) ve fourier-dönüşümlü nükleer manyetik rezonans (FT-NMR) kullanılarak değerlendirilmiştir.

Bulgular: PXRD analizi, aktif farmasötik bileşen ve konformer arasındaki etkileşimlerin ayrıntılarını sağlayan benzersiz kristalli tepe noktalarının varlığını ortaya çıkarmıştır. Ko-kristallerin termal davranışındaki değişiklikler DSC çalışmaları ile doğrulanmıştır. İlaç ve konformer arasında bir hidrojen bağı oluşumu, O-H grubunda FT-NMR spektrumlarının kimyasal kayma değerlerindeki bir değişiklikle doğrulanmıştır. Çözünürlük ve çözünme hızının karşılaştırmalı çalışmaları, elde edilen kristallerin çözünürlüğünün ve çözünme hızının, ana ilacınkinden neredeyse iki kat daha yüksek olduğunu ortaya koymuştur.

Sonuç: Ana ilacınkinden daha yüksek çözünürlük ve çözünme hızı ile RSC'nin yeni bir kristal formu elde edilmiştir ki bu da bu kokristaller için yeni uygulamalar anlamına gelmektedir.

Anahtar kelimeler: Kokristaller, rosuvastatin kalsiyum, çözünürlük, çözünme, çözücü buharlaşma ile kristalleşme

***Correspondence:** deepthichowdary438@gmail.com, **Phone:** +919032292387 **ORCID-ID:** orcid.org/0000-0003-3243-4340

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INTRODUCTION

Solid dispersions and cocrystals have emerged in the evolution of supramolecular systems, such as hybrid liquisolid systems, i modern investigations.[1,2] Crystal engineering is the process of creating supramolecules with specific structures and distinct physicochemical properties. Cocrystals are a general term for all products that have non-covalent or ionic intermolecular interactions between two or more dissimilar molecules with certain stoichiometric ratios in the crystal lattice and are prepared or developed using a crystal engineering approach;[3,4] one of the moieties selected must be an active moiety. Generally, the conformer, which often has no pharmacological efficacy, is chosen from the generally recognized as safe (GRAS) or everything added to food in the United States list.[5,6] Therefore, a practical approach is used for organizing the physicochemical properties that have no medical efficacy and diversity.[7,8] Over the past few decades, many studies have demonstrated a significant increase in the use of the cocrystallization approach and its feasibility in a formulation as an optimized approach for modulating physicochemical and biopharmaceutical properties of active moieties.[9,10]

Rosuvastatin calcium (RSC) is a synthetic biopharmaceutical classification system class-II drug that is used to treat hyperlipidemia by increasing high-density lipoprotein cholesterol and decreasing triglycerides, low-density lipoprotein cholesterol, and apolipoprotein. It can be used to decrease the progression of atherosclerosis and prevent coronary heart diseases.[11] Rosuvastatin, also known as "super statin", has demonstrated acceptable results in terms of potency and safety.[12,13] RSC exhibits low solubility in gastrointestinal fluids because it has low water solubility (0.33 mg/mL) due to its crystalline nature.[14,15] RSC has a 20% oral bioavailability due to pervasive first-pass hepatic biotransformation,[16] and high doses have been associated with increased hematuria, proteinuria, serum creatinine, and rhabdomyolysis.[17]

Cocrystallization of RSC may be a possible formulation path for increasing the bioavailability of molecules without changing the chemical integrity and upholding the physical stability. RSC cocrystals with coformers, such as sorbitol[18] and vanillin,[19] have been generated with improved bioavailability, resulting in their ability to modify physicochemical properties.[20] Ferrari et al.[21] reported methods for preparing RSC using three RSC cocrystals, such as rosuvastatin 2-aminopyrimidine hemihydrate, rosuvastatin pyrazine hydrate, and rosuvastatin quinoxaline.

In most cases, the choice of coformer should be based on the Cambridge structural database (CSD), functional/structural possessions, and risk-free GRAS status.[22,23] The chosen conformer must contain a group that can develop molecular synthons with active moieties. As an applicable companion, choosing amino acids that fall under the GRAS group is a beneficial option because they are inexpensive and have low toxicity. Researchers have previously reported that amino acids form salt when they react with several classes of therapeutic agents.[24,25]

This study aimed to use the solvent evaporation method to develop RSC cocrystals with the L-asparagine (ASN) conformer from the GRAS group in order to modify the physicochemical properties of the molecule. Figure 1 depicts the molecular structures of RSC and ASN. The obtained cocrystals were characterized by infrared (IR) spectroscopy, differential scanning calorimetry (DSC), powder X-ray diffraction (PXRD), and scanning electron microscopy (SEM). *In vitro* studies, such as apparent solubility studies, dissolution studies, and stability studies, were conducted on RSC and its cocrystals, and the results were compared.

MATERIALS AND METHODS

Materials

RSC was obtained from Apex laboratory Pvt ltd., Chennai. ASN and methanol (chromatographic grade) were purchasedfrom

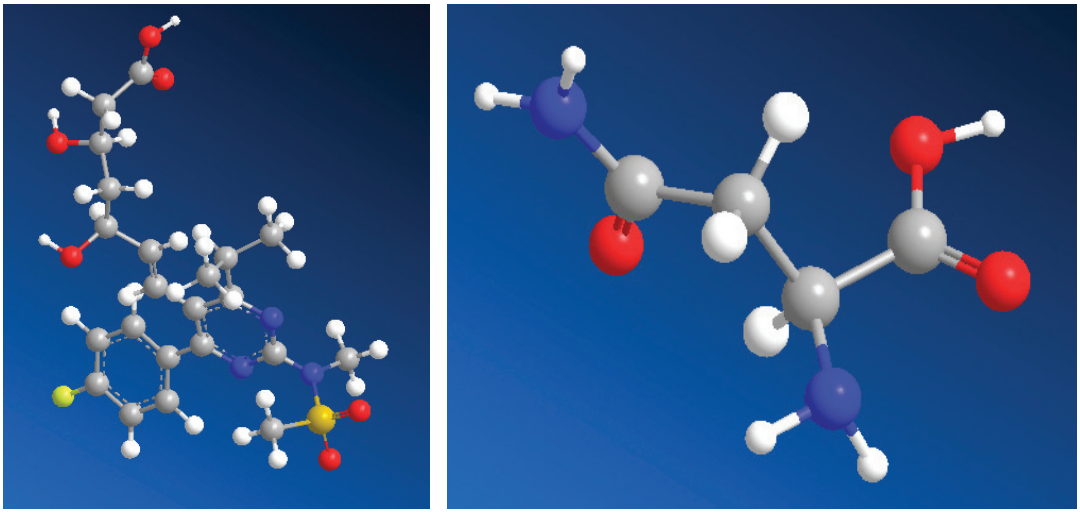

Figure 1. Molecular structures of the pure RSC and ASN

RSC: Rosuvastatin calcium, ASN: L-asparagine

Lotus chemicals. The chemicals used in the study were of analytical reagent grade.

Coformer selection

Amino acids may be the initial attraction in the formation of cocrystals. Their zwitterionic potential allows them to form zwitterionic cocrystals.[26,27] Based on the CSD,[28] structural research, and Scifinder literature scanning program, Tilborg et al.[27] provided a list of amino acids that indicate the formation of cocrystals in his mini-review, which may be useful to many researchers in choosing a suitable amino acid as a coformer. According to his findings, ASN does not carry any controversial side chains, allowing it to exist in a zwitterionic state and produce cocrystals.[29]. The stoichiometric ratio of the drug and coformer was selected for this study because of its safety and effectiveness in the preparation of the cocrystals.

Rosuvastatin-ASN cocrystal synthesis

The stoichiometric ratio of RSC (150 mg) and ASN (42 mg) was solubilized in 5 mL of methanol and 1.5 mL of water separately to form a clear solution using a sonicator. Both solutions were mixed in a magnetic stirrer at 900 rpm. The stirring process continued until the solvent evaporated completely at room temperature. To ensure the removal of the unbound drug and conformer, the obtained cocrystals were washed 3-5 times with methanol/water (5:1) and filtered using 0.45 µm membrane filters. The resulting product was dried overnight in a desiccator. The solvent evaporation method was used to produce large-scale samples for evaluation.

Aqueous solubility studies

Solubility analysis was conducted in distilled water and pH 1.2 buffer, pH 4.5 buffer, and pH 6.8 phosphate buffer solutions. Excess amounts of RSC (approx. 50 mg) and RSC-ASN (RSC-C) cocrystals (Eq. wt. of 50 mg of pure RSC) were placed in screw-capped vials containing 10 mL of distilled water and pH 6.8 phosphate buffer, with continuous stirring for 48 h in a water bath shaker. This was maintained at 37°C and 200 rpm and filtered using a 0.45 µm membrane filter. A quantitative analysis of RSC in the filtrate was performed spectrophotometrically using an ultraviolet (UV) spectrophotometer (V-630, Jasco, Japan) at 244 nm. Equilibrium analysis was conducted three times, and the residual solvents were centrifuged and analyzed by Fourier-transform IR (FTIR) spectroscopy.

In vitro dissolution studies

In vitro dissolution of RSC and the obtained cocrystals was determined using a 900 mL buffer solution (i.e., pH 6.8 phosphate buffer) at 37°C and 50 rpm as per U.S. Pharmacopeia (USP). This was performed using a USP Apparatus-I dissolution vessel (TDL-08L, Electrolab, India). The highest doses of RSC (40 mg) and cocrystals (Eq. wt. of RSC 40 mg) were weighed, and the intact powder was placed in a dissolution vessel. At time intervals of 0, 10, 20, 30, 40, 50, and 60 min, 5 mL of the samples were collected and replenished with a new buffer. The drug content in the filtered sample was measured spectrophotometrically using a UV spectrophotometer (V-630, Jasco, Japan).

