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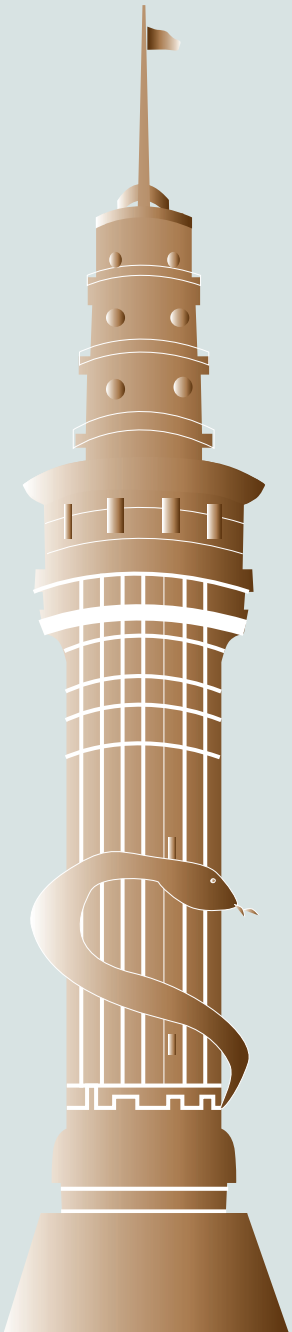

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VOLUME 54, ISSUE 1

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Publication Type: Periodical

İstanbul Journal of Pharmacy
APRIL 2024,
VOLUME 54, ISSUE 1

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AIMS AND SCOPE

İstanbul Journal of Pharmacy (Istanbul J Pharm) is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official publication of İstanbul University Faculty of Pharmacy and it is published triannually on April, August, and December. The publication language of the journal is English.

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The target audience of the journal includes specialists and professionals working and interested in all disciplines of pharmaceutical, also medicinal, biological and chemical sciences.

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publi- cation Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing).

İstanbul Journal of Pharmacy is currently indexed in Web of Science-Emerging Sources Citation Index, TUBITAK ULAKBIM TR Index and CAS database.

Processing and publication are free of charge with the journal. No fees are requested from the authors at any point throughout the evaluation and publication process. All manuscripts must be submitted via the online submission system, which is available at http://dergipark.gov.tr/iujp. The journal guidelines, technical information, and the required forms are available on the journal's web page.

All expenses of the journal are covered by the İstanbul University.

Statements or opinions expressed in the manuscripts published in the journal reflect the views of the author(s) and not the opinions of the İstanbul University Faculty of Pharmacy, editors, editorial board, and/or publisher; the editors, editorial board, and publisher disclaim any responsibility or liability for such materials.

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score taken from each article, the overall similarity score is generally expected to be less than 15 or 20%.

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Only those manuscripts approved by its every individual author and that were not published before in or sent to another journal, are accepted for evaluation.

Submitted manuscripts that pass preliminary control are scanned for plagiarism using iThenticate software. After plagiarism check, the eligible ones are evaluated by editor-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope.

The selected manuscripts are sent to at least two national/international external referees for evaluation and publica- tion decision is given by editor-in-chief upon modification by the authors in accordance with the referees' claims.

Editor-in-Chief evaluates manuscripts for their scientific content without regard to ethnic origin, gender, sexual orientation, citizenship, religious belief or political philosophy of the authors. He/She provides a fair double-blind peer review of the submitted articles for publication and ensures that all the information related to submitted manuscripts is kept as confidential before publishing.

Editor-in-Chief is responsible for the contents and overall quality of the publication. He/She must publish errata pages or make corrections when needed.

Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers. Only he has the full authority to assign a reviewer and is responsible

for final decision for publication of the manuscripts in the Journal.

Reviewers must have no conflict of interest with respect to the research, the authors and/or the research funders. Their judgments must be objective.

Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the editor if they are aware of copyright infringement and plagiarism on the author's side.

A reviewer who feels unqualified to review the topic of a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

The editor informs the reviewers that the manuscripts are confidential information and that this is a privileged interaction. The reviewers and editorial board cannot discuss the manuscripts with other persons. The anonymity of the referees must be ensured. In particular situations, the editor may share the review of one reviewer with other reviewers to clarify a particular point.

PEER REVIEW PROCESS

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Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers and is responsible for final decision for publication of the manuscripts in the Journal.

Reviewers' judgments must be objective. Reviewers' comments on the following aspects are expected while conducting the review.

- Does the manuscript contain new and significant information?
- Does the abstract clearly and accurately describe the content of the manuscript?
- Is the problem significant and concisely stated?
- Are the methods described comprehensively?
- Are the interpretations and consclusions justified by the results?
- Is adequate references made to other Works in the field?
- Is the language acceptable?

Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the editor if they are aware of copyright infringement and plagiarism on the author's side.

A reviewer who feels unqualified to review the topic of a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

The editor informs the reviewers that the manuscripts are confidential information and that this is a privileged interaction. The reviewers and editorial board cannot discuss the manuscripts with other persons. The anonymity of the referees is important.

PUBLICATION ETHICS AND MALPRACTICE STATEMENT

An approval of research protocols by the Ethics Committee in accordance with international agreements (World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects," amended in October 2013, www.wma.net) is required for experimental, clinical, and drug studies. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimen- tal research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manu script. It is the authors' responsibility to carefully protect the patients' anonymity. For photographs

that may reveal the identity of the patients, signed releases of the patient or of their legal representative should be enclosed.

All submissions are screened by a similarity detection soft- ware (iThenticate by CrossCheck) at any point during the peer-review or production process. Even if you are the au- thor of the phrases or sentences, the text should not have unacceptable similarity with the previously published data.

When you are discussing others' (or your own) previous work, please make sure that you cite the material correctly in every instance.

In the event of alleged or suspected research misconduct, e.g., plagiarism, citation manipulation, and data falsification/fabrication, the Editorial Board will follow and act in accordance with COPE guidelines.

Research Ethics

İstanbul Journal of Pharmacy adheres to the highest standards in research ethics and follows the principles of international research ethics as defined below. The authors are responsible for the compliance of the manuscripts with the ethical rules.

- Principles of integrity, quality and ransparency should be sustained in designing the research, reviewing the design and conducting the research.
- The research team and participants should be fully informed about the aim, methods, possible uses and requirements of the research and risks of participation in research.
- The confidentiality of the information provided by the research participants and the confidentiality of the respondents should be ensured. The research should be designed to protect the autonomy and dignity of the participants.
- Research participants should participate in the research voluntarily, not under any coercion.
- Any possible harm to participants must be avoided. The research should be planned in such a way that the participants are not at risk.
- The independence of research must be clear; and any conflict of interest or must be disclosed.
- In experimental studies with human subjects, written informed consent of the participants who decide to participate in the research must be obtained. In the case of children and those under wardship or with confirmed insanity, legal custodian's assent must be obtained.
- If the study is to be carried out in any institution or organization, approval must be obtained from this institution or organization.
- In studies with human subject, it must be noted in the method's section of the manuscript that the informed consent of the participants and ethics committee approval from the institution where the study has been conducted have been obtained.

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It is authors' responsibility to ensure that the article is in accordance with scientific and ethical standards and rules. And authors must ensure that submitted work is original. They must certify that the manuscript has not previously been published elsewhere or is not currently being considered for publication elsewhere, in any language. Applicable copyright laws and conventions must be followed. Copyright material (e.g. tables, figures or extensive quotations) must be reproduced only with appropriate permission and acknowledgement. Any work or words of other authors, contributors, or sources must be appropriately credited and referenced.

All the authors of a submitted manuscript must have di- rect scientific and academic contribution to the manuscript. The author(s) of the original research articles is defined as a person who is significantly involved in "conceptualization and design of the study", "collecting the data", "analyzing the data", "writing the manuscript", "reviewing the manuscript with a critical perspective" and "planning/conducting the study of the manuscript and/or revising it". Fund raising, data collection or supervision of the research group are not sufficient roles to be accepted as an author. The author(s) must meet all these criteria described above. The order of names in the author list of an article must be a codecision and it must be indicated in the **Copyright Agreement Form**. The individuals who do not meet the authorship criteria but contributed to the study must take place in the acknowledgement section. Individuals providing technical support, assisting writing, providing a general support, providing material or financial support are examples to be indicated in acknowledgement section.

All authors must disclose all issues concerning financial relationship, conflict of interest, and competing interest that may potentially influence the results of the research or scientific judgment. When an author discovers a significant error or inaccuracy in his/her own published paper, it is the author's obligation to promptly cooperate

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The Journal takes as principle to comply with the ethical standards of World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects and WMA Statement on Animal Use in Biomedical Research.

An approval of research protocols by the Ethics Committee in accordance with international standards mentioned above is required for experimental, clinical, and drug studies and for some case reports. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimental research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. It is the authors' responsibility to carefully protect the patients' anonymity. For photographs that may reveal the identity of the patients, signed releases of the patient or of their legal representative should be enclosed.

Conflict of Interest

İstanbul Journal of Pharmacy requires and encourages the authors and the individuals involved in the evaluation process of submitted manuscripts to disclose any existing or potential conflicts of interests, including financial, consultant, and institutional, that might lead to potential bias or a conflict of interest. Any financial grants or other support received for a submitted study from individuals or institutions should be disclosed to the Editorial Board. To disclose a potential conflict of interest, the ICMJE Potential Conflict of Interest Disclosure Form should be filled in and submitted by all contributing authors. Cases of a potential conflict of interest of the editors, authors, or reviewers are resolved by the journal's Editorial Board within the scope of COPE and ICMJE guidelines. The Editorial Board of the journal handles all appeal and complaint cases within the scope of COPE guidelines. In such cases, authors should get in direct contact with the editorial office regarding their appeals and complaints. When needed, an ombudsperson may be assigned to resolve cases that cannot be resolved internally. The Editor in Chief is the final authority in the decision-making process for all appeals and complaints.

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MANUSCRIPT PREPARATION

The manuscripts should be prepared in accordance with ICMJE-Recommendations for the Conduct, Reporting, Ed- iting, and Publication of Scholarly Work in Medical Journals (updated in December 2015 - http://www.icmje.org/icmje-recommendations.pdf). Authors are required to prepare manuscripts in accordance with the CONSORT guidelines for randomized research studies, STROBE guidelines for observational original research studies, STARD guidelines for studies on diagnostic accuracy, PRISMA guidelines for systematic reviews and meta-analysis, ARRIVE guidelines for experimental animal studies, and TREND guidelines for non-randomized public behavior.

Manuscripts can only be submitted through the journal's online manuscript submission and evaluation system, available at http://ijp.istanbul.edu.tr/en/_. Manuscripts submitted via any other medium will not be evaluated.

Manuscripts submitted to the journal will first go through a technical evaluation process where the editorial office staff will ensure that the manuscript has been prepared and submitted in accordance with the journal's guidelines. Submissions that do not conform to the journal's guidelines will be returned to the submitting author with technical correction requests.

Authors are required to submit the following:

- Copyright Agreement Form
- Author Form
- Title Page

during the initial submission.

The manuscript should be prepared in MS Word format by using Times New Roman font (12 pt) and double-spaced on one side of the paper with adequate margins (2.5 cm).

Preparation of the Manuscript

Title page: A separate title page should be submitted with all submissions and this page should include:

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- Name(s), affiliations, and highest academic degree(s) and ORCID ID(s) of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number) and fax numbers, and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

Abstract: An structured abstract should be submitted with Original Articles (Background and Aims, Methods, Results, Conclusion). Please check Table 1 below for word count specifications.

Keywords: Each submission must be accompanied by a minimum of three to a maximum of six keywords for subject indexing at the end of the abstract. The keywords should be listed in full without abbreviations. The keywords should be selected from the National Library of Medicine, Medical Subject Headings database (https://www.nlm.nih.gov/mesh/MBrowser.html).

Manuscript Types

Original Articles: This is the most important type of article since it provides new information based on original research. The main text of original articles should be structured with Introduction, Materials and Methods, Results, Discussion, and Conclusion subheadings. Results and Discussion sections can be combined under "Result and Discussion" heading. Please check Table 1 for the limitations for Original Articles.

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses with specified statistical software and descriptive details of the chemical used should be provided with a separate subheading under the Materials and Methods section.

Units should be prepared in accordance with the International System of Units (SI).

Editorial Comments: Editorial comments aim to provide a brief critical commentary by reviewers with expertise or with high reputation in the topic of the research article published in the journal. Authors are selected and invited by the journal to provide such comments. Abstract, Keywords, and Tables, Figures, Images, and other media are not included.

Review Articles: Reviews prepared by authors who have extensive knowledge on a particular field and whose scientific background has been translated into a high

Table 1. Limitations for each manuscript type

Type of manuscript	Word limit	Abstract word limit	Table limit	Figure limit
Original Article	3500	250 (Structured)	6	7 or total of 15 images
Review Article	5000	250 (Unstructured)	6	10 or total of 20 images
Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media

volume of publications with a high citation potential are welcomed. These authors may even be invited by the journal. Reviews should describe, discuss, and evaluate the current level of knowledge of a topic in clinical practice and should guide future studies. Please check Table 1 for the limitations for Review Articles.

Short Papers: Please check Table 1 for the limitations for Short Papers.

Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

Tables

Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to pro- vide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure sub- units. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 x 100 mm). Figure legends should be listed at the end of the main document.