PXRD analysis

A PXRD diffractometer (Philips Xpert MPD, Philips, Holland) was used to obtain diffractogram patterns of RSC, ASN, and the obtained product. The instrument was equipped with a Cu target X-ray tube source and a Xe-filled counteract or a proportional detector. The diffraction data were collected by maintaining tube voltage and current at 30 kV and 15 mA, respectively, for 2θ scan axes using a scan range of 5°-65°, a step width of 0.02°, and a scan speed of 10.00°/min. The JCPDF database software was used to determine the peak intensity of each sample.

FTIR spectroscopy

FTIR spectra of all the samples were obtained using the FTIR Azilent carry 360 series, which consists of a DLATGS detector with a 2 cm^{-1} spectra resolution. A powdered sample of 2-4 mg was kept in a sample holder and scanned over a range of 4000-400 cm^{-1}, and the obtained data were analyzed using the OPUS spectral software.

SEM analysis

Surface images of the pure RSC drug, ASN coformer, and obtained cocrystals were acquired at various magnifications using a SEM (XL30ESEM) with EDAX equipped with a secondary and backscattered electron detector. Samples were attached to carbon tabs, placed on aluminum pin stubs, and sputter-coated with gold/palladium under vacuum in preparation for SEM analysis.

DSC analysis

All the samples were measured using a Mettler Toledo DSC 821e instrument under nitrogen purge (30 mL/min). Powder samples (2-5 mg) were loaded into aluminum pans and sealed. The samples were scanned at temperatures of 30°C to 350°C at a heating rate of 10°C/min. The data was collected using the TAQ series advantage software.

^{1}H liquid fourier-transform nuclear magnetic resonance (FT-NMR) spectroscopy

The pure RSC drug and RSC-C cocrystals were dissolved in deuterated dimethyl sulfoxide, while the ASN conformer was dissolved in deuterated water for FT-NMR analysis. Chemical shifts were observed in the ^{1}H FT-NMR spectra of the RSC, ASN, and RSC-C cocrystals, which were recorded on a 400 MHz FT-NMR spectrometer (model: JNM- ECz 400S).

Product yield and drug content estimation:

The prepared cocrystals were collected and weighed accurately, and the product yield was calculated by dividing the actual weight of the obtained cocrystals by the total weight of the drug and coformer.

$$\text{Percentage yield} = \frac{\text{Weight of the cocrystals}}{\text{Total weight of the drug and coformer}} \times 100 \quad (1)$$

The prepared cocrystals were accurately weighed to be equivalent to 100 mg of the pure drug and dissolved in 100 mL of a pH 6.8 phosphate buffer solution. The solution was filtered, and the drug content was determined using a UV-visible spectrophotometer at 244 nm.

Micromeritic evaluation of the cocrystals

The micromeritic properties of the RSC-C cocrystals were compared to those of the pure RSC drug. The bulk density, tapped density, Hausner ratio, Carr's index, and repose angle of RSC and RSC-C were all measured. The bulk density was determined using USP method I, whereas the tapped density was determined using USP method II with a tapped density tester (Aymes, Turkey). The following equations were used to calculate the Hausner ratio, Carr's index, and repose angle.

$$\text{Hausner ratio} = \text{tapped density}/\text{bulk density} \quad (2)$$

$$\text{Carr's index\%} = (\text{tapped density} - \text{bulk density}) \times 100/\text{tapped density} \quad (3)$$

$$\text{Tan } \theta = 2h/D \quad (4)$$

Statistical analysis

The results of the percentage yield, micromeritic evaluations, solubility, and dissolution studies were expressed as mean (M) ± standard deviation.

RESULTS

Aqueous solubility analysis

Solubility analysis was conducted in distilled water and pH 1.2 buffer, pH 4.5 buffer, and pH 6.8 phosphate buffer solutions. The pure RSC concentrations in water, pH 1.2 buffer, pH 4.5 buffer, and pH 6.8 phosphate buffer were 0.836 mg/mL, 0.624 mg/mL, 1.143 mg/mL, and 1.427 mg/mL, respectively, after 48 h of continuous stirring. The solubility of the physical RSC-C mixture did not differ significantly from that of the pure active pharmaceutical ingredient (API). When compared to pure RSC, the RSC-C cocrystals exhibited a 2.17-fold, 2.21-fold, 2.29-fold, and 2.42-fold increase in the solubility in water, pH 1.2 buffer, pH 4.5 buffer, and pH 6.8 phosphate buffer, respectively (Table 1). The crystal arrangement and cocrystallization process could be the reasons for these outcomes.[30] Since RSC is poorly soluble in water,[14,15] the cocrystal solubility greatly relies on the solubility of its composition.[31] When compared to that of pure RSC, the improved solubility could also be due to its relatively small particle size. This establishes the possibility of RSC-C cocrystals to be used in new product formulations.

PXRD analysis

The diffractogram pattern of the RSC-C cocrystals exhibited unique crystalline peaks when compared to those of RSC and the coformer. Figure 2 shows the PXRD pattern of the pure drug, coformer, and obtained cocrystals.

The pure RSC showed a single 2θ scattering angle at 43.25°, indicating the amorphous nature of the drug.[32] The RSC-C cocrystals showed new characteristic peaks at 9.14°, 10.28°, 20.85°, 24.61°, 32.93°, and 40.06°, which were absent in the pure RSC and ASN diffractogram patterns. However, in the cocrystals, the ASN peaks of 11.85°, 17.70°, 18.23°, 19.91°, 27.84°, and 43.15° shifted to 11.71°, 17.53°, 18.05°, 19.73°, 27.72°, and 42.46°, respectively. When comparing the diffractogram pattern of the cocrystals to that of the pure drug and conformer, the appearance/disappearance and shifting of the peaks in the cocrystal diffractogram pattern indicate the evaluation of a new crystalline phase.

FTIR studies

FTIR spectroscopy was used to demonstrate the interaction between RSC and ASN. The FTIR spectra of RSC showed characteristic peaks corresponding to carboxylic O-H stretch at 3382 cm^{-1}, N-H stretch at 2968 cm^{-1}, C=C stretch at 1541 cm^{-1}, asymmetric vibration of CH_3 at 1436 cm^{-1}, C-F stretch at 1149 cm^{-1}, symmetric vibration of CH_3 at 1379 cm^{-1}, and C-H plane bending of the aromatic ring at 775 cm^{-1}.[33] ASN had characteristic peaks corresponding to O-H stretch at 3436 cm^{-1}, N-H stretch at 2924 cm^{-1}, and C=O stretch at 1716-1750 cm^{-1}.[34]

Figure 3 shows the IR spectra of RSC, ASN, and the RSC-C cocrystals. When compared to those of the pure RSC, the N-H group of the cocrystals shifts to 2931 cm^{-1}, while the O-H group

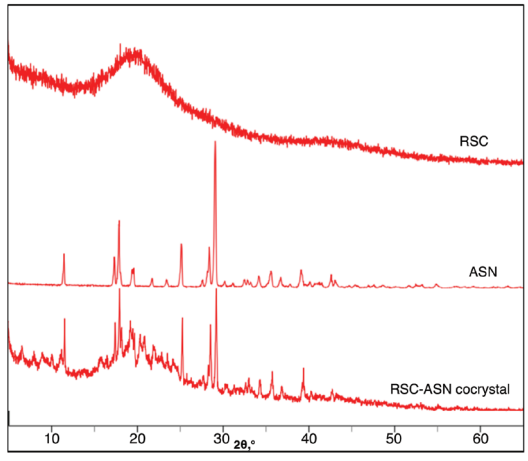

Figure 2. PXRD patterns of the pure RSC, ASN, and RSC-C cocrystals
PXRD: Powder X-ray diffraction, RSC: Rosuvastatin calcium, ASN: L-asparagine

Table 1. Aqueous solubility of rosuvastatin calcium

Chemical moiety	Solubility in water (mg/mL)	Solubility in pH 1.2 buffer (mg/mL)	Solubility in pH 4.5 buffer (mg/mL)	Solubility in pH 6.8 phosphate buffer (mg/mL)
Rosuvastatin calcium	0.836±0.036	0.624±0.052	1.143±0.058	1.427±0.034
Rosuvastatin + L-asparagine physical mixture	0.948±0.052	0.745±0.078	1.236±0.034	1.247±0.042
Rosuvastatin-asparagine cocrystals	1.817±0.066	1.379±0.065	2.612±0.087	3.466±0.057

Results are given as mean ± standard deviation, n=3

shifts to 3421 cm^{-1}, indicating new hydrogen bond formation in the cocrystals. Few additional peaks in the range of 1638-1750 cm^{-1} were observed in addition to the characteristic peaks of RSC because of the carboxylic acid moiety, which expresses the presence of C=O stretching in the cocrystals. When compared to the pure RSC, the cocrystals exhibited a change in the chemical environment, which was consistent with the PXRD and DSC results and confirmed the interaction between RSC and ASN.

SEM analysis

Figure 4 shows the SEM micrographs of RSC, ASN, and the RSC-C cocrystals obtained by solvent evaporation. RSC had irregular granular-shaped particles, with not much difference in the morphology of the pure RSC and physical RSC-C mixture. ASN exhibited a stick-shaped crystalline morphology. The cocrystals obtained via solvent evaporation underwent a phenomenal transformation into an irregular closely fitted crystalline structure. The formation of intermolecular hydrogen bonds between RSC and ASN could be the cause of the change.

DSC analysis

DSC data of the RSC-C cocrystals were obtained and compared with those of the pure drug and coformer, and the results are shown in Figure 5. The DSC thermogram of the pure RSC drug showed broad endothermic peaks at 80.7°C and 217.9°C, indicating that the drug substance had an amorphous form.[35] The thermal curve of ASN exhibited a sharp endothermic peak at

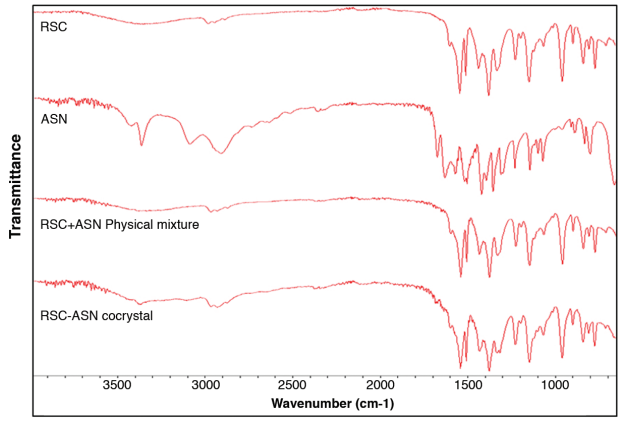

Figure 3. FTIR spectra of the pure RSC, ASN, RSC-C physical mixture, and RSC-C cocrystals

FTIR: Fourier transform infrared spectroscopy, RSC: Rosuvastatin calcium, ASN: L-asparagine

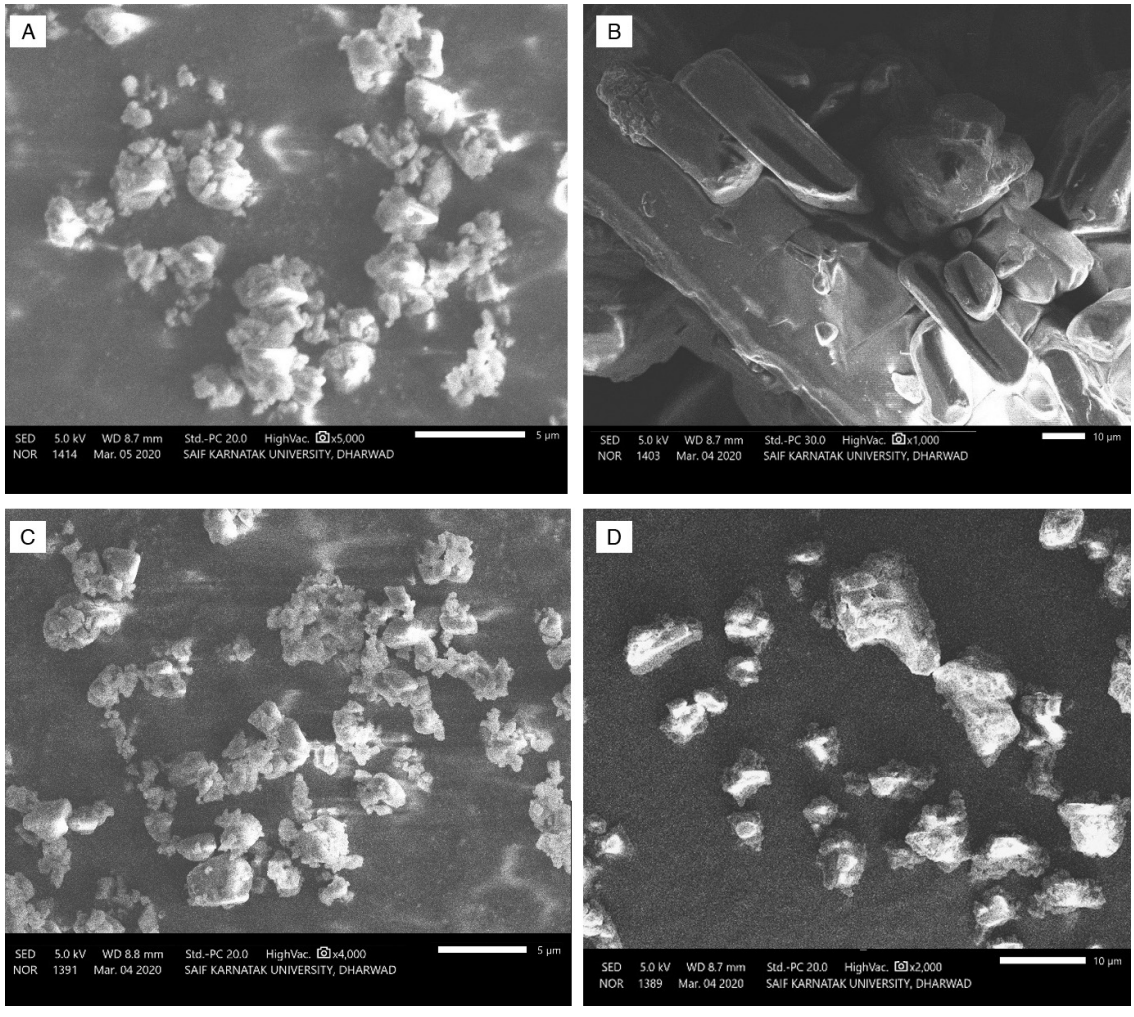

Figure 4. SEM micrographs of (A) pure RSC, (B) ASN, (C) RSC-C physical mixture, and (D) RSC-C cocrystals

SEM: Scanning electron microscopy, RSC: Rosuvastatin calcium, ASN: L-asparagine

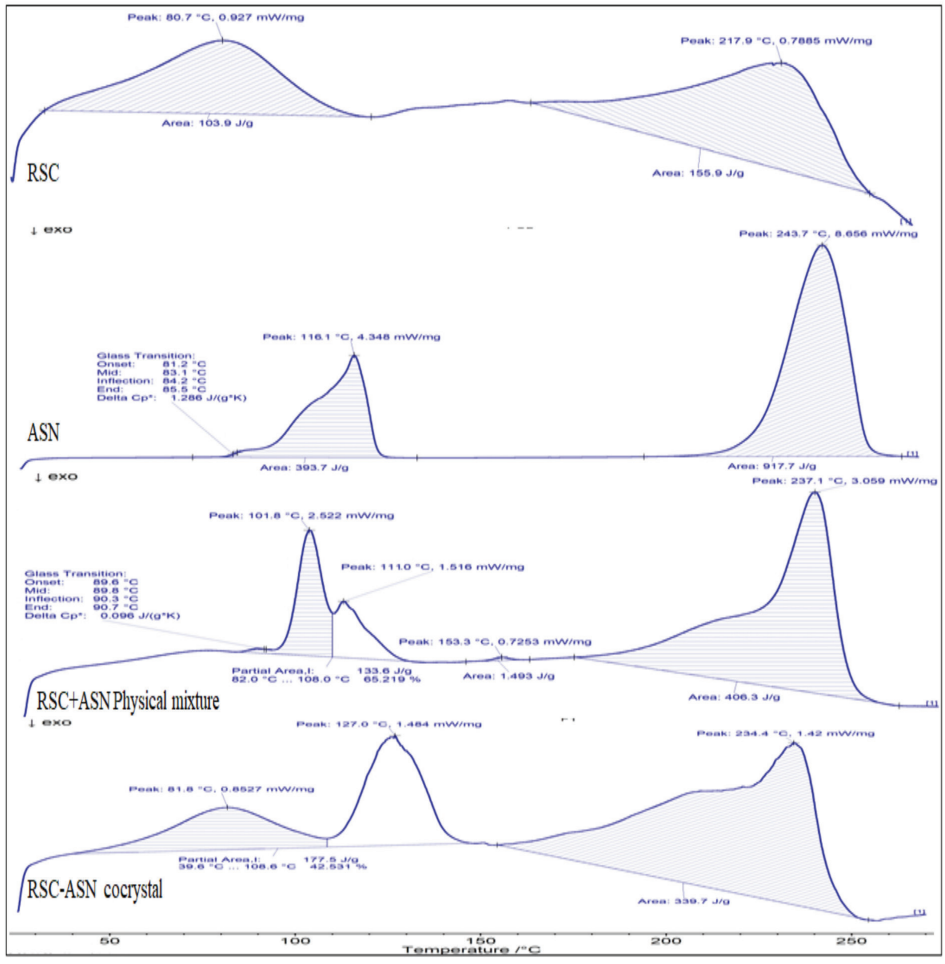

Figure 5. DSC thermograms of pure RSC, ASN, RSC-C physical mixture, and RSC-C cocrystals

DSC: Differential scanning calorimetry, RSC: Rosuvastatin calcium, ASN: L-asparagine

116.1°C and 237.1°C with high enthalpy. The physical mixture (1:1 ratio) of RSC and ASN exhibited endothermic peaks at 101.8°C and 237.1°C, with slight shifting and broadening of peaks. This may be due to the loss of purity of each compound when mixed together rather than a fundamental indication of incompatibility. The RSC-C cocrystal obtained by solvent evaporation had a unique characteristic sharp endothermic peak at 127°C with a high intensity that was different from those of the pure RSC and ASN coformer. According to many researchers, most cocrystals melt at a temperature that is different from that of their APIs and coformers. According to the Perlovich[36] study, the melting points of the obtained cocrystals are often in the middle of (55.3%), less than (38.9%), or higher than (14.5%) that of the starting materials. The RSC-C cocrystals exhibited middle endotherm when compared to the starting materials on the DSC profile, which is a very common phenomenon in 1:1 stoichiometric cocrystals. The changes in the crystalline structure and cocrystal formation are indicated by a change in the melting point. The PXRD and FTIR analyses confirmed these changes.