All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

For plant materials, herbarium name (or acronym), number, name and surname of the person who identified the plant materials should be indicated in the Materials and Methods section of the manuscript.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text. Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

REFERENCES

Reference Style and Format

İstanbul Journal of Pharmacy complies with APA (American Psychological Association) style 6th Edition for referencing and quoting. For more information:

- American Psychological Association. (2010). Publication manual of the American Psychological Association (6^{th} ed.). Washington, DC: APA.
- http://www.apastyle.org

Accuracy of citation is the author's responsibility. All references should be cited in text. Reference list must be in alphabetical order. Type references in the style shown below

Citations in the Text

Citations must be indicated with the author surname and publication year within the parenthesis.

If more than one citation is made within the same paranthesis, separate them with (;).

Samples:
More than one citation;
(Esin et al., 2002; Karasar, 1995)
Citation with one author;
(Akyolcu, 2007)
Citation with two authors;
(Sayıner & Demirci, 2007)
Citation with three, four, five authors;
First citation in the text: (Ailen, Ciambrune, & Welch, 2000) Subsequent citations in the text: (Ailen et al., 2000)
Citations with more than six authors;
(Çavdar et al., 2003)

Citations in the Reference

All the citations done in the text should be listed in the References section in alphabetical order of author surname without numbering. Below given examples should be considered in citing the references.

Basic Reference Types

Book

a) Turkish Book
Karasar, N. (1995). *Araştırmalarda rapor hazırlama* (8^{th} ed.) [Preparing research reports]. Ankara, Turkey: 3A Eğitim Danışmanlık Ltd.
b) Book Translated into Turkish
Mucchielli, A. (1991). *Zihniyetler* [Mindsets] (A. Kotil, Trans.). İstanbul, Turkey: İletişim Yayınları.
c) Edited Book
Ören, T., Üney, T., & Çölkesen, R. (Eds.). (2006). *Türkiye bilişim ansiklopedisi* [Turkish Encyclopedia of Informatics]. İstanbul, Turkey: Papatya Yayıncılık.
d) Turkish Book with Multiple Authors
Tonta, Y., Bitirim, Y., & Sever, H. (2002). *Türkçe arama motorlarında performans değerlendirme* [Performance evaluation in Turkish search engines]. Ankara, Turkey: Total Bilişim.
e) Book in English
Kamien R., & Kamien A. (2014). *Music: An appreciation*. New York, NY: McGraw-Hill Education.
f) Chapter in an Edited Book
Bassett, C. (2006). Cultural studies and new media. In G. Hall & C. Birchall (Eds.), *New cultural studies: Adventures in theory* (pp. 220–237). Edinburgh, UK: Edinburgh University Press.
g) Chapter in an Edited Book in Turkish
Erkmen, T. (2012). Örgüt kültürü: Fonksiyonları, öğeleri, işletme yönetimi ve liderlikteki önemi [Organization culture: Its functions, elements and importance in leadership and business management]. In M. Zencirkıran (Ed.), *Örgüt sosyolojisi* [Organization sociology] (pp. 233–263). Bursa, Turkey: Dora Basım Yayın.
h) Book with the same organization as author and publisher
American Psychological Association. (2009). *Publication manual of the American psychological association* (6^{th} ed.). Washington, DC: Author.

Article

a) Turkish Article
Mutlu, B., & Savaşer, S. (2007). Çocuğu ameliyat sonrası yoğun bakımda olan ebeveynlerde stres nedenleri ve azaltma girişimleri [Source and intervention reduction of stress for parents whose children are in intensive care unit after surgery]. *İstanbul University Florence Nightingale Journal of Nursing, 15*(60), 179–182.
b) English Article
de Cillia, R., Reisigl, M., & Wodak, R. (1999). The discursive construction of national identity. *Discourse and Society, 10*(2), 149–173. http://dx.doi.org/10.1177/0957926599010002002
c) Journal Article with DOI and More Than Seven Authors
Lal, H., Cunningham, A. L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S.-J. ... Heineman, T. C. (2015). Efficacy of an adjuvanted herpes zoster subunit

vaccine in older adults. *New England Journal of Medicine, 372*, 2087–2096. http://dx.doi.org/10.1056/NEJMoa1501184

d) Journal Article from Web, without DOI

Sidani, S. (2003). Enhancing the evaluation of nursing care effectiveness. *Canadian Journal of Nursing Research, 35*(3), 26-38. Retrieved from http:/ / cjnr.mcgill.ca

e) Journal Article wih DOI

Turner, S. J. (2010). Website statistics 2.0: Using Google Analytics to measure library website effectiveness. *Technical Services Quarterly, 27*, 261–278. http:/ / dx.doi.org/10.1080/07317131003765910

f) Advance Online Publication

Smith, J. A. (2010). Citing advance online publication: A review. *Journal of Psychology*. Advance online publication. http://dx.doi.org/10.1037/a45d7867

g) Article in a Magazine

Henry, W. A., III. (1990, April 9). Making the grade in today's schools. *Time, 135*, 28–31.

Doctoral Dissertation, Master's Thesis, Presentation, Proceeding

a) Dissertation/Thesis from a Commercial Database

Van Brunt, D. (1997). *Networked consumer health information systems* (Doctoral dissertation). Available from ProQuest Dissertations and Theses database. (UMI No. 9943436)

b) Dissertation/Thesis from an Institutional Database

Yaylalı-Yıldız, B. (2014). *University campuses as places of potential publicness: Exploring the politicals, social and cultural practices in Ege University* (Doctoral dissertation). Retrieved from Retrieved from: http://library.iyte.edu.tr/tr/hizli-erisim/iyte-tez-portali

c) Dissertation/Thesis from Web

Tonta, Y. A. (1992). *An analysis of search failures in online library catalogs* (Doctoral dissertation, University of California, Berkeley). Retrieved from http://yunus.hacettepe.edu.tr/~tonta/yayinlar/phd/ickapak.html

d) Dissertation/Thesis abstracted in Dissertations Abstracts International

Appelbaum, L. G. (2005). Three studies of human information processing: Texture amplification, motion representation, and figure-ground segregation. *Dissertation Abstracts International: Section B. Sciences and Engineering, 65*(10), 5428.

e) Symposium Contribution

Krinsky-McHale, S. J., Zigman, W. B., & Silverman, W. (2012, August). Are neuropsychiatric symptoms markers of prodromal Alzheimer's disease in adults with Down syndrome? In W. B. Zigman (Chair), *Predictors of mild cognitive impairment, dementia, and mortality in adults with Down syndrome*. Symposium conducted at the meeting of the American Psychological Association, Orlando, FL.

f) Conference Paper Abstract Retrieved Online

Liu, S. (2005, May). *Defending against business crises with the help of intelligent agent based early warning solutions*. Paper presented at the Seventh International Conference on Enterprise Information Systems, Miami, FL. Abstract retrieved from http://www.iceis.org/iceis2005/abstracts_2005.htm

g) Conference Paper - In Regularly Published Proceedings and Retrieved Online

Herculano-Houzel, S., Collins, C. E., Wong, P., Kaas, J. H., & Lent, R. (2008). The basic nonuniformity of the cerebral cortex. *Proceedings of the National Academy of Sciences, 105*, 12593–12598. http://dx.doi.org/10.1073/pnas.0805417105

h) Proceeding in Book Form

Parsons, O. A., Pryzwansky, W. B., Weinstein, D. J., & Wiens, A. N. (1995). Taxonomy for psychology. In J. N. Reich, H. Sands, & A. N. Wiens (Eds.), *Education and training beyond the doctoral degree: Proceedings of the American Psychological Association National Conference on Postdoctoral Education and Training in Psychology* (pp. 45–50). Washington, DC: American Psychological Association.

i) Paper Presentation

Nguyen, C. A. (2012, August). *Humor and deception in advertising: When laughter may not be the best medicine*. Paper presented at the meeting of the American Psychological Association, Orlando, FL.

Other Sources

a) Newspaper Article

Browne, R. (2010, March 21). This brainless patient is no dummy. *Sydney Morning Herald, 45*.

b) Newspaper Article with no Author

New drug appears to sharply cut risk of death from heart failure.(1993, July 15). *The Washington Post*, p. A12.

c) Web Page/Blog Post

Bordwell, D. (2013, June 18). David Koepp: Making the world movie-sized [Web log post]. Retrieved from http://www.davidbordwell.net/blog/page/27/

d) Online Encyclopedia/Dictionary

Ignition. (1989). (2^{nd} ed.). Retrieved from http://dictionary.oed.com

Marcoux, A. (2008). Business ethics. In E. N. Zalta (Ed.). *The Stanford encyclopedia of philosophy*. Retrieved from

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http://plato.stanford.edu/entries/ethics-business/

e) Podcast

Dunning, B. (Producer). (2011, January 12). *inFact: Conspiracy theories* [Video podcast]. Retrieved from http://itunes.apple.com/

f) Single Episode in a Television Series

Egan, D. (Writer), & Alexander, J. (Director). (2005). Failure to communicate. [Television series episode]. In D. Shore (Executive producer), *House*; New York, NY: Fox Broadcasting.

g) Music

Fuchs, G. (2004). Light the menorah. On *Eight nights of Hanukkah* [CD]. Brick, NJ: Kid Kosher..

REVISIONS

When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be cancelled. If the sub- mitting author(s) believe that additional time is required, they should request this extension before the initial 30- day period is over.

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Original Article

The evaluation and classification of drug-related problems by a clinical pharmacist in an internal diseases intensive care unit: A prospective cohort 7-month study*

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*This study was accepted as a Clinical Pharmacy Specialization thesis within the scope of the İnönü University Pharmacy Specialization Program on February 3, 2023.

ABSTRACT

Background and Aims: Drug-related problems can cause morbidity and mortality as well as increase health-care costs. Clinical pharmacists provide many benefits to healthcare systems by detecting, decreasing, and preventing drug-related problems. It was aimed to determine and classify drug-related problems and determine risk factors for drug-related problems.

Methods: Drug-related problems were evaluated prospectively between August 16, 2021, and March 16, 2022, in 257 patients during their hospital stay who were hospitalized in the internal diseases intensive care unit and took at least one drug. Patients who were not administered any drug or who were younger than 18 were excluded from the study. The Pharmacetical Care Network Europe v.9 method was utilized to classify these problems. Clinical and demographic characteristics of patients with and without drug-related problems were compared by statistical analysis. Risk factors of drug-related problems were determined by logistic regression analysis.

Results: At least one drug-related problem was detected in 157 of the 257 patients and a total of 399 drug-related problems were recorded. 399 recommendations were made, and 349 (87.5%) of these were accepted and 50 (12.5%) were not accepted. Drug selection (C1) was the most common cause of drug-related problems at 42.2%, and dose selection (C3) followed this by 41.5%. The results of regression analysis showed that atrial fibrillation (OR: 2.985, CI: 1.158-7.692), hematopoietic stem cell transplantation (OR: 3.883, CI: 1.256-11.999), antibacterial drugs (OR: 3.285, CI: 1.563-6.904), or polypharmacy (OR: 3.955, CI:1.207- 11.071) were risk factors of drug-related problems.

Conclusion: The most common drug-related problem category was found as treatment safety and the causes of them were found as drug selection and dose selection. Clinicians should pay attention when prescribing new drugs to patients with atrial fibrillation and a history of hematopoietic stem cell transplantation. Furthermore, clinicians and clinical pharmacists should pay attention if polypharmacy and antibacterial drugs are present in medical therapies.

Keywords: Clinical pharmacy, drug-related problem, intensive care unit

INTRODUCTION

Drugs have many benefits to patient care, but they could also have detrimental effects such as adverse drug reactions, and drug-drug interactions. These drug-related problems (DRPs) can cause morbidity and mortality as well as increase healthcare costs (Ruths, Viktil, & Blix, 2007). Drug-related problem as defined to be an event or circumstance involving drug therapy that actually or potentially interferes with desired health outcomes by Pharmaceutical Care Network Europe Association (Pharmaceutical Care Network Europe Association, 2023). There were many risk factors for DRPs such as polypharmacy, polymorbidity, anticoagulant usage, renal failure, and hepatic failure (Kaufman, Stämpfli, Hersberger, & Lampert, 2015). Pharmaceutical care by clinical pharmacists is aimed at detecting, decreasing, and preventing DRPs (Viktil & Blix, 2008). Detection and classification of DRPs have many advantages. Raising awareness about the frequency and source of DRPs; increasing the knowledge of using medicine with care among clinicians, pharmacists, and patients by feedback; developing pharmaceutical

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care practice and research by documentation and classification of DRPs; being the evidence of the benefits of clinical pharmacy services by documentation of DRPs; these items can be considered as the advantages (Horvat & Kos, 2016). It is known that the management of critically ill patients is very difficult because of having comorbidities and abrupt changes in organ function. Furthermore, polypharmacy in intensive care units (ICUs) is a cause of adverse drug reactions, complications, and drug-drug interactions (Aljbouri et al. 2013). The study by Tharanon et al. has reported that the too-frequent daily drug order changes, too-frequent use of intravenous drugs, polypharmacy, and multiple organ failure increased the risk of DRPs in ICUs (Tharanon, Putthipokin, & Sakthong, 2022). Studies in the literature have shown that clinical pharmacists in ICUs led to a decrease in the frequency of DRPs, so that the healthcare were improved (Lee et al., 2019; Reinau, Furrer, Stämpfli, Bornand, & Meier, 2019; Tasaka et al., 2018).