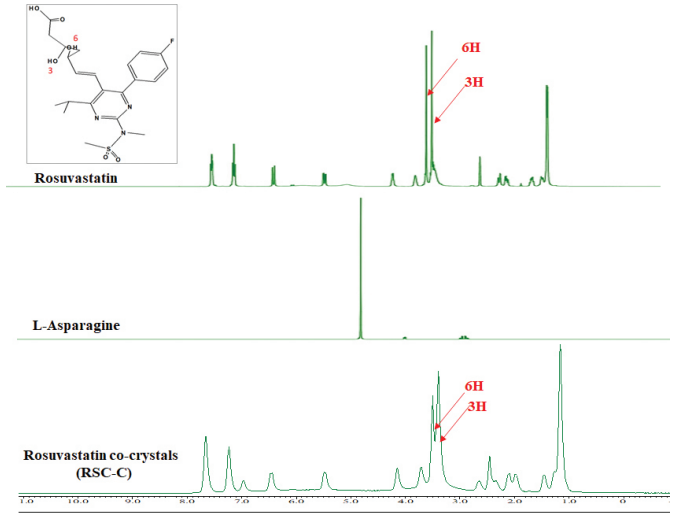

Figure 6. FT-NMR spectra of the pure RSC, ASN conformer, and RSC-C cocrystals

FT-NMR: Fourier-transform nuclear magnetic resonance, RSC: Rosuvastatin calcium, ASN: L-asparagine

^{1}H liquid FT-NMR spectroscopy

Figure 6 shows the ^{1}H FT-NMR spectra of RSC, ASN, and the RSC-C cocrystals. When the NMR spectra of the RSC-C cocrystals were compared with the pure drug (RSC) spectra, changes in the chemical shift values were observed at 3H from 3.395 to 3.428 and 6H from 3.500 to 3.533, respectively. The chemical shift value confirmed cocrystal formation between the drug and conformer, which was probably due to interactions between the free hydroxyl group of RSC and the amine moiety of ASN. Furthermore, the FT-NMR analysis results were in total agreement with the PXRD, DSC, and FTIR analysis results.

In vitro dissolution analysis

The cumulative dissolution rate was determined for the pure RSC and RSC-C cocrystals obtained by solvent evaporation. The dissolution rate was measured in pH 6.8 buffer, and the results are presented in Figure 7. RSN showed a 0.86% dissolution rate immediately after the addition of a solvent, whereas RSC-C cocrystal presented 3.8%. After 10 min, the cocrystals had a dissolution rate of 23.23%, while the pure drug had a 9.42% dissolution rate. The dissolution rate of the Pure RSC increased slowly and reached 42.64% within 2 h, whereas that of the cocrystals increased quickly, reaching 91.02% within 2 h. The increase in the dissolution rate of the RSC-C cocrystals is because of the change in the crystal morphology and the tiny particle size, as shown in the SEM analysis.[37] A change in the melting point of the DSC profile indicates more dissolution.[38,39] The FTIR, PXRD, SEM, DSC, solubility, and dissolution analyses revealed altered dissimilarities between the cocrystal properties and the pure API properties, which are due to the change in the crystalline structure.

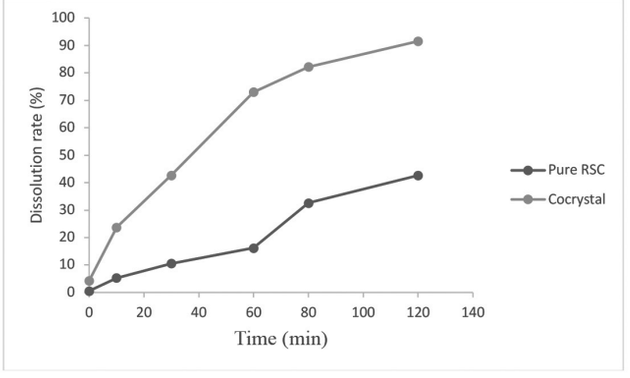

Figure 7. Dissolution rate (%) of the pure RSC and RSC-C cocrystals
RSC: Rosuvastatin calcium

Percentage yield and drug content estimation

The percentage yield and drug content estimation of the obtained cocrystals were conducted in triplicate. The percentage yield of the cocrystals was found to be 95.75%±0.25%, while that of the drug content was estimated to be 98.4%±0.43%. The results indicated that the obtained cocrystals had a good product yield with an acceptable drug content.

Micromeritic evaluation of the cocrystals

Table 2 shows the micromeritic properties of RSC and RSC-C. The repose angle of the RSC-C cocrystal was 29˚.81'±0.45, whereas that of the pure RSC was 32˚.31'±0.52. In comparison to the pure RSC, this shows that the cocrystals have good flow properties. The compressibility of the RSC-C cocrystals was found to be 6.32%±0.68%, whereas that of the pure RSC was 11.24%±0.46%. The result demonstrated in comparison to the pure RSC, the cocrystals have excellent compressibility. The Hausner ratios of RSC-C and RSC were found to be 1.06%±0.02% and 1.18%±0.21%, respectively, indicating that RSC-C had better flow properties and compression strength than RSC.

DISCUSSION

The functional groups present in the drug and coformer molecules were the primary determinants of cocrystal formation in the synthon method.[40] The RSC consists of two oxydrillic groups that are bound to asymmetric carbon atoms. Therefore, ASN, which contains an amide group, was chosen as a coformer. The structural characterization confirmed the expected structural modification. The SEM analysis results revealed that the irregular granular nature of pure RSC changed to a closely fitted crystalline structure in the cocrystals. The average particle size of the RSC-C cocrystals was 4.3 µm, which is in between the average particle size of pure RSC and ASN. The alteration in the crystalline habit and the average particle size may have influenced the formation of a hydrogen bond between the drug and conformer. In the FTIR analysis, alterations in chemical structure included the broadening of the O-H stretching peak at 3382 cm^{-1} in the RSC-C cocrystals when compared to RSC, confirming the hydrogen bond interaction. The broad endothermic peak of RSC changed to a sharp endothermic peak in the RSC-C cocrystals with different melting points, indicating partial crystallization. According to Perlovich's[41] investigation, 28.9% of the cocrystal formulation had a lower melting point than the parent molecules. The decrease in the melting point and enthalpy indicates the formation of a weak crystalline structure.[42-44] According to the PXRD diffractogram, RSC had an amorphous form. This was confirmed by the broad peaks in the DSC profile.[45] However, new characteristic peaks

Table 2. Micromeritic evaluation of the cocrystals

Micromeritic evaluation	Pure RSC	RSC-C cocrystals
Angle of repose (Ø)	32.31±0.56	2.81±0.45
Carr's index (%)	11.24±0.46	6.32±0.68
Hausner's ratio	1.18±0.21	1.06±0.02

Results are given as mean ± standard deviation, n=3, RSC: Rosuvastatin calcium

other than RSC and ASN peaks were observed in the RSC-C cocrystals, indicating a change in the chemical environment and transition from a semi-crystalline form to a crystalline form. The ^{1}H liquid FT-NMR spectra revealed an exact shift in two oxydrillic groups to an up-field nature, indicating the transfer of electrons from the hydroxyl group during hydrogen bond interaction. All these results confirm the formation of cocrystals between the drug and coformer because of interactions between the free hydroxyl group of the drug and the amine group of the coformer. Afterward, comparative studies on the solubility and dissolution rate were conducted, and the results showed that the cocrystals had almost 2-fold higher solubility and dissolution rates than the parent molecule. The cocrystals demonstrated better micromeritic properties (flow properties, compressibility, and compression strength) than RSC, providing them an advantage in pelleting and tableting properties.

CONCLUSION

RSC-C cocrystals were successfully prepared using the solvent evaporation cocrystallization technique. The solubility and dissolution rates of the obtained cocrystals were almost 2-fold higher than those of the parent molecule. This demonstrates the removal of impediments to RSC entering the bloodstream, such as low solubility and low dissolution rates. The formation of a new crystalline form was affirmed by FTIR, PXRD, and FT-NMR analyses. These results were supported by a change in the melting point in the DSC thermogram compared to that of the pure RSC and ASN. The enhanced physicochemical properties of RSC contribute to the viability of novel formulations. According to the results, the micromeritic properties of the cocrystals provide the advantages of pelleting and tableting properties. The strategy for transforming a cocrystal idea into an application has been effectively explained in the literature, which is mainly helpful for the development of the process in future investigations.[46-48] However, the *in vivo* performance of RSC cocrystals, such as AUC and C_{max}, was not covered in this study, but it will be included in the next study. The results clearly indicate that this cocrystallization method improved the dissolution and solubility of RSC and caused a change in its chemical structure.

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REVIEW

Isoflavones in Soybean as a Daily Nutrient: The Mechanisms of Action and How They Alter the Pharmacokinetics of Drugs

Günlük Besin Olarak Soya Fasulyesindeki İzoflavonlar: Etki Mekanizmaları ve İlaçların Farmakokinetiğini Değiştirmeleri

Amelia SOYATA[1], Aliya Nur HASANAH[2], Taofik RUSDIANA[1]*

[1]Padjadjaran University Faculty of Pharmacy, Department of Pharmaceutics and Pharmaceutical Technology, Sumedang, Indonesia
[2]Padjadjaran University Faculty of Pharmacy, Department of Analysis of Pharmaceutical and Medical Chemistry, Sumedang, Indonesia

ABSTRACT

Soybeans [*Glycine max* (L.)] are a good source of isoflavones. The main isoflavone components of soybean are daidzein, genistein, and glycitein. World soybean production is very high. Because of its pharmacological activity, soy isoflavone intake over a long period of time may result in interactions with the drugs. This review summarizes soy isoflavone-drug interactions based on the pharmacokinetic parameters. Soy isoflavones have pharmacokinetic interactions with celecoxib, theophylline, paclitaxel, midazolam, imatinib, carbamazepine, valproic acid, repaglinide, omeprazole and danofloxacin. This is due to the changes in the area under the curve, maximum serum concentration, time that a drug is present at the maximum concentration in serum, clearance and half-life of the drugs when delivered together with soy isoflavones. The mechanisms of pharmacokinetic interactions occurs through the inhibition/induction of drug metabolizing cytochrome P450 (CYP450) enzymes such as CYP3A4, CYP2A1, and CYP2C9 or through the inhibition of drug transporters such as P-glycoprotein and breast cancer resistance protein. Thus, the consumption of soybean, soy isoflavones or soy products with drugs needs to be reconsidered.

Key words: Soybean, isoflavones, pharmacokinetic ınteraction, drug metabolizing enzyme, drug transporter

ÖZ

Soya fasulyesi [*Glycine max* (L.)] iyi bir izoflavon kaynağıdır. Soya fasulyesinin ana izoflavon bileşenleri daidzein, genistein ve glisitindir. Dünyada soya üretimi çok yüksektir. Farmakolojik aktivitesi nedeniyle, uzun süre soya izoflavon alımı, ilaçlarla etkileşime neden olabilir. Bu derleme, farmakokinetik parametrelere dayalı olarak soya izoflavon-ilaç etkileşimlerini özetlemektedir. Soya izoflavonları, selekoksib, teofilin, paklitaksel, midazolam, imatinib, karbamazepin, valproik asit, repaglinid, omeprazol ve danofloksasin ile farmakokinetik etkileşimlere sahiptir. Bunun nedeni, soya izoflavonları ile birlikte verildiğinde ilaçların eğrinin altındaki alan, maksimum serum konsantrasyonu, ilacın maksimum serum konsantrasyonunda bulunduğu süre, klirens ve yarılanma ömründe yaptığı değişikliklerdir. Farmakokinetik etkileşimlerin mekanizmaları ilaç metabolize eden sitokrom P450 (CYP450) enzimlerinin (örneğin; CYP3A4, CYP2A1 ve CYP2C9) inhibisyonu/indüklenmesi veya P-glikoprotein ve meme kanseri direnç proteini gibi ilaç taşıyıcılarının inhibisyonu yoluyla gerçekleşir. Bu nedenle soya fasulyesi, soya izoflavonları veya soya ürünlerinin ilaçlarla tüketimi yeniden gözden geçirilmelidir.

Anahtar kelimeler: Soya fasulyesi, izoflavonlar, farmakokinetik etkileşim, ilaç metabolize eden enzim, ilaç taşıyıcısı

***Correspondence:** t.rusdiana@unpad.ac.id, **Phone:** +62227796200, **ORCID-ID:** orcid.org/0000-0002-3321-2179
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INTRODUCTION

Soybeans [*Glycine max* (L.)] are a source of isoflavones in the daily meals. In 2016, global soybean production amounted to be 34,894,085 tons; with 293,414,006 tons from the Americas, 28,808,950 tons from Asia, 10,488,759 tons from Europe, and 2,119,814 tons from Africa. In 2016, total of 89.05% of soybean production was from five countries: India (4.18%), China (3.57%), Argentina (17.56%), Brazil (28.75%), and the USA (34.99%).[1] Soybeans contain non-steroidal polyphenol compounds[2] with a chemical structure similar to that of oestradiol-17β, so these compounds may have a similar effect to that of the estrogen.[3,4] The main isoflavone content of soybean is in aglycone form, including genistein, daidzein, and glycitein; the glycosidic forms are genistin, daidzin, and glycitin, which are precursors of the metabolic process which forms daidzein and genistein aglycones.[5] The total glycitein and glycoside content in soybeans is only 5-10% of the total isoflavones, while the remaining is comprised of daidzein and genistein.[6] Isoflavones have effects on postmenopausal nutrition,[7] relief of postmenopausal vasomotor symptoms,[8] osteoporosis,[9] inflammation,[10] and cardiovascular disease.[11] The compounds also have antioxidant activity,[12] increase the efficacy of cancer therapy,[13] and inhibit the cancer cell proliferation.[14]

Based on this pharmacological activity, soy isoflavones could be used as a dietary nutrition over a long period of time. Soybean consumption continued to increase in 2011.[15] Fonseca et al.[16] showed that the amount of isoflavones taken in by the infants fed with soy-based formula is 0.8 mg/day/kg of body weight; this number is two-fold higher than the level of isoflavones consumed by the adults in Japan. The daily intake of isoflavones is related to how much soy is consumed and differs in each country, [i.e., it is much higher in east and south Asian countries (20-50 mg/day), than in Europe (0.49-1 mg/day)].[17,18] To fulfill the daily nutrient needs, the Chinese government has recommended that every citizen consumes 50 mg of soy food daily. Simple processed soy foods from Asia usually contain 3.5 mg of isoflavones in every gram. Large studies performed in the United States showed that each adult there consumes 2.5 mg of isoflavones per day, but other research data shows different results where the consumption of isoflavones per day may reach the range of 30-50 mg. In China, the average daily consumption of isoflavones is 40.8±28.7 mg/day.[19]

Isoflavone consumption patterns in this community therefore raise the possibility of drug interactions when used together, so their use must be monitored. Drug interactions occur when other substances affect the activity of a drug.[20] These interactions may occur with the soy isoflavones. Soy extracts, soy products, and soy isoflavones have interactions with the drugs such as: Warfarin,[21] tamoxifen,[22] levodopa,[23] and ciprofloxacin.[24] The mechanism of the drug-isoflavone interaction is by the inhibition or induction of drug metabolizing enzymes (DMEs) or drug transporters.[25]

Almost all the drug biotransformation reactions need a metabolic enzyme, and the enzymes most often used to process the drugs are the liver microsomal cytochrome P450 (CYP450) enzymes. The CYP enzymes involved in the drug metabolism are CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5.[26] Drugs or bioactive compounds such as isoflavonoids interact with these enzymes, and change the efficacy and action of the drug.[27] Soybean products (infusions) have an inhibitory effect on human CYP enzymes, including CYP2C9, CYP2C19, CYP3A4, and CYP2D6.[28] It has also been reported that soy isoflavones reduce the hepatic CYP2E1 and CYP3A activities related to acetaminophen metabolism.[29]

Furthermore, drug transporters could be involved in the drug interactions, because drug transporters mediate the absorption, distribution, and excretion of the drugs in the transport process across the plasma membrane.[30] There are two classifications of these drug transporters: The ATP-binding cassette (ABC) family and the solute carrier (SLC) family. P-glycoprotein (P-gp) is a member of the ABC family, and could be induced by various factors, including clinical drugs, environmental xenobiotics, and dietary compounds;[31] which is known to be involved in the drug interactions. There are reports that genistein from soy inhibits the efflux of the P-gp substrates cimetidine[32] and paclitaxel.[33] The efflux of vinblastine in KB-V1 cells highly expressing P-gp, and the P-gp substrate paclitaxel could be inhibited by genistein at some doses.[34] In addition to the P-gp, interactions may occur through other drug transporters. So, DMEs and drug transporters play important roles in the absorption, distribution, metabolism, and the excretion (ADME) of the drugs, and are involved in the interactions that will affect the pharmacokinetics and pharmacodynamics of the drugs.

These pharmacokinetic interactions could be seen by assessing the pharmacokinetic parameters including the area under the curve (AUC), maximum concentration (C_{max}), volume of distribution (Vd), half-life ($t_{1/2}$), and clearance. Nagashima et al.[23] found that soybean increases the AUC of levodopa. Soybean also reduces the AUC and C_{max} of losartan.[35] These differences in pharmacokinetic parameters depend on the mechanism. Until now, there has been no summary to explain how soy isoflavones could affect the pharmacokinetic profile of a drug and the mechanisms involved. This is needed as a reference regarding the safety of using soy isoflavones as daily nutrients with the co-administration of the drugs.

Methods

This review is based on the literature collected from the internet through Google Scholar, Elsevier, PubMed, and NCBI, using the keywords soybean, soy products, soy isoflavones, soy drug interaction, isoflavone content, daidzein, genistein, isoflavone interaction, pharmacokinetic parameter, and the pharmacokinetic interaction. In total, 181 articles were collected, but only 99 articles were included based on the inclusion criteria. The inclusion criteria were articles with a publication year before 2000, containing a description of pharmacokinetic parameter values, describing interactions with soybeans, containing isoflavone content data, or related to isoflavones, soybeans, and pharmacokinetic interactions. The flowchart of the search is illustrated in Figure 1.

Soy isoflavones

Isoflavones are bioactive metabolites and include a group of phytoestrogens. Isoflavones have structures like those of mammalian estrogens. The largest source of isoflavones is soybean. Soy isoflavones are present in 12 different isoforms, divided into four chemical forms: Acetylglucoside (acetylgenistin, acetylglycitin, acetyldaidzin), malonylglucoside (malonylgenistin, malonyldaidzin, malonylglycitin), glucoside (genistein, daidzin, and glycitin), and aglycone (genistein, daidzein, and glycitein).[36] After the metabolism process in the human gut, glucoside isoflavones become aglycones through the effect of gastrointestinal enzymes.[5] Genistein, daidzein, and glycitein comprise approximately 50%, 40%, and 10% of the isoflavones in soybean.[37] The isoflavone content is influenced by several factors; in this article, we summarize the content of genistein, daidzein, and glycitein in soybeans, as seen in Table 1.[37-47] The amount of isoflavones is in the order genistein › daidzein › glycitein, and the content of the glycoside form is lower than that of the aglycone form; differences arise based on the variety, location of the production, humidity etc.[38] Sources of isoflavones include soy products such as traditional soy foods (such as tofu and soy milk), isolated soy protein, soybean paste, soy flakes, soy flour, fermented soybean products (such as tempeh, miso, and natto), and soy sauce.[39]