In this study, it was aimed to reveal the clinical pharmacist's contribution to patient care in the ICU by determining DRPs, classifying DRPs, and making interventions to solve DRPs. Furthermore, our secondary aim was to determine the risk factors for DRPs in the Internal Diseases ICU.

MATERIALS AND METHODS

This study was conducted at the Internal Diseases ICU of a university hospital between August 16, 2021, and March 16, 2022. In this ICU, patients were hospitalized according to the departments of internal diseases (hematology, nephrology, gastroenterology, endocrinology, and medical oncology). Inclusion criteria were those who were hospitalized in the internal diseases ICU, took at least one drug, and were evaluated by the clinical pharmacist. Inform consent was obtained from all individual participants included in the study or from their relatives. The exclusion criteria were that those patients who were not administered any drug, were younger than 18, weren't evaluated by the clinical pharmacist, or were not approved the patient consent form by the patient or their relatives. Patients' medical therapies were evaluated daily during their stay in hospital. DRPs were being evaluated without any restriction on patients' length of hospital stay. If patients were hospitalized and discharged within the days without the clinical pharmacist, they were not included in the study. DRPs of 257 patients' medical therapy were evaluated prospectively by the clinical pharmacist who was in a clinical pharmacy specialist training program and the interventions were shared with the responsible physician and/or other healthcare staff. Patients were divided into two groups with DRPs and without DRPs. Clinical and demographic characteristics of patients with and without DRPs were recorded. The clinical pharmacist attended clinic visits in the ICU together with the clinicians and the DRPs that had been detected were discussed and determine. Then, the clinical pharmacist followed up the process to see if the problems were resolved. Pharmaceutical Care Network Europe (PCNE) v.9 classification system was utilized to classify these problems. The detected problems that didn't comply with the explanatory categories were classified as the "unclear problem" or "other" problem category. We did not calculate the study size because all patients who met the inclusion criteria between the specified date were evaluated. We included all patients who matched the inclusion criteria during the study period to prevent the probability of bias.

Ethical approval for the study was obtained from the non-interventional ethics committee of İnönü University on 29.06.2021 (Decision no:2021\2267).

Evaluation and Definitions

Sex, age, comorbidity, department of the ICU, clinical features, drugs used before admission, drugs used in the ICU, and daily laboratory data of the patients were recorded on a patient profile form.

Intubation was recorded if the patient was dependent on a mechanical ventilator device for more than 48 hours. The Glasgow coma scale (GCS), which was calculated at the admission of the ICU by clinicians, was recorded. Polypharmacy is defined as the presence of 5 or more drug usage in a patient (Masnoon, Shakib, Kalisch-Ellet, & Caughey, 2017). Fluid and nutrition support and topical dosage forms weren't included in the total number of drugs. The estimated glomerular filtration rate (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formulary (Napier et al., 2022). Chronic kidney disease stages, acute kidney injury status, and hemodialysis status were recorded. There were no patients with continuous renal replacement therapy. Appropriateness of dosage regimen and drug-drug interactions were checked with the Lexicomp© drug information database and the interventions were recommended according to Uptodate© and Lexicomp© databases. Only these databases were used to evaluate the patients'medical therapies. Drug-drug interactions that could be the clinical significance were grouped as DRPs and recommendations were made based on them. DRPs and interventions were shared with the responsible physician and/or other healthcare staff. The interventions and status of DRPs were classified by the PCNE v.9 classification system (Pharmaceutical Care Network Europe Association, 2023).

Statistical Analysis

The IBM SPSS (Statistical Package for Social Sciences) 23.0 software program was used for statistical analysis. Normality tests of quantitative data were analyzed by Kolmogorov Smirnov test and were found to be non-normally distributed, so were given as median. Quantitative data from 2 groups were analyzed by Mann Whitney U test. Kruskal-Wallis H test was used for the comparison of quantitative data of >2 groups.

Rates of characteristics and PCNE v9 classification categories were given as numbers and percentages. The relationship of qualitative data between the two groups was determined by the Chi-Square Test. The multivariate analysis was performed using binary logistic regression analysis. The variance explained by the model was shown with the Nagelkerke R2 value. The Hosmer-Lemeshow test was used to show the data fit the model well. The risk factors were explained with the odds ratio. A *p*-value smaller than 0.05 was considered statistically significant.

RESULTS

During the 7 months of study protocol, there were 265 hospitalizations in the ICU and 8 were excluded because of no drug usage. 161 (62.6%) of the patients were male among 257 patients. The patients' median age was 67 and the interquarter range (IQR) was 54-76. Female patients' median age was 66 (IQR: 49-76), and male patients' was 67 (IQR: 56-76.5). The median of the total number of drugs was 10 (IQR: 7-13) for each patient. Patients with DRPs had a significantly higher rate of drug number (median:12, IQR:9-15) than patients without DRPs (median:7, IQR: 5-10) ($p<0.05$). Demographics, GCS, and eGFR were not found in a relationship with the presence of DRPs according to the Mann-Whitney U test ($p>0.05$). The longer hospital stays in the ICU were found to be associated with DRPs detection ($p<0.05$). The mortality rate was significantly higher in patients with DRPs. ($p<0.05$). DRPs detection in hematology and nephrology ICU departments was found significantly higher than in the gastroenterology ICU department ($p<0.05$) (Table 1). 399 DRPs were detected among 257 patients and 399 interventions were made. The number of patients with DRPs was found 157 (61%) and the number of DRPs per patient was found 1.55. The accepted ratio of the interventions was found as 87.5%.

Intubation, polypharmacy, antibacterials, electrolytes, noradrenaline/dopamine, corticosteroids, antifungals, insulin, antivirals, and sedatives/analgesics were found in a link to DRPs detection ($p<0.05$). In multivariate analysis, polypharmacy (OR: 3.955, CI:1.207-11.071) and antibacterials (OR: 3.285, CI: 1.563-6.904) were found to be risk factors for DRPs detection (Table 2).

Comorbidities and their relationship with DRPs detection are given in Table 3. Atrial fibrillation (OR: 2.985, CI: 1.158-7.692) and hematopoietic stem cell transplantation (OR: 3.883, CI: 1.256-11.999) were found to be risk factors for DRPs detection (Table 3).

Treatment safety (P2) (43.4%) was detected to be the most common problem (Table 4). Drug selection (C1) (42.2%) was detected to be the most seen cause of DRPs and dose selection (C3) (41.5%) followed this. The most seen interventions belonged to the "at prescriber level" (I1) (53.4%) category as seen in Table 4. The interventions' accepted status was classified, and the accepted interventions were found by 87.5%. The outcomes of the interventions were classified as not known, solved, partially solved, and not solved as seen in Table 4.

DISCUSSION

Demographic and Clinical Characteristics of Patients

In the study by Albayrak et al., female patients consisted of 37.1% similar to our study (37.4%) (Albayrak, Başgut, Bıkmaz, & Karahalil, 2022). The median age of the patients was found (67), likely to the studies of Albayrak et al. (69) and Ayhan et al. (62.5) (Albayrak et al., 2022; Ayhan, Karakurt, & Sancar, 2022). In our study, the mortality rate was found as 52.1% which differed from the other studies that were found as 77%, 28.7%, and 15% (Albayrak et al., 2022; Ayhan et al., 2022; Johansen, Haustreis, Mowinckel, & Ytrebø, 2016), respectively. We suggested those studies were conducted at different times such as at the new coronavirus disease pandemic season or not, and patients' comorbidities may led to differences of the mortality rates. In the ICU setting studies, there were no relationships between demographics and DRPs detection, similar to our study (Albayrak et al., 2022; Martins, Silva, & Lopes, 2019). In contrast to our study, age is a risk factor for DRPs in a non-ICU setting study, so, this difference could be due to different settings (Lenssen et al., 2016).

We found that the length of hospital stay was significantly higher in patients with DRPs similar to the other ICU setting studies (Albayrak et al., 2022; Martins et al., 2019). Moreover, DRPs detection was found higher in patients with mortality than in discharged patients, similar to the other ICU setting study (Albayrak et al., 2022). In addition, we determined that, DRPs were experienced higher in patients who were ordered antibacterials, antifungals, antivirals, corticosteroids, or sedatives/analgesics. While, the study by Ayhan et al. reported that the presence of antibacterials was found in a relationship with DRPs detection (Ayhan et al., 2022). Furthermore, Greeshma et al. have found that antibacterials and corticosteroids increased the risk of DRPs detection and Martins et al. have reported midazolam to be a risk factor for DRPs detection (Greeshma, Lincy, Maheswari, Tharanath, & Viswam, 2018; Martins et al., 2019).

Polypharmacy was found to be a risk factor for DRPs detection in our study. Similarly, >5 drug usage has also been reported to be a risk factor for DRPs in the literature (Greeshma et al., 2018). A non-ICU setting study has reported that the number of drugs was a risk factor for DRPs (Lenssen et al., 2016). Furthermore, we found that the presence of antibacterials was a risk factor for DRP detection, similar to another ICU setting study (Ayhan et al., 2022).

When we evaluated patients' comorbidities, atrial fibrillation and the history of hematopoietic stem cell transplantation were found to be risk factors for DRPs detection. It was stated in

Table 1. Patient characteristics and their relationship with drug-related problem detection.

Variables	Total (%)	Patients with DRPs n(%)	Patients without DRPs n(%)	*p*
Male	161 (62.6)	99 (63.1)	62 (62)	0.864*
Female	96 (37.4)	58 (36.9)	38 (38)	
Age median (IQR)	67 (54-76)	68 (54-76.5)	66 (55-76)	0.774**
Number of drugs median (IQR)	10 (7-13)	12 (9-15)	7 (5-10)	*<0.001***
GCS median (IQR)	14 (11-15)	13 (11-15)	14 (12-15)	0.227**
eGFR median (IQR)	47 (19-90)	40 (18-76)	50 (23.25-94.75)	0.112**
Length of stay, days median (IQR)	6 (3-11)	8 (4.5-16)	4 (3-7)	*<0.001***
Causes of Leaving the hospital[x]				
Discharge[a]	26 (10.1)	9 (5.7)	17 (17)	*0.01**
Discharge on own responsibility[a,b]	10 (3.9)	7 (4.5)	3 (3)	
Transfer to another service[a,b]	87 (33.9)	50 (31.8)	37 (37)	
Mortality[b]	134 (52.1)	91 (58)	43 (43)	
Departments of the ICU[x]				
Nephrology [b]	79 (30.7)	57(36.3)	22 (22)	*0.002**
Gastroenterology[a]	70 (27.3)	33(21)	37 (37)	
Hematology [b]	59 (23)	43(27.4)	16 (16)	
Medical Oncology [a,b]	43 (16.7)	22(14)	21 (21)	
Endocrinology[a,b]	6 (2.3)	2(1.3)	4 (4)	

[x]The same superscript letters indicate there weren't any differences between groups statistically.
*Pearson Chi-Square test **Mann Whitney U test
Significant values were given in italics.

the literature that clinical pharmacists provide healthcare benefits to patients with these related diseases (Clemmons, 2020; Ritchie, Penson, Akpan, Lip, & Lane, 2022). In a previous study, the number of DRPs per patient was found high in a pediatric hematopoietic stem cell transplantation service which led to the thought that clinical pharmacists have an important role in the management of DRPs in this patient population (Ozdemir, Celiker, Kuskonmaz, Okur, & Cetinkaya, 2019).

The classification of DRPs

The ICU setting studies by Albayrak et al and Martins et al. demonstrated that the mostly reported DRPs are belonged to the P2.1 "adverse drug events (possible) occurring" category by 77.18% and 68.64%, respectively (Albayrak et al., 2022; Martins et al., 2019). Our study showed similarity to these studies as P2.1 was found to be the most common DRPs (43.4%). In contrast to these studies Toukhy et al. have found the P2 category at the rate of 17.5% as the third seen problem while, they found P1 "treatment effectiveness" as the most commonly seen problem by 50.2% (Toukhy, Fayed, Sabry, & Shawki, 2021).

In an ICU setting study by Li X-x et al., the effectiveness of drug therapy (%34.2), the safety of drug therapy (%31.1), and others (%34.7) were detected as categories of DRPs (Li, et al., 2020). In our study, the incidence of the problem categories of DRPs were partially similar to this study in that we found P1 "treatment effectiveness" (26.8%), P2 "treatment safety" (43.4%), and P3 "other" (29.8%). Furthermore, they have reported that adverse drug reactions and concerns about treatment safety stemmed from high drug doses or low drug doses/not frequently enough drug regimens similar to our study (Li, et al., 2020). An ICU setting study by Zaidi et al. reported that unnecessary drug treatment was detected by 37% which differed from our study because of the different DRP classification systems (Zaidi, Hassan, Postma, & Seiw Hain, 2003).

Table 2. Clinical characteristics and drug groups used in the patients, and their relationship with drug-related problem detection.

		Univariate analysis			Multivariate analysis	
Clinical characteristics and used drug groups	**Total n(%)**	**Patients with DRP n(%)**	**Patients without DRP n(%)**	***p***	**OR (CI)**	***p*^x**
Intubation	53 (20.6)	40 (25.5)	13 (13)	*0.016**	0.402 (0.059-2.722)	0.350
Polypharmacy	230 (89.4)	152 (96.8%)	78 (78)	*<0.001**	3.955 (1.207-11.071)	*0.022*
GCS (3-8)	40 (15.6)	27 (17.2)	13 (13)		-	-
GCS (9-13)	88 (34.2)	55 (35)	33 (33)	0.539*	-	-
GCS (14-15)	129 (50.2)	75 (47.8)	54 (54)		-	-
Renal dysfunction	152 (59.14)	98 (62.4)	54 (54)	0.181*	-	-
G3b (GFR<45-30)	5 (1.9)	4 (2.5)	1 (1)		-	-
G4 (GFR<30-15)	11 (4.3)	6 (3.8)	5 (5)	0.4*	-	-
G5 (GFR<15)	4 (1.6)	3 (1.9)	1 (1)		-	-
AKI	92 (35.8)	55 (35)	37 (37)		-	-
AKI on CKD	2 (0.8)	1 (0.6)	1 (1)		-	-
Dialysis	38 (14.8)	29 (18.5)	9 (9)		-	-
Proton pump inhibitors	248 (96.5)	153 (97.5)	95(95)	0.317**	-	-
Antibacterials	207 (80.2)	142 (90.4)	65 (65)	*<0.001**	3.285 (1.563-6.904)	*0.002*
Electrolytes	128 (49.8)	86 (54.8)	42 (42)	*0.046**	0.881 (0.483-1,606)	0.678
Antihypertensives	117 (45.5)	77 (49)	40 (40)	0.156*	-	-
Anticoagulants	106 (41.2)	72 (45.9)	34 (34)	0.06*	-	-
Norepinephrine/dopamine	95 (37)	69 (43.9)	26 (26)	*0.004**	1.514 (0.760-3.017)	0.238
Corticosteroids	71 (27.6)	51 (32.5)	20 (20)	*0.029**	1.397 (0.715-2.730)	0.328
Antifungals	61 (23.7)	49 (31.2)	12 (12)	*<0.001**	1.892 (0.827-4.329)	0.131
Insulin	52 (20.2)	40(25.5)	12 (12)	*0.009**	1.985 (0.925-4.259)	0.058
Sedatives/analgesics	50 (19.5)	39 (24.8)	11 (11)	*0.006**	2.94 (0.332-18.165)	0.379
Antivirals	42 (16.3)	33 (21)	9 (9)	*0.011**	1.483 (0.588-3.740)	0.404
Oral antidiabetics	11 (4.3)	7(4.5)	4 (4)	1**	-	-

*Pearson Chi-Square test **Fischer's Exact test ^xLogistic regression
GCS: Glasgow coma scale, AKI: Acute kidney injury, CKD: Chronic kidney disease, DRP: Drug-related problem, OR: Odds ratio, CI: Confidence interval.
Significant values were given in italics.

Another ICU setting study classified DRPs with a method that consists of 11 categories and found "the drug dose too high" and "unnecessary drug treatment" at rates of 28% and 13%, respectively (Johansen et al., 2016). Our study differed from this study as "the drug dose was too high" and "unnecessary drug treatment" were found at the rates of 10.8% and 24.5%. Although the problems and causes were defined in the PCNE method, only the problems were defined in the classification system used in that study (Johansen et al., 2016).

The most common causes of DRPs were "drug selection" by 42.2% and "dose selection" by 41.5% in our study. Similar to our study, Albayrak et al have reported these categories as the most seen causes of DRPs in the ICU by 40.29% and 54.36% (Albayrak et al., 2022). Furthermore, two different ICU setting studies have reported that the most common causes of DRPs were "drug selection" with 51.1% and 60% according to the PCNE method (Ayhan et al., 2022; Martins et al., 2019). The study by Li X-x et al. found that DRPs mostly stemmed from drug selection (41.3%) and dose selection (29%) (Li et al., 2020). Another ICU setting study found "drug selection" as the most common cause of DRPs at 50.7% and "dose selection" as the third cause at %19.5 (Toukhy, 2021).We determined that C1.3 "no indication for drug" (12%), C1.6 "no or incomplete drug treatment in spite of existing indication" (9%), and C1.2 "inappropriate drug" (8.1%) were the most seen subcategories of C1 "drug selection". In the study by Martins et al., DRPs

Table 3. Frequency of comorbidities and their relationship with drug-related problem (DRP) detection.

		Univariate analysis			Multivariate analysis	
Comorbidity	**Total n(%)**	**Patients with DRP n(%)**	**Patients without DRP n(%)**	***p***	**OR (CI)**	***p*^x**
Hypertension	145 (56.4)	91 (58)	54 (54)	0.532*	-	-
Diabetes	75 (29.2)	42 (26.8)	33 (33)	0.283*	-	-
Solid organ tumor	69 (26.8)	40 (25.5)	29 (29)	0.534*	-	-
Coronary artery disease	62 (24.1)	45 (28.7)	17 (17)	*0.033**	1.743 (0.903-3.367)	0.098
Hematologic malignancy	54 (21)	39 (24.8)	15 (15)	0.059*	-	-
BPH	41 (16)	26 (16.6)	15 (15)	0.739*	-	-
Atrial fibrillation	34 (13.2)	28 (17.8)	6 (6)	*0.006**	2.985 (1.158-7.692)	*0.024**
Cirrhosis	34 (13.2)	20 (12.7)	14 (14)	0.771*	-	-
CP A	2 (0.8)	0	2 (2)	0.350*	-	-
CP B	7 (2.7)	4 (2.5)	3 (3)		-	-
CP C	25 (9.7	16 (10.2)	9 (9)		-	-
COPD	23 (8.9)	15 (9.6)	8 (8)	0.670*	-	-
Hearth failure	23 (8.9)	19 (12.1)	4 (4)	*0.027**	2.275 (0.709-7.3)	0.167
Hematopoietic stem cell transplantation	22 (8.6)	18 (11.5)	4 (4)	*0.037**	3.883 (1.256-11.999)	*0.018**
Vascular disease	19 (7.4)	11 (7)	8 (8)	0.767*	-	-
Cerebrovascular disease	17 (6.6)	15 (9.6)	2 (2)	0.18*	-	-
Epilepsy	17 (6.6)	14 (8.9)	3 (3)	0.109***	-	-
Hypothyroid	14 (5.4)	9(5.7)	5 (5)	0.801*	-	-
Alzheimer's disease	13 (5.05)	9 (5.7)	4 (4)	0.537*	-	-
Asthma	12 (4.7)	8 (5.1)	4 (4)	0.770**	-	-
Autoimmune disease	9 (3.5)	8 (5.1)	1 (1)	0.160**	-	-
Hyperthyroidism	2 (0.8)	1 (0.6)	1 (1)	1**	-	-

*Pearson Chi-Square test **Fischer's Exact test ***Continuity Correction *x*Logistic regression
BPH: Benign prostate hypertrophy, CP: Child pugh, COPD: Chronic obstructive pulmonary disease, DRP: Drug-related problem, OR: Odds ratio, CI: Confidence interval
Significant values were given in italics.

were classified with the PCNE method and C1.6 was found by 6.36%, and C1.2 by 9.09% (Martins et al., 2019). The other ICU setting study reported the conditions that "the drug wasn't given in spite of an existing indication" and "drug was given in spite of no indication" by 33.2% and 14.3% (Al-Jazairi et al., 2008). Contrary categorizing the problems and causes of DRPs in our study, in that study the problems were categorized but the causes of DRPs were not categorized, so this difference may have occurred.

The subcategories of C3 "dose selection"; C3.4 "dosage regimen was too frequent", C3.2 "drug dose too high" and C3.1 "drug dose too low" were found by 15.7%, 10.8%, and 9.5% respectively. Similar to our study, Ayhan et al. have reported C3.2 and C3.1 by 12.4% and 6.2%, respectively (Ayhan et al., 2022). Although similar to our study, Martins et al. found C3.2 by 13.18%, in contrast, they didn't find any DRPs that belong to the C3.4 and C3.1 categories (Martins et al., 2019). In contrast to our study, the study by Albayrak et al. found C3.4, C3.2, and C3.1 by 10.19%, 24.7%, and 14.56% respectively (Albayrak et al., 2022). As seen, there is no linearity in the dose selection of DRPs in the literature and we suggested that this difference may stem from the clinicians' ordering practice. C6.1 "inappropriate timing of administration and dosing intervals" was found the most and only seen subcategory of C6 and showed similarity to the other ICU setting studies (Albayrak et al., 2022; Martins et al., 2019). We found that most of the interventions were made at I1 "at the prescriber level" and I3 "at drug level" category, similar to the other three ICU setting studies (Albayrak et al., 2022; Ayhan et al., 2022; Martins et al., 2019). We found I1.3 "intervention proposed to the prescriber" had a higher rate than the I1.4 "intervention discussed to the prescriber" similar to the other two ICU setting studies (Albayrak et al., 2022; Martins et al., 2019). Toukhy et al. have reported that I1 "at the prescriber level" and I3 "at drug level" categories by 57.4% and 40% as the most made interventions in the ICU, similarly the rates were 53.4% and 44.6% in our study (Toukhy et al., 2021).

Table 4. The classification of the drug-related problems and planned interventions.

The problems	**n (%)**
P1 Treatment effectiveness	107 (26.8)
P1.1. No effect of drug treatment	4 (1)
P1.2. Effect of drug treatment not optimal	67 (16.8)
P1.3. Untreated symptoms or indication	36 (9)
P2 Treatment safety	173 (43.4)
P2.1. Adverse drug (possibly) occurring	173 (43.4)
P3 Other	119 (29.8)
P3.1. Problem with cost-effectiveness of the treatment	2 (0.5)
P3.2. Unnecessary drug-treatment	98 (24.5)
P3.3. Unclear problem/complaint. Further clarification necessary	19 (4.8)
The causes	**n (%)**
C1 Drug selection	183 (42.2)
C1.1. Inappropriate drug according to guidelines/formulary	10 (2.3)
C1.2. Inappropriate drug (within guidelines but otherwise contraindicated)	35 (8.1)
C1.3. No indication for drug	52 (12)
C1.4. Inappropriate combination of drugs, or drugs and herbal medications, or drugs and dietary supplements	7 (1.6)
C1.5. Inappropriate duplication of therapeutic group or active ingredient	33 (7.6)
C1.6. No or incomplete drug treatment in spite of existing indication	39 (9)
C1.7. Too many drugs prescribed for indication	7 (1.6)
C2 Drug form	14 (3.2)
C2.1. Inappropriate drug form (for this patient)	14 (3.2)
C3 Dose selection	180 (41.5)
C3.1. Drug dose too low	41 (9.5)
C3.2. Drug dose too high	47 (10.8)
C3.3. Dosage regimen not frequent enough	24 (5.5)
C3.4. Dosage regimen too frequent	68 (15.7)
C4 Treatment duration	8 (1.8)
C4.2. Duration of treatment too long	8 (1.8)
C6 Drug use process	11 (2.5)
C6.1. Inappropriate timing of administration or dosing intervals	6 (1.4)
C6.2. Drug under-administered	2 (0.5)
C6.3. Drug over-administered	1 (0.2)
C6.4. Drug not administered at all	1(0.2)
C6.5 Wrong drug administered	1(0.2)
C9 Other	38 (8.8)
C9.1. No or inappropriate outcome monitoring	15 (3.5)
C9.2. Other cause; specify	21 (4.8)
C9.3. No obvious cause	2 (0.5)
The planned interventions	**n (%)**
I1 At prescriber level	398 (53.4)

Table 4. The classification of the drug-related problems and planned interventions. (Continued)

I1.3. Intervention proposed to prescriber	396 (53.1)
I1.4. Intervention discussed with prescriber	2 (0.3)
I3 At drug level	332 (44.6)
I3.1. Drug changed to …	12 (1.6)
I3.2. Dosage changed to …	145 (19.5)
I3.3. Formulation changed to …	4 (0.5)
I3.4. Instructions for use changed to …	8 (1.1)
I3.5. Drug paused or stopped	131 (17.6)
I3.6. Drug started	32 (4.3)
I4 Other intervention or activity	15 (2)
I4.1. Other intervention (specify)	15 (2)
Acceptance of the intervention proposals	**n (%)**
A1 Intervention accepted	349 (87.5)
A1.1. Intervention accepted and fully implemented	348 (87.2)
A1.2. Intervention accepted, partially implemented	1 (0.3)
A2 Intervention not accepted	50 (12.5)
A2.1. Intervention not accepted: not feasible	1 (0.25)
A2.2. Intervention not accepted: no agreement	49 (12.3)
Status of the DRP	**n (%)**
O1 Problem totally solved	348 (87.2)
O2 Problem partially solved	2 (0.5)
O3 Not solved	49 (12.3)
O3.2. Problem not solved, lack of cooperation of prescriber	48 (12)
O3.4. No need or possibility to solve problem	1 (0.3)

In the current study, the most seen planned interventions' subcategory of I3, was found I3.2 "dosage changed to . . . " by 19.5%. Similar to our study, Martins et al. have reported the I3.2 by 24.09% as the most seen subcategory of the I3 category (Martins et al., 2019). In contrast to our study, Al-Azzam et al. classified only the interventions and found the interventions about drug dose changes by 7.3% (Al-azzam, Shara, Alzoubi, Almahasneh, & Iflaifel, 2013). It was reported in the study by Bourne et al. that the most made interventions were "new drug addiction", "drug dose change", "drug administration changes", and "stopping the drug", respectively (Bourne, Choo, & Dorwarb, 2014). We thought that these differences originated from the different drug classification systems. The I3.6 "drug started" subcategory was found by 4.3% in our study which differed from the study by Jiang et al. (16.6%), and Kubas and Halboup (18.9%) (Jiang, Chen, Zhang, Lu & Zhao, 2014; Kubas & Halboup, 2020). We thought that these differences occurred since the classification systems differed from each other and our study.