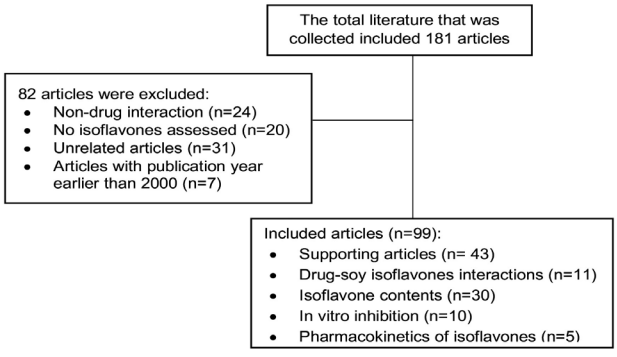

Figure 1. Flow chart of the literature review

In each type of soybean product containing different soy isoflavones, we summarize the isoflavone content focusing only on the aglycone form, (i.e., genistein, daidzein, and glycitein in various soy products from the several studies, presented in Table 2).[48-62] It appears that soy tablets commercially contain the highest levels of isoflavones, because soy tablets are usually used as additional nutrients so the soy isoflavone content is adjusted to nutritional requirements. Of the soy products shown Table 2, sufu has the highest content compared to others. Sufu is a traditional food from China, and it is made of fermented soybean curd.[48] Other fermented foods that also have high soy isoflavones content are natto, tempeh, and miso. The fermentation process influences the isoflavone content. Fermentation can increase aglycone isoflavones from black soybean pulp[49] in tempeh and tofu.[50,51] Another study reported a 75% increase in aglycone isoflavones in soybean flour after fermentation.[52] The fermentation process is also influenced by the several factors such as time and temperature.[53,54]

The differences in the isoflavone content of soybean products also leads to variations in the pharmacokinetic profile of isoflavones, as presented in Table 3. There are variations in the different levels, caused by many factors, [i.e., differences in the test subjects used (human, rats, or mice), variations in the age, the hydrolysis process of glycosides by the gut bacteria or gut wall enzymes, uptake, ethnicity, etc].[44] The content of daidzein and genistein in soy products depends on the raw material and the conditions while processing, Faughnan et al.[63] found that urinary recovery of equol from tempeh is higher than the soymilk, although the solid food matrix and fermentation may increase the production of equol. Equol is a metabolite of daidzein produced by the intestinal bacteria; the level of equol production has been linked to the consumption, and the content

Table 1. Summary of isoflavone contents in soybean

Sample	Genistein	Daidzein	Glycitein	Genistein	Daidzin	Glycitin	Reference
Soybean extract	36.55 µg/g	88.87 µg/g	34.42 µg/g	-	-	-	40
Soybean extract	1260 µg/g	849 µg/g	174 µg/g	-	-	-	41
Soybean	0.126 µg/g	0.71 µg/g	-	-	-	-	37
Soybean	330 µg/g	100 µg/g	50 µg/g	100 µg/g	69 µg/g	-	42
Soybean (culture origin)	3771 µg/g	3366 µg/g	-	-	-	-	43
Soybean (market origin)	2971 µg/g	2579 µg/g	-	-	-	-	43
Soy sprout	232 µg/g	177 µg/g	-				43
Soy flour	-	-	-	700 µg/g	620 µg/g		44
Soybean	42 µg/g	47.8 µg/g	2.7 µg/g	-	-	-	45
Isogen (refined soy isoflavones)	368 µg/g	782 µg/g		-	-	-	46
Soybean seed	-	-	-	465.78 µg/g	251.64 µg/g	108.25 µg/g	47

of the isoflavone daidzein. The solid food matrix of tempeh may protect isoflavones from degradation, so they could reach the large intestine and metabolized into equol by the gut bacteria. This indicates that tempeh contains more daidzein than soymilk. Information about pharmacokinetics is very important to evaluate the safety and understand the efficacy. For example, from the $t_{1/2}$, we could predict that how long isoflavones are still present in the body, so that its consumption time could be regulated by the medication.

It turns out that not only isoflavone tablets are high in isoflavones, but daily food processed from the soy also contains quite high levels of isoflavones, and may interact if taken together with certain drugs. Thus, there is a need for careful monitoring. An assessment of the pharmacokinetic profile of several other processed soybean products needs to be done, for example tofu, to obtain more information.

The mechanism of drug-isoflavone pharmacokinetic interactions

Drug interactions not only occur between drugs, but also occur between drugs and herbal or natural compounds, such as isoflavones. Isoflavones are a component of dietary foods or herbal supplements, so there is a possibility of long-term exposure together with the drugs. This simultaneous use may lead to the drug-isoflavone interactions. This is supported by Laurenzana et al.[64], who found that the content of natural materials such as flavones, isoflavones, and tangeretin affects the activity of human CYP enzymes when given orally together with the drugs. These changes in ADME will certainly affect the pharmacokinetic parameters of the drugs, because of the interactions with DMEs and interaction with the drug transporters.

In this article, the pharmacokinetic interactions between isoflavones and some drugs and their mechanisms of interaction have been summarized, focusing on the enzymes and drug transporters, as shown in Table 4. It has been reported that the co-administration of soy isoflavones (genistein or daidzein), soy tablets, or soybean extract with drugs results in changes in the pharmacokinetic parameters of the drug, which indicates an interaction. These effects include the changes in the AUC, C_{max}, and the clearance. These changes may be either an increase or

Table 2. Isoflavone contents of soybean products

Sample	Genistein	Daidzein	Glycitein	References
Isoflavin tablet	31.863 mg	12.803 mg	-	43
Novasoy tablet	19.9 mg	24.9 mg	3.4 mg	55
Soy nut	0.039 µg /mL	0.032 µg/mL	-	37
Soy milk	0.043 µg/mL	0.027 µg/mL	-	37
Soy milk	25.86 µg/mL	8.25 µg/mL	-	56
Soy milk	47.6 µg /mL	47.3 µg /mL	-	57
Soy milk	26.46 µg/mL	-	-	58
Soy milk	22.3 µg/g	19.6 µg/g	22 µg/g	59
Soy milk	71.1 µg/g	67.9 µg/g	11 µg/g	45
Soy milk	56 µg/mL	52 µg/mL	-	46
Tempeh	0.0196 µg/mL	0.0107 µg/mL	-	37
Tempeh	186.4 µg/g	137.1 µg/g	22.1 µg/g	59
Raw tempeh	280 µg/g	260 µg/g	-	60
Fried tempeh	310 µg/g	350 µg/g	-	60
Firm tofu	4.916 µg/g	7.306 µg/g	-	61
Tofu	14.5 mg	24.6 mg	-	57
Tofu	98.7 µg/g	104.9 µg/g	18.8 µg/g	45
Soybean meal	92.4 µg/g	109.2 µg/g	13.8 µg/g	45
Natto	224 µg/g	411 µg/g	-	57
Natto	147.4 µg/g	234.4 µg/g	8.8 µg/g	45
Fermented soybean miso	145 µg/g	166.8 µg/g	17 µg/g	45
Sufu	617.7 µg/g	536.9 µg/g	103.2 µg/g	45
Fermented tofu	321 µg/g	319 µg/g	-	62
Sufu	99.98 mg	65.48 mg	16.42 mg	48

a decrease in the pharmacokinetic parameters, depending on the mechanism. The mechanisms that will be discussed here involve enzymes and drug transporters.

Effects of soy isoflavones on drug metabolizing enzymes

Soybeans influence the metabolism of the drugs, and affect ADME through the interactions with phase-I or phase-II DMEs. The enzymes involved in phase-I metabolism are the CYP450 families, while the enzymes involved in phase-II metabolism are sulfotransferases, uridine diphosphate glucuronosyltransferases (UDPGT/UGTs), N-acetyl transferases (UDPGT/UGTs), glutathione-S-transferases, and methyltransferases.[25]

Phase-I metabolism enzymes

CYP450 are the main group of enzymes that catalyze the oxidative biotransformation of the drugs and other lipophilic xenobiotics.[27] The enzymes involved in the drugs metabolism are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.[26] The enzymes that are influenced by soy isoflavone are discussed below based on the pharmacokinetic interaction mechanism of some drugs (Table 4). To support the discussion, we have summarized the inhibitory effect of soy isoflavones on CYP enzymes in Table 5.

CYP2C9

Pharmacokinetic interactions may occur through the inhibition or induction of DMEs. Around 15% of all drug biotransformation is metabolized by the CYP2C9.[78] An interaction between celecoxib and genistein has been reported;[65] as shown in Table 4, there is an increase in C_{max} and AUC is almost 2.7 times higher than celecoxib alone, because of the inhibition of the CYP2C9 enzyme by genistein. Thus, the metabolism of celecoxib is reduced, clearance also decreases, and celecoxib accumulates in the body. This mechanism is also in line with the results of Kopecna-Zapletalova et al.[77] based on *in vitro* studies (Table 5) showing that genistein may inhibit CYP2C9 at doses of 35.96 mmol/L and 100 μM.[65] The flavone structure of genistein (4,5,5,7-trihydroxyisoflavone) may suppress CYP2C9 by interacting with the active site of CYP2C9.[79]

CYP1A2

The same effect was also seen by Peng et al.[66] when theophylline was given with soy isoflavones such as daidzein at a dose of 200 mg twice a day to healthy volunteers. There was an increase in the AUC and C_{max}. Theophylline is mainly excreted through the hepatic metabolism pathway, and CYP1A2 catalyzes all these pathways; thus, the inhibition of CYP1A2 will inhibit the metabolism of this drug. This is also related to the

Table 3. Pharmacokinetics of isoflavones after oral administration in humans

Sample	Isoflavones	C_{max}	t_{max}	$t_{1/2}$	AUC	Vd/F	Cl/F	References
Soy milk	Daidzein	2.19 μmol/L.mg dose	6.1 h	8 h	22.09 μmol.h/L mg dose	1.53 L/kg	8.47 L/h	59
Tempeh	Daidzein	2.33 μmol/L.mg dose	8.4 h	9.4 h	15.28 μmol.h/L mg dose	2.07 L/kg	9.86 L/h	59
Soy beverage	Daidzein	96.31 ng/mL	5.92 h	7.68 h	11.50 ng.h/mL	-	-	44
Soy extract capsule	Daidzein	96.02 ng/mL	6.25 h	6.67 h	1211.93 ng.h/mL	-	-	44
Soy isoflavones (isogen)	Daidzein	230 ng/mL	3.78 h	9.75 h	2629 ng.h/mL	211.4 L	12.2 L/h	46
Fermented soybean	Daidzein	214 ng/mL	2.88 h	9.54 h	2594 ng.h/mL	295.4 L	12.9 L/h	46
Soymilk	Daidzein	211.2 ng/mL	3.71 h	5.92 h	2101 ng.h/mL	131.4 L	19 L/h	46
Soymilk	Genistein	4.07 μmol/L.mg dose	5.6 h	9.9 h	50.01 μmol.h/L mg dose	0.72 L/kg	3.31 L/h	59
Soymilk	Genistein	231.1 ng/mL	4.86 h	5.64 h	2326 ng.h/mL	104 L	13.5 L/h	46
Tempeh	Genistein	2.35 μmol/L.mg dose	7.2 h	9.4 h	32.28 μmol.h/L mg dose	1.12 L/kg	6.58 L/h	59
Soy beverage	Genistein	116.37 ng/mL	5.75 h	7.61 h	1437.23 ng. h/mL	-	-	44
Soy extract capsule	Genistein	261.84 ng/mL	7 h	7.96 h	3259.54 ng. h/mL	-	-	44
Soy isoflavones (isogen)	Genistein	160 ng/mL	4.67 h	8.53 h	2356 ng.h/mL	226 L	15.1 L/h	46
Fermented soybean	Genistein	195.7 ng/mL	3.5 h	8.22 h	2279 ng.h/mL	347 L	17.4 L/h	46

C_{max}: Maximum concentration, t_{max}: Time-to-maximum, $t_{1/2}$: Half-life, AUC: Area under the curve, Vd: Volume of distribution

Table 4. Interaction of soy isoflavones with drugs based on pharmacokinetic parameters

Sample	Pharmacokinetic parameters					Significant effect	Mechanism	Method	Route of administration	Reference
	C_{max}	t_{max}	$t_{1/2}$	AUC	Clz/F					
Celecoxib	1380 µg/L	2.6 h	4.34 h	11455 µg/L.h	3.49 L/kg.h	Increased C_{max} and AUC, decreased Clz/F	Inhibitor of CYP2C9	Rats	Oral	65
Celecoxib + genistein 100 mg/kg	3756.71 µg/L	3.4 h	2.93 h	30835.89 µg/L. h	1.64 L/kg.h					
Theophylline	1.33 µg/ mL	2.77 h	9.88 h	18.52 µg h/mL	-	Increased C_{max}, AUC, $t_{1/2}$	Inhibitor of CYP1A2	Human	Oral	66
Theophylline + daidzein	1.63 µg/mL	2.65 h	12.01 h	24.41 µg h/mL	-					
Midazolam	48.86 ng/mL	0.83 h	2.01 h	209.18 ng.h/mL	1.68 L/h	Decreased C_{max}, AUC, increased Cl/F	Inducer of CYP3A4	Human	Oral	67
Midazolam + genistein tablet 1000 mg	36.25 ng /mL	1.13 h	1.67 h	180.59 ng.h/mL	3.98L/h					
Imatinib	14511 mg/L	2.6 h	2.89 h	109010 mg.h/L	300.125 L/kg	Decreased C_{max} and AUC	Inducer of CYP3A4	Rats	Oral	68
Imatinib + genistein 50 mg/kg	10810 mg/L	2.8 h	2.29 h	79070 mg.h/L	406.776 L/kg					
Carbamazepine	634 ng/mL	1.83 h	7.95 h	6087.77 ng/L.h	0.7791 L/h	Decreased C_{max}, AUC, t_{max}, increase Cl/F	Inducer of CYP3A4	Rats	Oral	69
Carbamazepine + soybean	320.16 ng/mL	1 h	6.69 h	1928 ng/L.h	0.9086 L/h					
Paclitaxel	36.8 ng/mL	1 h	14.7 h	702 ng.h/mL	712 mL/min.kg	Increased C_{max}, AUC, decreased Cl/F	Inhibitor of CYP3A4 and inhibitor of P-gp	Rats	Oral	33
Paclitaxel + genistein 10 mg/kg	70.6 ng/mL	0.5 h	16.2 h	1086 ng.h/mL	461 mL/min.kg					
Repaglinide	70.8 ng/mL	0.7 h	1.13 h	134.89 ng.h/mL	3.06 L/kg.h	Increased C_{max} and AUC	Inhibitor of P-gp	Rats	Oral	70
Repaglinide + genistein 10 mg/kg	124.71 ng/mL	0.75 h	1.39 h	245.71 ng.h/mL	2.23 L/kg.h					
Omeprazole	2007.33 ng/mL	0.5 h	1.31 h	1586.25 ng/L.h	0.156 L/h	Increased C_{max} and AUC, decreased Clz /F	Inhibitor of Pg-p	Rats	Oral	69
Omeprazole + soybean	3242.33 ng/ mL	0.5 h	2.21 h	7115.83 ng/L.h	0.134 L/h					
Danofloxacin	2.72 µg/mL	4.5 h	-	9.58 µg.h/mL	-	Decreased C_{max} and AUC	Inhibitor of BCRP	Sheep	Oral	71
Danofloxacin + soy diet	1.16 µg/mL	2.6 h	-	4.9 µg.h/mL	-					
Valproic acid	216.94 µg/mL	0.08 h	3.95 h	656.579 µg.h/mL	88.02 mL /h.kg	Decreased C_{max}, AUC, increased Cl/F, $t_{1/2}$	Inducer of UGT	Rats	Intravenous	72
Valproic acid + soy 500 mg	143.64 µg/mL	0.08 h	4.98 h	456.491 µg.h/mL	118.97 mL/h.kg					

C_{max}: Maximum concentration, t_{max}: Time-to-maximum, $t_{1/2}$: Half-life, AUC: Area under the curve, CYP: Cytochrome, P-gp: P-glycoprotein

results of Anderson et al.[76] who showed that soybean extract inhibits the CYP1A2 enzyme *in vitro*; one of the isoflavones contained in soy extract is daidzein.

CYP3A4

Inhibition of CYP3A4 will increase the drug levels, as shown by Li and Choi.[33] *In vivo*, genistein may increase the value of AUC and C_{max} of paclitaxel through the inhibition of CYP3A4; this is also supported by *in vitro* studies. It has been widely reported that genistein from soybean inhibits CYP3A4.[28,76,77] *In vitro* studies have reported that genistein inhibits CYP3A4 at a concentration of 0.5 mM/well, supported by Kopecna-Zapletalova et al.[77] from 2016 showing that genistein may inhibit CYP3A4 at a concentration of 23.25 mM. Another trial using different cells, namely V_{79} cells, showed the inhibitory activity of genistein on CYP3A4.[75] The inhibitory effect of isoflavones on CYP3A4 is classified as moderate inhibition and is non-competitive.[77]

In addition to the inhibitory effect described above, some studies show that the mechanism of isoflavones also may alter the pharmacokinetics of drugs by the induction of enzymes. This may decrease the AUC and C_{max} and increase clearance. Studies by Xiao et al.[67] showed there is a change in the value of midazolam pharmacokinetic parameters after the patients were given genistein tablets (1000 mg) for 14 days; the same thing was also found for imatinib[80] and carbamazepine.[69] Midazolam and imatinib are primarily metabolized by CYP3A4 after oral administration.[67] Imatinib is metabolized into N-desmethyl imatinib by CYP3A4[80,81] and is a prodrug. This means that there is a different mechanism for the prodrug. Prodrugs are activated by a CYP, so it is important to know if metabolism or the activation of enzymes may alter CYP activity.[82] Genistein increases the C_{max} and AUC of N-desmethyl imatinib by the induction of CYP3A4. In the future, to clarify the mechanism, it will be necessary to carry out a deeper investigation related to the effect of soy isoflavones on prodrugs.

Induction of xenobiotic-mediated *CYP3A* genes in humans is known to be regulated by pregnane X receptors (PXR), constitutive and immune receptors, glucocorticoid receptors, and other receptors.[83] PXR is the main regulator of xenobiotic-induced *CYP3A* gene expression. Previous research has found that genistein may significantly activate the human PXR, and induce the human CYP3A4 luciferase reporter activity.[84] According to this study, we consider that genistein acts as an inducer of CYP3A4 in humans. However, the $_{CYP3A4}$ induction mechanism is contrary to *in-vitro* studies (Table 4) because many studies report that soy, and its isoflavones have an inhibitory effect rather than induction, so there is no *in vivo*/*in vitro* correlation related to the effect of soybean on CYP3A4.