The accepted interventions were found at the rate of 87.5% in our study, similar to other ICU setting studies that were found by Albayrak et al. (90.8%), Johansen et al (87%), and Martins et al. (85.45%) (Albayrak et al., 2022; Johansen et al., 2016; Martins et al., 2019). It is possible to say that clinical pharmacist interventions' were accepted at a higher rate independent of the studies' geography. The number of DRPs per patient was found (1.55) similar to other studies in the literature that were found as 1.8 and 1.36 (Johansen et al., 2016; Albayrak et al., 2022; Martins et al., 2019).

Polymorbidity, polypharmacy, and anticoagulant, antibacterial, and corticosteroid usage were found to be risk factors for DRPs (Kaufman et al., 2015). Polypharmacy and antibacterial usage were found to be risk factors in our study harmoniously with the literature. Differently, atrial fibrillation and hematopoietic stem cell transplantation were found to be independent risk factors for DRPs among comorbidities. Clinicians should pay attention when prescribing new drugs to patients with atrial fibrillation and a history of hematopoietic stem cell transplantation. Furthermore, clinicians and clinical pharmacists should pay attention if polypharmacy and antibacterial drugs are present in medical therapy.

DRPs are seen commonly in ICUs and clinical pharmacists

recommend solution proposals to clinicians. In the literature treatment safety was the most seen DRPs category, and drug selection and dose selection were the most seen causes of DRPs (Albayrak et al., 2022; Ayhan et al., 2022; Martins et al., 2019).

The most commonly seen DRP categories (treatment safety) and their causes (drug selection and dose selection) were found similar to the literature, so clinical pharmacists and clinicians should pay attention to these topics. The literature and our study promote clinical pharmacists' beneficial roles in ICUs as well as all services of hospitals. We thought that one or more clinical pharmacists should work in all services of hospitals, especially in ICUs so the World's healthcare system will take an important step. In the literature, there are many studies about the evaluation of the clinical pharmacist's implementation in the ICUs. However, this study is one of the most comprehensive studies among a few studies which were conducted in internal diseases ICUs. New studies should be done in specific departments of internal diseases ICUs (Nephrology, Gastroenterology, Endocrinology, Hematology, Medical Oncology, etc.)

Strengths and Limitations

The limitations of our study were being one single-center study, not specifying an ICU department, and evaluation of DRPs by one clinical pharmacist; and while inclusion criteria were determined to prevent potential bias, the homogeneity of the study might have not been achieved. Additionally, we could count as a limitation that some DRPs did not comply with the explanatory categories of the PCNE method, so they were classified under the "unclear problem" of the "other" problem category. The strengths of our study were having a prospective design, a large patient population for 7 months, and a comparison of different science departments in the ICU.

CONCLUSION

Atrial fibrillation and hematopoietic stem cell transplantation were found to be independent risk factors for DRPs among comorbidities offering new data to the literature. The presence of antibacterials and polypharmacy were found to be independent risk factors for DRPs as supporting to the literature.

The most common DRP categories (treatment safety) and their causes (drug selection and dose selection) were found similar to the other studies, thus, supporting the literature.

It was concluded in this study that; clinicians should pay attention when prescribing new drugs to patients with atrial fibrillation and a history of hematopoietic stem cell transplantation. Clinical pharmacists and clinicians should pay attention to "drug selection" and "dose selection" areas when reviewing patients' therapy in ICUs. Furthermore, this study attracts attention to the evaluation of clinical pharmacy services in specific areas of internal diseases ICUs.

The literature and our study promote clinical pharmacists' beneficial roles in ICUs as well as in all services of hospitals. We suggest that one or more clinical pharmacists should work in all services of hospitals, especially in ICUs.

List of Abbreviations

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration
DRP: Drug-related problem
eGFR: estimated glomerular filtration rate.
GCS: Glasgow coma scale
ICU: Intensive care unit
PCNE: Pharmaceutical Care Network Europe

Ethics Committee Approval: Ethical approval for the study was obtained from the non-interventional ethics committee of İnönü University on 29.06.2021 (Decision no:2021267).

Informed Consent: Inform consent was obtained from all individual participants included in the study or from their relatives.

Peer-review: Externally peer-reviewed.

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REFERENCES

Albayrak, A., Başgut, B., Bıkmaz, G, A., & Karahalil, B. (2022). Clinical pharmacist assessment of drug-related problems among intensive care unit patients in a Turkish university hospital. *BMC Health Service Research, 22*(1), 1-7. https://doi.org/10.1186/s12913-022-07494-5

Aljbouri, T. M., Alkhawaldeh, M. S., Abu-Rumman, K.,Hasan, T. A., Khattar, H. M., & Abu- Oliem, A. S. (2013). Impact of clinical pharmacist on cost of drug therapy in the ICU. *Saudi Pharmaceutical Journal, 21*(4), 371-374. https://doi.org/10.1016/j.jsps.2012.12.004

Al-azzam, S. I., Shara, M., Alzoubi, K. H., Almahasneh, F. A., & Iflaifel, M. H. (2013). Implementation of clinical pharmacy services at a university hospital in Jordan. The In-

ternational *Journal of Pharmacy Practice, 21*(5), 337-340. https://doi.org/10.1111/ijpp.12009

Al-Jazairi, A. S., Al-Agil, A. A., Asiri, Y. A.,Al-Kholi, T. A., Akhras, N. S., & Horanieh, B. K. (2008).The impact of clinical pharmacist in a cardiac-surgery intensive care unit. *Saudi Medical Journal, 29*(2), 277-81.

Ayhan, Y. E., Karakurt, S., & Sancar, M. (2022). The effect of the clinical pharmacist in minimizing drug-related problems and related costs in the intensive care unit in Turkey: A non- randomized controlled study. *Journal of Clinical Pharmacy and Therapeutics, 47*(11), 1867- 1874. https://doi.org/10.1111/jcpt.13784

Bourne, R. S., Choo, C. L., & Dorward, B. J. (2014). Proactive clinical pharmacist interventions in critical care: effect of unit speciality and other factors. *The International Journal of Pharmacy Practice, 22*(2), 146-154. https://doi.org/10.1111/ijpp.12046

Clemmons, A. (2020). The Hematopoietic Cell Transplant Pharmacist: A Call to Action. *Pharmacy, 8*(1), 1-7. https://doi.org/10.3390/pharmacy8010003

Greeshma, M., Lincy, S., Maheswari, E., Tharanath, S., & Viswam, S. (2018). Identification of drug related problems by clinical pharmacist in prescriptions with polypharmacy: A prospective interventional study. *Journal of Young Pharmacists, 10*(4), 460-465. https://doi.org/10.5530/jyp.2018.10.100

Horvat, N., & Kos, M. (2016). Development and validation of the Slovenian drug-related problem classification system based on the PCNE classification V 6.2. *International Journal of Clinical Pharmacy, 38*(4), 950-959. https://doi.org/10.1007/s11096-016-0320-7

Jiang, S-P., Chen, J., Zhang, X-G., Lu, X-Y., & Zhao, Q-W. (2014). Implementation of pharmacists' interventions and assessment of medication errors in an intensive care unit of a Chinese tertiary hospital. *Therapeutics and Clinical Risk Management, 10*, 861-866. https://doi.org/10.2147/TCRM.S69585

Johansen, E. T., Haustreis, S. M., Mowinckel, A. S., & Ytrebø, L. M. (2016). Effects of implementing a clinical pharmacist service in a mixed Norwegian ICU. *European Journal of Hospital Pharmacy, 23*(4), 197-202. https://doi.org/10.1136/ejhpharm-2015-000751

Kubas, M. A., & Halboup, A. M. (2020). Implementation of clinical pharmacist recommendations and services at a University Hospital in Yemen. *International Journal of Clinical Pharmacy, 42*(1), 51-56. https://doi.org/10.1007/s11096-019-00936-x

Kaufmann, C. P., Stämpfli, D., Hersberger, K. E., &Lampert, M. L. (2015). Determination of risk factors for drug-related problems: A multidisciplinary triangulation process. *BMJ Open,5*(3), e006376-82. https://doi.org/10.1136/bmjopen-2014-006376

Lee, H., Ryu, K., Sohn, Y.,Kim, J., Suh, G. Y., &Kim, E. Y. (2019). Impact on patient outcomes of pharmacist participation in multidisciplinary critical care teams: A systematic review and meta-analysis. *Critical Care Medicine, 47*(9), 1243-1250. https://doi.org/10.1097/CCM.0000000000003830

Lenssen, R., Heidenreich, A., Schulz, J. B., Trautwein, C., Fitzner, C., Jaehde, U., & Eisert, A. (2016). Analysis of drug-related problems in three departments of a German University hospital. *International Journal of Clinical Pharmacy, 38*(1), 119-126. https://doi.org/10.1007/s11096-015-0213-1

Li, X-x., Zheng, S-q., Gu, J-h., Huang, T., Liu, F., Ge, Q-g., . . . Shi, L-w. (2020). Drug-related problems identified during pharmacy intervention and consultation: Implementation of an intensive care unit pharmaceutical care model. *Frontiers in Pharmacology. 11*, 1-13 (571906). https://doi.org/10.3389/fphar.2020.571906

Martins, R. R., Silva, L. T., & Lopes, F. M. (2019). Impact of medication therapy management on pharmacotherapy safety in an intensive care unit. *International Journal of Clinical Pharmacy, 41*(1), 179-188. https://doi.org/10.1007/s11096-018-0763-0

Masnoon, N., Shakib, S., Kalisch-Ellett, L., & Caughey, G. E. (2017). What is polypharmacy? A systematic review of definitions. *BMC Geriatrics, 17*(230), 1-10. https://doi.org/10.1186/s12877-017-0621-2

Napier, K., Lim, D., Thomas, E., Boyd, J., Chakera, A., Williamson, J., . . . Robinson, S. (2022). Impact of routine reporting of estimated glomerularfiltration rate using the European Kidney Function Consortium and Chronic Kidney Disease Epidemiology Collaboration equations in a Western Australian community population. *Nephrology, 27*(10), 823-833. https://doi.org/10.1111/nep.14083

Ozdemir, N., Celiker, A., Kuskonmaz, B. B., Okur, F. V., & Cetinkaya, D. U. (2019). Evaluation of Drug-Related Problems in a Pediatric Bone Marrow Transplantation Unit Identified by a Clinical Pharmacist in-training in a 7-Month Period. *Clinical and Experimental Health Sciences, 10*(1), 21-26. https://doi.org/10.33808/clinexphealthsci.590213

Pharmaceutical Care Network Europe Association. (20.04.2023). Accessed address: https://www.pcne.org/upload/files/410_PCNE_classification_V9-0m.pdf.

Reinau, D., Furrer, C., Stämpfli, D.,Bornand, D., & Meier, C. R. (2019). Evaluation of drug- related problems and subsequent clinical pharmacists' interventions at a Swiss university hospital. *Journal of Clinical Pharmacy and Therapeutics, 44*(6), 924-931. https://doi.org/10.1111/jcpt.13017

Ritchie, L. A., Penson, P. E., Akpan, A. A., Lip, G. Y. H., & Lane, D. A. (2022) Integrated Care for Atrial Fibrillation Management: The Role of the Pharmacist. *The American Journal of Medicine, 135*(12), 1410-1426. https://doi.org/10.1016/j.amjmed.2022.07.014

Ruths, S., Viktil, K. K., &Blix, H. S. (2007). Classification of drug-related problems. *The Journal of the Norwegian Medical Association,127*, 3073-3076.

Tasaka, Y., Tanaka, A., Yasunaga, D.,Asakawa, T., Araki, H., &Tanaka, M. (2018). Potential drug-related problems detected by routine pharmaceutical interventions: Safety and economic contributions made by hospital pharmacists in Japan. *Journal of Pharmaceutical Health Care and Sciences, 4*(33), 1-11. https://doi.org/10.1186/s40780-018-0125-z

Tharanon, V., Putthipokin, K., &Sakthong, P. (2022). Drug-related problems identified during pharmaceutical care interventions in an intensive care unit at a tertiary university hospital. *SAGE Open Medicine, 10*, 1-10. https://doi.org/10.1177/20503121221090881

Toukhy, A., Fayed, S., Sabry, N., & Shawki M. (2021). The Impact of an Established Pharmaceutical Care Pathway on Drug Related Problems in an Intensive Care Unit. *The American Journal of Medical Sciences, 362*(2), 143-153. https://doi.org/10.1016/j.amjms.2021.03.007

Viktil, K. K., & Blix, H. S. (2008). The impact of clinical pharmacists on drug-related problems and clinical outcomes. *Basic & Clinical Pharmacology & Toxicology,102*(3), 275-280. https://doi.org/10.1111/j.1742-7843.2007.00206.x

Zaidi, S. T. R., Hassan, Y., Postma, M. J., & Seiw Hain, N. (2003). Impact of pharmacist recommendations on the cost of drug therapy in ICU patients at a Malaysian hospital. *Pharmacy World and Science, 25*(6), 299-302. https://doi.org/10.1023/B:PHAR.0000006524.52076.2f

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Original Article

The effects of chard extract against streptozotocin-induced erectile dysfunction in rats

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ABSTRACT

Background and Aims: To analyze the potential therapeutic effects of chard against streptozotocin (STZ) -induced erectile dysfunction (ED) and oxidative damage in the corpus cavernousum in rats.

Materials and Methods: In this study, Sprague-Dawley rats (250-300g) were allocated into groups as follows: control, diabetic, diabetic + chard, and diabetic + insulin. In order to induce diabetes, rats were given 65 mg/kg intraperitoneal streptozotocin. Chard extract was given orally at a dose 2 g/kg for 45 days beginning on 15th days. Sixty days after STZ injection, intracavernosal pressure (ICP) was measured and rats were decapitated. Blood samples were obtained for glucose, asymmetric dimethylarginine (ADMA)levels, and lactate dehydrogenase (LDH) activity while cavernous tissues were taken to analyze luminol and lucigenin chemiluminescence (CL), malondialdehyde and glutathione and along with histological analysis.

Results: The results revealed that diabetes caused significant decreases in cavernosal tissue glutathione levels, while luminol and lucigenin CL, and malondialdehyde levels were significantly elevated. Plasma glucose, ADMA levels, and LDH activity were also found to be increased in diabetic group. On the other hand, both chard extract and insulin treatment reversed these biochemical parameters significantly. Furthermore, it was found that the ICP value examined for evaluating erectile functions were lower in the diabetic group, but increased in both treatment groups which were similar to the control values.

Conclusion: According to our results, chard extract, similar to insulin, reduced diabetes-induced oxidative damage in cavernosal tissue and protected erectile functions. This effects may be attributed its hypoglycemic and antioxidant properties.

Keywords: Chard extract, diabetes mellitus, erectile dysfunction, oxidative damage

INTRODUCTION

Increased reactive oxygen species (ROS) increase the risk of atherosclerosis and cardiovascular disease in diabetic patients (Kesavulu et al., 2001).Erectile dysfunction is one of the main complications developing in male diabetes patients and has a negative impact on quality of life. *In vivo* experimental studies report that in diabetic ED, endothelial dysfunction and reduction in nitric acid synthase (NOS) activity are mediated by ROS (Saenz de Tejada, Goldstein, Azadzoi, Krane, & Cohen, 1989; Way & Reid, 1999; Taylor, 2001). A hyperglycemic environment has been linked to increased formation of free oxygen radicals due to glucose auto-oxidation and also protein glycation. When the antioxidant capacity of the body is exceeded, the increased free radicals cannot be scavenge, and in this case, the tissue damage begins due to radicals harmful effects (Hunt, Smith, &Wolff, 1999). The main aim of diabetes treatment is to control hyperglycemia, as well as to prevent complications caused by high blood sugar.

Research on new agents with antidiabetic effects, especially those of natural origin, is still ongoing, and it is also being investigated whether these agents are protective against diabetic complications (Neef, Declercq, & Laekeman, 1995; Grover, Yadav, & Vats, 2002; Gezginci-Oktayoglu, et al., 2014).Chard (*Beta vulgaris* L. var. *cicla*) [Chenopodiaceae] is one of phy-

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totheraupetic and hypoglycemic agents used by diabetic patients in Turkey (Yanardağ & Çolak, 1998). Chard contains phospholipids, ascorbic acid, glycolipids, polysaccharides, folic acid, fatty acids, vitamin E, carotenoids and minerals (Ertik, Sacan, Kabasakal, Sener &Yanardag, 2021). Various Beta vulgaris species contain saponins and flavonoids, which were reported to have hypoglycemic effects (Bozkalfa, Eşiyok, &Kaygisiz Aşçioğul, 2016). Moreover flavonoids, natural polyphenolic substances, can act as antioxidants in biological systems.

In previous studies, hypoglycemic and antioxidant properties of chard extract were studied in various tissues of diabetic rats (Bolkent, Yanardag, Tabakoglu-Oguz, & Ozsoy-Sacan, 2000; Ozsoy-Sacan, Karabulut-Bulan, Bolkent, Yanardag, & Ozgey, 2004; Sacan & Yanardag, 2010; Oztay et al., 2015; Ustundag et al., 2016; Sacan et al., 2018; Tunali, Cimen, & Yanardag, 2020; Ozel et al., 2021). However, the effects of this extract on the cavernous tissue are unknown. In this study, the effect of chard extract on the cavernous tissues of rats with diabetes mellitus with STZ was investigated functionally and biochemically.

MATERIAL AND METHODS

Biological plant material

Chard (*Beta vulgaris* L. var. *cicla*) leaves were obtained from traditional markets in Istanbul and identified by Prof.Dr. Neriman Ozhatay. Chard leaves were rinsed with cold tap water and then washed with cold distilled water in order to remove salts and other contaminants. Leaves were sliced, defoliated at 20 centigrade and then stored in well-sealed plastic bags.

Preparation of aqueous plant extract

Five hundred mL of distilled water was added to the shade-dried and powdered chard leaves (40g) and boiled for 30 minutes. The resulting extract was filtered and the water was removed under pressure. Before administration to rats, extract was dissolved in distilled water.

Animals

Male Sprague-Dawley rats (250-300g) were obtained from Marmara University Experimental Animals Research Center. The ethical committee approval was taken from the Marmara University Animal Care and Use Committee (68.2008.mar). Animals were divided into 4 groups containing 8 rats. Groups were as follows; control, diabetic, diabetic + chard treated, and diabetic + insulin treated.

Diabetes model

Diabetes was established in rats with a single dose and intraperitoneal (ip) administration of 65 mg/kg STZ (Sigma Chemical Co., Louis, MO). STZ was dissolved in citrate buffer, pH 4.5. Rats with plasma glucose levels of 200 to 250 mg/dL after 24 hours were careful diabetic. Chard extract was dissolved in 1 mL of distilled water. Beginning on the 15th day of the experiment, the diabetic + chard group was administered chard extract at a dose of 2 g/kg daily via gavage through an intragastric tube for 45 days, while insulin was given subcutaneously at a rate of 6U/kg/day(Junod, Lambert, Stauffacher, & Renold, 1969).At the end of the experimental period, they were subjected to intracavernosal pressure (ICP) measurement and thereafter decapitated to obtain blood and cavernosal tissue samples.

Plasma assays

An automated analyzer (Bayer Opera Biochemical Analyzer, Germany) was used to detect plasma LDH activities, while ADMA determination was performed with an ELISA kit (Immunodiagnostic AG). Blood glucose levels were measured using glucometer (OneTouch Ultra, LifeScan).

Measurement of erectile responses

On the 60th days of the study, erectile function was evaluated through both ICP and mean arterial pressure (MAP) measurements. Under anaesthesia with pentobarbital (30 mg/kg) trachea was cannulated with PE-240 polyethylene tubing to provide the patency of the airway. MAP in the cannulated left internal carotid artery was measured with an amplifier unit, data acquisition system and Biopac software system. ICP was measured via a 24-gauge needle transducer inserted the left crus of the penis. Isolated cavernosal nerve (CN) stimulation was achieved with a stainless steel bipolar electrode with parallel hooks placed around the nerve. A four-sided rhythm stimulation enthralled CN. Each rat received CNS at a frequency of 15 Hz with a pulse width of 30 seconds. To achieve significant erectile responses, CNS was administered at 2.5, 5, and 7.5 V. The inspiration time was one minute, with a three-to-five-minute rest interval between CNSs. The maximum ICP/MAP ratio is calculated by dividing the recorded highest ICP by the corresponding MAP and expressed as a percentage (Bivalacqua et al., 2003).

Cavernosal tissue malondialdehyde (MDA) and glutathione (GSH) measurement

1 g of tissue after adding 10 mL of 10% TCA was cooled with ice and homogenized in Ultra Turrax tissue homogenizer. Malondialdehyde (MDA) levels in tissue were determined by thiobarbituric acid method (Beuge & Aust, 1978). Cavernosal tissues were homogenized in cold 0.9% NaCl with glass equipment to obtain 10% (w/v) homogenate. Glutathione levels in tissue homogenate were analyzed according to the modified Ellman procedure (Beutler & Gelbart, 1986).

Luminol and lucigenin chemiluminescence (CL) measurements

Luminol and lucigenin chemiluminescence measurements are the best indicators of the amount of ROS formed in the tissues. Junior LB 9509 luminometer (EG&G Berthold, Germany)was used and measurements were done at room temperature (Haklar et al., 2002).

Histological assay

Tissues were washed in water after 10% formalin was applied and water was removed with increasing alcohol concentrations. It was then incubated in paraffin at 60°C overnight and the next day the tissue was embedded in paraffin blocks. The 6 mm thickness tissue sections embedded in paraffin were subjected to a series of treatments, colored with Hematoxylin-Eosin dyes and examined under the light microscope.

Statistical analysis

Data was analyzed using GraphPad Prism 5.0 statistical program (GraphPad Software, San Diego, CA, USA). Groups were compared with analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $p<0.05$ was considered statistically significant.

RESULTS

Blood glucose, body weight and ADMA values, and LDH activities of the groups were summarized in Table 1. Blood glucose values were found to be significantly higher in the diabetic rats while chard extract treatment decreased the glucose levels. Although insulin treatment also decreased the glucose levels; it was still higher than the control. ADMA level and LDH activity were found to be increased due to diabetes induction but both treatments decreased ADMA level and LDH activity significantly. Diabetes caused significant increase in both luminol and lucigenin CL and MDA levels in corpus cavernosum. On the other hand, GSH levels were decreased in the diabetic group ($p<0.05$-0.001). Chard extract and insulin treatment significantly reversed the elevations in the luminol, lucigenin CL and MDA levels, while reduced GSH levels were returned to the control levels ($p<0.05$-0.001) (Fig.1).

Erectile responses were assessed in control, diabetic, diabetic + chard and diabetic + insulin groups in a voltage dependent manner by ICP measurements. The ICP trace obtained with stimulation of the CNS at a setting of 7.5 V for 1 minute is shown in Figure 2. The mean ICP and ICP/MAP values recorded after CNS stimulation for 1 minute in all groups were given in Figure 3. In diabetic rats, these values were found to be significantly lower than in control, diabetic + chard and diabetic + insulin groups. On the other hand, chard treatment restored the erectile responses significantly ($p<0.05$-0.001).

In histological examination, regular cavernous structures were observed in the control group. Moderate irregularities and fibrotic structures were observed in the cavernous structures in the diabetic group and slight damage was observed in the endothelial structures. The damage in corpus cavernosum regressed indiabetic + chard and diabetic + insulin groups (Fig.4).

DISCUSSION

The results of the present study demonstrate that diabetes causes oxidative tissue damage in the corpus cavernosum of the rats, as evaluated by augmented luminol and lucigenin CL and lipid peroxidation, and reduced GSH levels. Treatment with chard extract reduces oxidant production thereby lipid peroxidation, and refills GSH content in the tissues, confirming the possible defensive effect of chard in contradiction of oxidative injury. Furthermore, chard extract protecting cavernosal tissue, erectile dysfunction seen in diabetic group was also improved. Since ED that is caused by diabetes significantly affects quality of life in male patients, chard extract may be an alternative option to prevent diabetic complications.

In the past, the vast majority of ED was thought to be due to nonspecific physiologic causes, but nowadays 50-80% of the affected males have detected organic etiology (Bivalacqua, Champion, Hellstrom, & Kadowitz, 2000). Organic ED is most often linked to vascular risk factors such as arteriosclerosis, hyperlipidemia, hypertension, and diabetes mellitus. ED is more common in diabetic men than in the normal population and treatment is often more difficult this subgroup (Rendell, Rajfer, Wicker, & Smith, 1999). Currently, there are various medications and medical applications in ED treatment. However, the treatment of ED in diabetic patients should be related to diabetes induced ED pathophysiology, which may increase the treatment success. Furthermore, when side effects of drugs in ED treatment are taken into account, plant-based treatment alternatives are even more remarkable.

Chard (*Beta vulgaris* var. *cicla*) is one of the phytotherapeutic used as another hypoglycemic medicine by diabetic patients in Turkey. The hypoglycemic effect of chard was initially described by Yanardağ & Çolak (1998). Experimental studies have shown that the chard regenerates pancreatic beta cells, protects kidney functions in diabetic animals, and prevents oxidative stress caused by hyperglycemia in skin, heart, and aorta (Tunali et al., 1998; Sener, Saçan, Yanardağ, & Ayanoğlu-Dülger, 2002; Yanardağ, Bolkent, Özsoy-Saçan, & Karabulut-Bulan, 2002). Therefore, chard can be used as an alternative treatment for type 1 and type 2 diabetes due to its regarenative effect in pancreatic beta cells and its hypoglycemic effect with saponins and flavonoids (Bozkalfa et al., 2016).

ADMA, is an endogenous inhibitor of nitric oxide synthase

Table 1. Blood glucose, body weight and ADMA levels and LDH activities of all groups.