According to Cheng et al.[48] soybean contains 42 µg/g genistein and 4.78 µg/g daidzein, while some have reported extracts containing and 1260 µg/g genistein and 849 µg/g daidzein,[41] or 36.55 µg/g genistein and 88.87 µg/g daidzein.[40] These variations in the content may be caused by the differences in the soybean variety assessed, the location of growth, plant age, etc. Other than that, processed soybean foods such as tofu, tempeh, soy milk, natto, miso, and sufu also have variable contents, which could be seen in Table 3. For example, fried

Table 5. Summary of the inhibitory effects of soy isoflavones on CYP enzymes

Sample	Method	CYP450	References
Standardized soybean extract containing 37% isoflavones	*In vivo* (rat)	CYP3A1 (homologue to human CYP3A4)	73
Soybean 100 mg/kg	*In vivo* (rat)	CYP3A1 (homologue to human CYP3A4) CYP2D2 (homologue to human CYP2D6)	74
Soy 129 mg/day	*In vivo* (monkey)	CYP3A4	75
Soybean powder 375 µg/mL	*In vitro*	CYP3A4	28
Genistein 0.5 mM/well	*In vitro*	CYP3A4	28
Isoflavones	*In vitro* (V79 cells)	CYP3A4	75
Soy extract 12.2 µg/mL	*In vitro*	CYP3A4	76
Genistein 23.25 mmol/L	*In vitro*	CYP3A4	77
Genistein 35.95 mmol/L	*In vitro*	CYP2C9	77
Daidzein 60.56 mmol/L	*In vitro*	CYP2C9	77
Soy extract 2.6 µg/mL	*In vitro*	CYP2C9	76
Genistein 100 µM	*In vitro*	CYP2C9	65
Genistein 62.73 mmol/L	*In vitro*	CYP2C19	77
Genistein 20.97±1.27 mmol/L	*In vitro*	CYP2C8	77
Soy extract 23.6 µg/mL	*In vitro*	CYP1A2	76

CYP450: Cytochrome P450

tempeh contains 310 µg/g genistein and 350 µg/g daidzein.[60] In natto, the level of genistein is 224 µg/g and that of daidzein is 411 µg/g[57], but soymilk has a lower content of 56 µg/mL and 52 µg/mL, respectively.[46] When linked with experimental data *in vitro* from the various studies, it appears that soybean extract may inhibit the CYP3A4 enzyme at a concentration of 12.2 µg/mL, CYP2C9 at 2.6 µg/mL, and CYP1A2 at 23.6 µg/mL.[76] This means that consuming 1 gram of soybean extract may influence these enzymes. The same thing is also the case with soybeans and soybean products, because the content of genistein and daidzein (shown in Table 3) in each gram exceeds the inhibitory dose reported by Kopecna-Zapletalova et al.[77] However, further *in vivo* studies in human subjects need to be performed, as *in vivo* studies have only been conducted on mice with soybean doses that inhibit CYP3A1 (the homologue to CYP3A4 in humans), (i.e., 100 mg/kg).[74] If simplified, the dose is equivalent to 100 µg/g, so based on this the consumption of soymilk, tofu, soybeans could be said to be safe, but again further research is needed to obtain more accurate results.

Phase-II metabolism enzymes

Uridine diphosphate glucuronosyltransferases

Soybean increases the phase-II metabolism of drugs to increase the detoxification and clearance of potentially carcinogenic intermediaries. The results of Marahatta et al.[72] report that the administration of 500 mg for 5 days could affect valproic acid (VPA) in terms of its pharmacokinetic parameters. Specifically, the C_{max} decreased by 65%, but time-to-maximum (t_{max}) was not significantly different. AUC decreased by 69%. There were significant differences in C_{max}, $t_{1/2}$, AUC, and clearance between the treatment and control groups.[72] Soybean contributes to VPA excretion, which is very effective as it increases VPA glucuronidation. Valproate glucuronide is the main metabolite of VPA in urine and is metabolized by UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15. The metabolism and elimination of VPA is affected by glucuronidation, especially by uridine 59-diphosphate-glucuronosyltransferase. Similarly, previous studies have shown that soy induces the UGT enzyme, an important component of glucuronidation.[85] Daidzein may stimulate glucuronidation.[86] Similarly, genistein has been reported to induce UGT activity.[87] The inhibition or induction of important enzymes for drugs that require therapeutic drug monitoring and food-drug interactions depend on the therapeutic index of each drug.[72]

Effects of soy ısoflavones on drug transporters

Drug transporters have an important role in the ADME of drugs and xenobiotics.[88] Drug transporters are also related to disposition of drug and drug interactions.[89] Drug transporters are classified as uptake and efflux transporters. Uptake transporters play a role in facilitating the translocation of drugs into cells such as organic anion transporting polypeptides (OATP; SLCO)[90], organic anion transporters (OAT; SLC22A)[91], and organic cation transporters (OCT; SLC22A)[92], while efflux transporters transfer or remove drugs from the intracell to the extra cell, for example the ABC group and SLC transporters. The ABC family includes transporters for the elimination of drugs likes P-gp [multidrug resistance protein 1 (MDR1); ABCB1], certain members of the multidrug resistance-associated protein (MRP; ABCC) family, and breast cancer resistance protein [(BCRP); ABCG]. These drug transporters are expressed in the intestine or liver, two main locations that affect how much drug will enter the body after the administration of an oral dose. Thus, the effect of isoflavones on drug transporters is important because it will affect the pharmacokinetic profile of a drug.[93] As shown in Table 4, the pharmacokinetic interaction mechanisms of some drugs occur only through efflux transporters.

Efflux drug transporters

P-glycoprotein

P-gp is a product of the *MDR1* gene, which is an efflux transporter that is widely studied and known for its ability to limit the entry of drugs into various organ compartments. P-gp functions as an efflux pump, such that it facilitates the transfer of intracellular drugs to the extracellular space.[26] Genistein may influence the administration of the drugs by modulating efflux proteins such as MDR1 and P-gp. P-gp is expressed mainly in the apical membrane of the intestine. MDR1 has been reported to increase the elimination of drugs in the intestinal lumen.[34] This mechanism is shown in Figure 2. Genistein inhibits P-gp and causes pharmacokinetic interactions with repaglinide at a genistein concentration of 10 mg/kg, characterized by an increase in the repaglinide AUC of 53% and C_{max} by 36%.[70] Genistein affects P-gp by increasing intestinal absorption. Li and Choi.[33] found an increase in the paclitaxel plasma concentration with a mechanism of P-gp inhibition, similar to what was also found with midazolam.[69] To confirm, Li et al.[54] tested Caco-2 cells and IEC-6 cells to investigate further repaglinide absorption in human cells and in mice, resulting in significantly increased intracellular repaglinide accumulation with genistein administration.[70] This means that P-gp transporters, which are supposed to carry drugs to the extracellular are blocked by genistein, resulting in the intracellular accumulation of repaglinide.

The mechanism by which genistein inhibits P-gp was revealed by molecular docking studies. The basic structure of P-gp includes four main core regions, with two nucleotide-binding

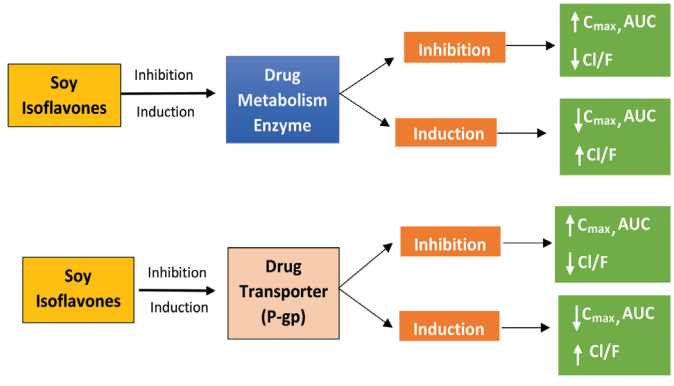

Figure 2. Mechanisms of pharmacokinetic interactions between drugs and soy isoflavones (based on Table 4)

C_{max}: Maximum concentration, AUC: Area under the curve, P-gp: P-glycoprotein, CL/F: Oral clearance

domains (NBD) located in the cytoplasm and two hydrophobic transmembrane domains (TMD).[94] The TMD serve as a channel to facilitate drug transport, whereas the NBD located in the cytoplasm have binding sites for ATP, used as the energy supply for drug transport.[95] 6C0V was chosen as a P-gp molecule with a three-dimensional structure combined with NBD simulation; it was found that genistein has a certain binding affinity for NBD and shares several binding sites with ATP in the corresponding functional area, which affects the energy supply when the drug is transported by P-gp. This is what causes the inhibition of the efflux function of P-gp.[70]

BCRP

Drug interactions that lead to the inhibition of efflux transporters can cause changes in the pharmacokinetics of the drug. For example, in the case of BCRP, several drugs are secreted into milk, such as danofloxacin as shown in a study performed in sheep given a soy diet to see its effect on drug levels in milk. A change was observed in the pharmacokinetic parameters of danofloxacin, namely a 50% decrease in C_{max} and AUC.[71] *BCRP* inhibitors administered with drugs that are substrates of the transporter could have effects on *in vivo* ADME, as well as the presence of drugs in milk.[96,97] A soy diet contains daidzein and genistein, which are BCRP inhibitors.[98,99]

From what has been discussed above, we could see that the pharmacokinetic interaction of soy isoflavones with drugs occurs through the several mechanisms, (i.e., through DMEs or drug transporters). These interactions will affect the bioavailability of drugs in the blood. The mechanisms are summarized and illustrated in Figure 2.

CONCLUSION

Soybeans are a good source of isoflavones. The isoflavone content of soybean is mainly in the aglycone form as daidzein, genistein, and glycitein. Soybean products also contain variable levels of isoflavones. Co-administration of soy isoflavones with the drugs may cause pharmacokinetic interactions. These interactions may cause changes in the AUC, C_{max}, t_{max}, and $t_{1/2}$ of the drugs. These interactions occur through mechanisms related to the inhibition/induction of DMEs, namely CYP3A4, CYP2C9, CYP1A2, and UGT or the inhibition/induction of drug transporters, such as P-gp and BCRP. Thus, the consumption of soy, soy isoflavones, or soy products together with the drugs needs to be considered because this diet may affect the efficacy of the drugs. Furthermore, the timing and consumption of soy isoflavones with the drugs should be monitored.

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