	Control	Diabetic	Diabetic+chard	Diabetic+insulin
Glucose (mg/dL)				
t0	91 ± 8.1	90 ± 5.2	94 ± 6.3	95 ± 7.1
24th hours	95 ± 5.1	310 ± 30.6 ***	331 ± 21.7 ***	333 ± 24.1 ***
15th days	96 ± 5.3	371 ± 31.3 ***	351 ± 26.3 ***	383 ± 23.4 ***
60th days	97 ± 5.4	362 ± 25.5 ***	143 ± 9.8 $^{+++}$	164 ± 14.2 *, $^{+++}$
Body weight				
t0	311 ± 5.3	310 ± 9.4	322 ± 8.6	309 ± 8.2
60th days	405 ± 6.3	289 ± 6.9	404 ± 7.1	419 ± 10.5
ADMA (µmol/L)	1.03 ± 0.1	3.42 ± 0.3 ***	1.65 ± 0.2 $^{+++}$	2.08 ± 0.2 *, $^{++}$
LDH (U/L)	76 ± 4.2	133 ± 10.5 ***	95.8± 5.1 $^{++}$	103± 7.2 $^{+}$

Each group consists of 8 rats. Values are represented as mean ± SEM.
* p<0.05, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01^{+++}p<0.001; vs vehicle-treated diabetic group.

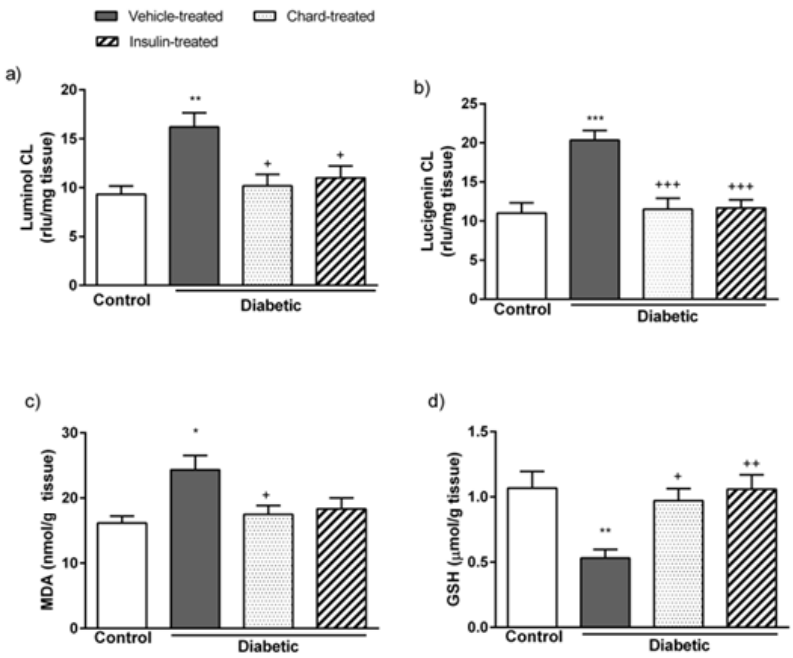

Figure 1. a) Luminol CL, b) Lucigenin CL, c) Malondialdehyde and d) Glutathione (GSH) values of control, diabetic, diabetic + chard and diabetic + insulin groups.Standards are signified as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01^{+++}p<0.001; vs vehicle-treated diabetic group.

(NOS) and augmented plasma levels of ADMA causes endothelial and thereby cardiovascular dysfunction. However, the relation of ADMA to diabetes, glycemic control, and renal function, specially early diabetic hyperfiltration, remains unknown (Kielsteinet et al., 2003). Asymmetric dimethylarginine activating renin-angiotensin system and NAD(P)H oxidase leads to increase in production of $O2^{-\bullet}$, which then delays with the bioavailability of NO, subsequent in reduced dilation and augmented arteriolar tone (Veresh, Racz, Lotz, & Koller, 2008). Therefore, increased ADMA levels in our study may have led to reduce NO bioavailability and may have led to erectile dys-

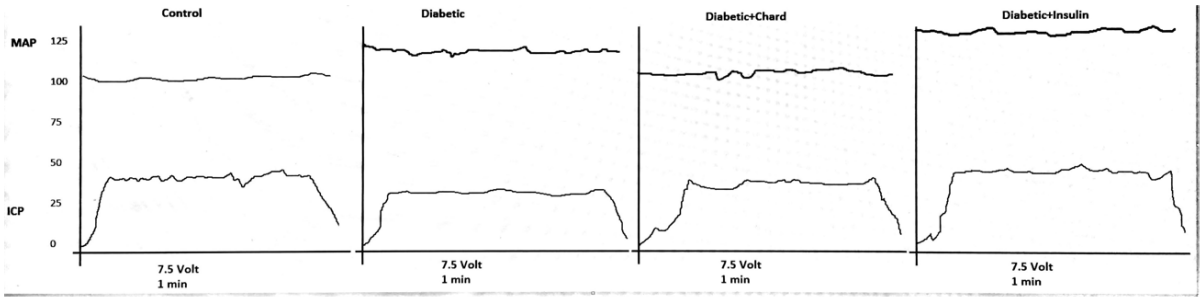

Figure 2. Representative ICP and MAP tracing after CNS at 7.5 V setting for 1 minute in control, , diabetic, diabetic + chard and diabetic + insulin groups.

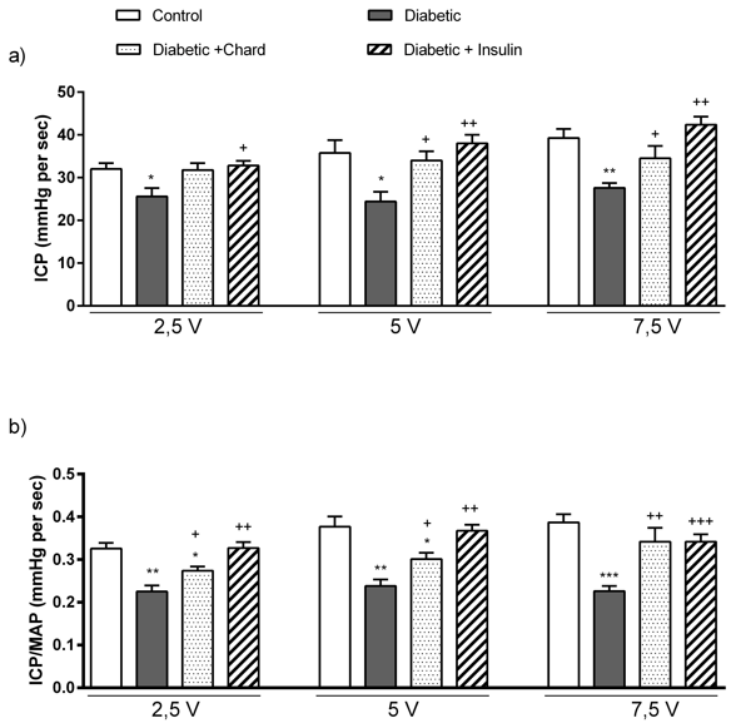

Figure 3. a) ICP and b) ICP/MAP values of control, diabetic, diabetic + chard and diabetic + insulingroups. Values are represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001; vs vehicle-treated diabetic group. (ICP: Intracavernosal pressure, MAP: Mean arterial pressure).

function. In contrast chard extract, suppressed ADMA levels and thus protected erectile function.

Increased ROS levels in diabetic human corpus cavernosum tissue compared to controls may designate that ROS formation theatres a role in the pathogenesis of diabetic endothelium and ED (Seftel et al.,1997). In the present study oxidant making was evaluated with luminol and lucigenin CL measurements. Luminol notices a group of reactive species, i.e. hypochlorous acid (HOCL), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-),while lucigenin is discerning for superoxide anion($O2^-$) (Haklar et al., 2002). Our results demonstrated that diabetes increased these oxidant species in the cavernosal tissue since both CL values were significantly increased. On the other hand, chard extract effectively reduced the production of oxidants and there by lipid peroxidation. Free radicals, which are promoted by increased protein glycosylation and glucose auto-oxidation, may increase lipid peroxidation in some organs. A significant increase in the level of MDA from lipid peroxidation products resulting from oxidative stress in the erectile tissues of diabetic rats and a decrease in the level of GSH responsible for antioxidation of free radicals has been reported (Ryu et al.,2003). Similar to Ryu et al. study, in our study, lipid peroxidation product MDA increased in diabetic animals and GSH levels responsible

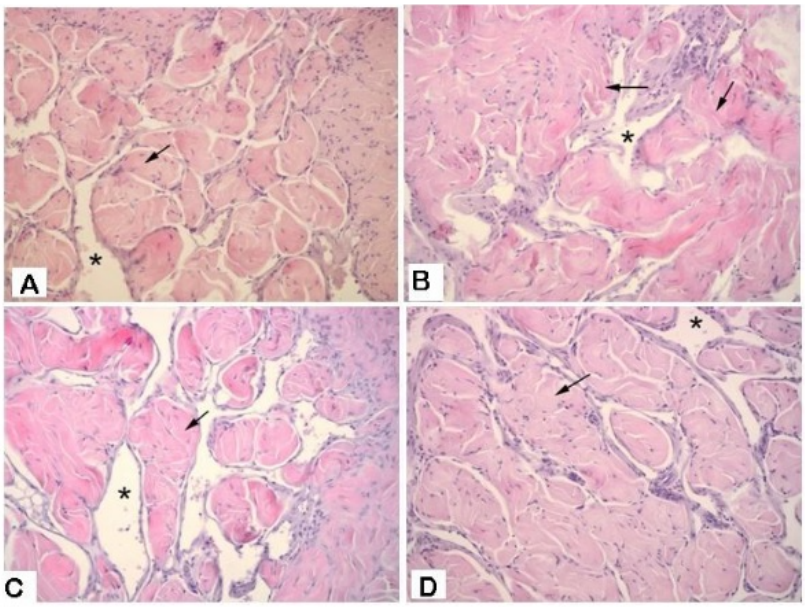

Figure 4. Regular cavernous structures were observed in the control group (A). Regular cavernous structure (arrow) and endothelium (*). In diabetic group (B) moderate irregularities and fibrotic structures (arrows) were observed in cavernous structures and slight damage (*) was observed in endothelial structures. The damage in corpus cavernosum regressed in diabetic + chard (C) and diabetic + insulin (D) groups. Cavernous structure (arrow), regular endothelium (magnification, X200).

for antioxidation decreased significantly. This result confirms the characterization of diabetic corpus cavernosum with overexpression of free radicals and inadequate elimination. In this study, chard treatment increased GSH levels significantly in diabetic rats. In parallel with this finding, the lipid peroxidation in diabetic + chard group was significantly lower than the diabetic group ($p<0.05$). Also, this value was similar to the control group. This finding showed the strong antioxidative activity of chard in diabetes mellitus. Chard extract treatment reduced oxidative stress by showing antioxidant activity in the penis as in other tissues, but also significantly improved erectile answer to cavernous nerve stimulation in vivo.

The overproduction of free radicals and the attenuation of antioxidant systems competing with them may be responsible for the structural and functional damage of the corporal endothelium and smooth muscle responsible for ED (Ryu et al., 2003). Ahn et al. (2005) showed that Transforming Growth Factor-β1 (TGF-β1) expression and corporal fibrosis increased in diabetic rats, and fibrosis regressed with phosphodiesterase type 5 (PDE5) inhibitor treatment. In present study, moderate irregularity and fibrotic structures were observed in the cavernous structures in the diabetes group and moderate damage to the endothelial structures was observed. It was observed that the damage was regressed with both chard and insulin treatment (Ahn et al., 2005). Moreover, this information supports the therapeutic use of antioxidants or free-radical protectants in diabetic ED.

Men with diabetic ED are a difficult group to treat and are often resistant to treatment with standard oral PDE5 inhibitors. In the general population, the effect of PDE5 inhibitors is shown between 70-89%; however, the effect is just over 50% in the diabetic population (Rendell et al.,1999). One of the possible causes of this relative resistance has been reported to be oxidative stress (De Young, Yu, Freeman, & Brock, 2003). It has been reported that a significant increase in sildenafil response was observed in diabetic rats given vitamin E (De Young, Yu, Freeman, & Brock, 2004). In our study, chard showed an antioxidant effect, in addition to the normoglycemic effect. Based on these data, antioxidant treatments such as chard may be useful as an alternative treatment in patients with diabetic ED. In addition, this study is the first in the literature to evaluate the effects of chard on the penile tissue. Further studies are needed to confirm these findings.

CONCLUSIONS

The results of this study indicated that chard extract exhibited protective activity against diabetes-induced erectile dysfunction. This effect may be related with augmented activity of anti-oxidant protection system and decrease in oxidative stress in the penile corporal cavernousa of rats. Chard leaf extract with having strong antioxidant properties, can protect the cavernosal tissue against diabetes-induced oxidative damage and thus regulate erectile dysfunction.

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REFERENCES

Ahn, G.J., Sohn, Y.S., Kang, K.K., Ahn, B.O., Kwon, J.W., Kang, S.K.,& Wang, W.S. (2005). The effect of PDE5 inhibition on the erectile function in streptozotocin-induced diabetic rats. *International Journal of Impotence Research, 17*, 134-141. https://doi.org/ 10.1038/sj.ijir.3901295.

Beuge, J.A., & Aust, S.D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302–311. https://doi.org/ 10.1016/s0076-6879(78)52032-6.

Beutler, E., &Gelbart, T. (1986). Improved assay of the enzymes of glutathione synthesis: Gamma-glutamylcysteine synthetase and glutathione synthetase. *Clinica Chimica Acta, 158*, 115-123. https://doi.org/ 10.1016/0009-8981(86)90122-1.

Bivalacqua, T.J., Champion, H.C., Hellstrom, W.J., & Kadowitz, P.J. (2000). Pharmacotherapy for erectile dysfunction. *Trends Pharmacological Sciences, 21*, 484-489. https://doi.org/ 10.1016/s0165-614(00)01587-x.

Bivalacqua, T.J., Usta, M.F., Champion, H.C., Adams, D., Namara, D.B., Abdel-Mageed, A.B., &Hellstrom, W.J.G. (2003). Gene transfer of endothelial nitric oxide synthase partially restores nitric oxide synthesis and erectile function in streptozotocin diabetic rats. *Journal of Urology, 169*, 1911-1917. https://doi.org/ 10.1097/01.ju.0000051881.14239.4a.

Bolkent, S., Yanardağ, R., Tabakoğlu-Oğuz, A., &Ozsoy-Saçan, O. (2000). Effects of chard (*Beta vulgaris* L. var. cicla) extract on pancreatic B cells in streptozotocin-diabetic rats: a morphological and biochemical study.*Journal of Ethnopharmacology, 73*, 251-259. https://doi.org/ 10.1016/s0378-8741(00)00328-7.

Bozkalfa, M., Eşiyok, D., &Kaygisiz-Aşçioğul, T. (2016). Diversity pattern among agromorphological traits of the Swiss chard (*Beta vulgaris* L. subsp. vulgaris) genetic resources of Turkey. *Turkish Journal of Agriculture and Forestry, 40*, 684-695. https://doi.org/10.3906/tar-1512-80.

De Young, L., Yu, D., Freeman, D., & Brock, G.B. (2003). Effect of PDE5 inhibition combined with free oxygen radical scavenger therapy on erectile function in a diabetic animal model. *International Journal of Impotence Research, 15*, 347–354. https://doi.org/ 10.1038/sj.ijir.3901026.

De Young, L., Yu, D., Bateman, R.M., & Brock, G.B. (2004). Oxidative stress and antioxidant therapy: their impact in diabetes-associated erectile dysfunction. *Journal of Andrology, 25*, 830–836. https://doi.org/ 10.1002/j.1939-4640.tb02862.x.

Ertik,O.,Sacan,O., Kabasakal, L.,Sener,G.,&Yanardag, R. (2021). Protective effect of chard extract on glycoprotein compounds and advanced oxidation protein product levels in diabetic rat livers. *Experimed,11*, 27-32. https://doi.org/ 10.26650./experimed.2021.879204.

Gezginci-Oktayoglu, S., Sacan, O., Bolkent, S., Ipci, Y., Kabasakal, L., Sener, G., &Yanardag, R. (2014). Chard (*Beta vulgaris* L. var. cicla) extract ameliorates hyperglycemia by increasing GLUT2 through Akt2 and antioxidant defense in the liver of rats. *Acta Histochemica, 116*, 32-39. https://doi.org/ 10.1016/j.acthis.2013.04.016.

Grover, J.K., Yadav, S., & Vats, V. (2002). Medicinal plants of Indiana with anti-diabetic potential. *Journal of Ethnopharmacology, 81*, 81-100. https://doi.org/ 10.1016/s0378-8741(02)0059-4.

Haklar, U., Yüksel, M., Velioglu, A., Turkmen, M., Haklar, G., &Yalçın, A.S. (2002). Oxygen radicals and nitric oxide levels in chondral or meniscal lesions or both. *Clinical Orthopaedics and Related Research, 403, 135-142*. https://doi.org/ 10.1097/00003086-200210000-00021.

Hunt, J.V., Smith, C.C., & Wolff, S.P. (1999). Autooxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes, 3*, 1420-1424. https://doi.org/ 10.2337/diab.39.11.1420.

Junod, A., Lambert, A.E., Stauffacher, W., & Renold, A.E. (1969). Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *Journal of Clinical Investigation, 48*, 2129-2139. https://doi.org/ 10.1172/JCI106180.

Kesavulu, M.M., Rao, B.K., Giri, R., Vijaya, J., Subramanyam, G., &Apparao, C. (2001). Lipid peroxidation and antioxidant enzyme status in Type 2 diabetics with coronary heart disease. *Diabetes Research and Clinical Practice, 53*, 33-39. https://doi.org/ 10.1016/S0168-8227(01)00238-8.

Kielstein, J.T., Bode-Böger, S.M., Frölich, J.C., Ritz, E., Haller, H., &Fliser, D. (2003).Asymmetric dimethylarginine, blood pressure, and renal perfusion in elderly subjects. *Circulation, 107*, 1891-1895. https://doi.org/ 10.1161/01.CIR.0000060496.23144.A7.

Neef, H., Declercq, P., & Laekeman, G. (1995). Hypoglycemic activity of selected European plants. *Phytotherapy Research, 9*, 45-48.

Ozel, A.B., Cilingir-Kaya, O.T., Sener, G., Ozbeyli, D., Sen, A., Sacan, O.,Yanardag, R., & Yarat, A. (2021). Investigation of possible neuroprotective effects of some plant extracts on brain in bile duct ligated rats. *Journal of Food Biochemistry, 45, e 13835*.

https://doi.org/10.1111/jfbc.13835.

Ozsoy-Sacan, O., Karabulut-Bulan, O., Bolkent, S., Yanardag, R., &Ozgey, Y. (2004). Effects of chard (*Beta vulgaris* var. cicla) on the liver of the diabetic rats: a morphological and biochemical study. *Bioscience and Biotechnology Biochemistry, 68*, 1640-1648. https://doi.org/10.1271/bbb.68.1640.

Oztay, F., Sacan, O., Kayalar, O., Bolkent, S., Ipci, Y., Kabasakal, L.,& Yanardag R. (2015). Chard (*Beta vulgaris* var. cicla) extract improved hyperglycemia-induced oxidative stress and surfactant-associated protein alterations in rat lungs. *Pharmaceutical Biology, 53*, 1639-1646. https://doi.org/10.3109/13880209.

Rendell, M.S., Rajfer, J., Wicker, P.A., & Smith, M.D. (1999). Sildenafil for treatment of erectile dysfunction in men with diabetes: a randomized controlled trial. Sildenafil Diabetes Study Group. *Journal of American Medical Association, 281*, 421-426. https://doi.org/10.1001/jama.281.5.421.

Ryu, J.K., Kim, D.J., Lee, T., Kang, Y.S., Yoon, S.M., & Suh, J.K. (2003). The role of free radical in the patho-genesis of impotence in streptozotocin-induced diabetic rats. *Yonsei Medical Journal, 44*, 236-241. https://doi.org/10.3349/ymj.2003.44.2.236.

Sacan, O., & Yanardag, R. (2010). Antioxidant and anti-acetylcholinesterase activities of chard (*Beta vulgaris* L. var. cicla). *Food and Chemical Toxicology, 48*, 1275-1280. https://doi.org/10.1016/j.fct.2010.02.022.

Sacan, O., Ertik, O., Ipci, Y., Kabasakal, L., Sener, G., & Yanardag, R. (2018). Protective effect of chard extract on glycoprotein compounds and enzyme activities in streptozotocin-induced hyperglycemic rat lungs. *Bulgarian Chemical Communications, 50*, 119-123.

Saenz de Tejada, I., Goldstein, I., Azadzoi, K., Krane, R.J., & Cohen, R.A. (1989). Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *New England Journal of Medicine, 320*, 1025–1030. https://doi.org/10.1056/NEJM198904203201601. Retrived from http://bcc.bas.bg/BCC_Volumes/Volume_50_Number_1_2018/BCC-50-1-2018-119-123-4450.

Seftel, A.D., Vaziri, N.D., Ni, Z., Razmjouei, K., Fogarty, J., Hampel, N., &Haas, C. (1997). Advanced glycation end products in human penis: elevation in diabetic tissue, site of deposition, and possible effect through iNOS or eNOS. *Urology, 50*, 1016-1026. https://doi.org/10.1016/S0090-4295(97)00512-8.

Sener, G., Saçan, O., Yanardağ, R., &Ayanoğlu-Dülger, G. (2002). Effects of chard (*Beta vulgaris* L. var. cicla) extract on oxidative injury in the aorta and heart of streptozotocin-diabetic rats. *Journal of Medicinal Food, 5*, 37-42. https://doi.org/10.1089/109662002753723205.

Taylor, A.A. (2001). Pathophysiology of hypertension and endothelial dysfunction in patients with diabetes mellitus. *Endocrinology and Metabolism Clinics of North America, 30*, 983–997. https://doi.org/10.1016/s0889-8529(05)70223-1.

Tunali, T., Yarat, A., Yanardağ, R., Ozçelik, F., Ozsoy, O., Ergenekon, G., & Emekli, N. (1998). The effect of chard (*Beta vulgaris* L. var cicla) on the skin of streptozotocin-induced diabetic rats. *Pharmazie, 53*, 638-640. Retrieved from https://pubmed.ncbi.nlm.nih.gov/9770212.

Tunali, S., Cimen, E.S., & Yanardag, R. (2020). The effects of chard on brain damage in valproic acid-induced toxicity. *Journal of Food Biochemistry, 44*, e 13382. https://doi.org/ 10.1111/jfbc.13382.

Ustundag, U.V., Tunali, S., Alev B., İpekci, H., Emekli-Alturfan, E., Tunali,T,.Yanardag, R.,&Yarat, A.(2016). Effects of chard (*Beta vulgaris* L. var. cicla) on cardiac damage in valproic acid-induced toxicity. *Journal of Food Biochemistry, 40, 132-139*. https://doi.org/10.1111/jfbc.12202.

Veresh, Z., Racz, A., Lotz, G., & Koller, A. (2008). ADMA impairs nitric oxide-mediated arteriolar function due to increased superoxide production by angiotensin II-NAD(P)H oxidase pathway. *Hypertension, 52*, 960-966 https://doi.org/10.1161/HYPERTENSIONAHA.108.116731.

Way, K.J., & Reid, J.J. (1999). The effects of diabetes on nitric oxide-mediated responses in rat corpus cavernosum. *European Journal of Pharmacology, 376*, 73–82. https://doi.org/10.1016/s0014-2999(99)00347-7.

Yanardağ, R., & Çolak, H. (1998). Effect of chard (*Beta vulgaris* L. var cicla) on blood glucose levels in normal and alloxan-induced diabetic rabbits. *Pharmacy and Pharmacology Communications, 4*, 309-311. https://doi.org/10.1111/j.2042-7158-1998.tb00702.x.

Yanardağ, R., Bolkent, Ş., Özsoy-Saçan, Ö., & Karabulut-Bulan, Ö. (2002). The effect of chard (*Beta vulgaris* L. var cicla) extract on the kidney tissue, serum urea and creatinine levels of diabetic rats. *Phytotherapy Research, 16*, 758-761. https://doi.org/10.1002/ptr.1041.

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Original Article

Preparation of lidocaine hydrochloride containing chitosan-based buccal films for mucositis: *In-vitro* evaluation and cytotoxicity assay

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ABSTRACT

Background and Aims: The aim of this study was to prolong the anesthetic effect of lidocaine (LC) in the oral cavity for use in the treatment of oral mucositis and to compare the *in vitro* characteristics of the film formulations prepared by using either chitosan extracted from *Metapenaeus stebbingi (M. stebbingi)* or commercial chitosan.

Methods: In this study, the *in vitro* properties of the film formulations extracted and prepared with commercial chitosan were successfully compared with the addition of different types and amounts of plasticizer and cross-linking agent. In the evaluation of the formulations, different parameters such as structure, thickness, degree of swelling, moisture content, drug content, texture profile analysis, release kinetics according to the *in vitro* drug release, and cytotoxicity evaluation were taken into consideration.

Results: Films prepared using chitosan extracted with 5% glycerol addition showed the highest strength and lowest elongation properties compared to other films (p<0.05). The thickness of the films varied between 500-1400 µm in all formulations. While it was observed that formulations prepared with medium molecular weight commercial chitosan had high surface roughness, the lowest swelling degree was observed for these formulations (77.41 ± 3.65-84.76 ± 6.34). The highest degree of swelling was calculated for the formulations prepared with extracted chitosan (137.23 ± 7.86). The *in vitro* dissolution rate results demonstrated that the increase in the molecular weight of chitosan caused a decrease in the release rate of lidocaine, while at the same time, formulations with added crosslinking agents exhibited a slower release profile. Cytotoxicity studies revealed cell viability at different polymer concentrations.

Conclusion: All the *in vitro* characterization results showed that extracted chitosan from M. stebbingi shells can be a good alternative for pharmaceutical use.

Keywords: Buccal film, chitosan, lidocaine hydrochloride, mucositis, MTT.

INTRODUCTION

Mucositis is a common complication that can cause severe ulcers and, it is characterized by ulceration and inflammation of the entire gastrointestinal tract, often leading to reduced oral intake, weight loss, decreased quality of life, unpredictable interruptions in treatment, and even a life-threatening pathological condition with the development of secondary inflammations (Pulito et al., 2020). One of the primary causes of oral mucositis is the epithelial mucosal inflammatory response to chemo and/or radiotherapy cytotoxic effects as a severe side effect of antineoplastic therapies. Approximately 40% of chemotherapy patients develop oral mucositis, and this figure rises to 90% in patients who have head and neck cancer treatment. Among these patients, around 19% need hospitalization due to experiencing a delay in high-grade mucositis treatment (Elad, Yarom, Zadik, Kuten-Shorrer, & Sonis, 2022; Pulito et al., 2020).

The current management of oral mucositis is mainly symptomatic (Hosseinjani et al., 2017). Prioritizing the prevention and/or treatment of dry mouth, infections, and pain is crucial. Topical anesthetics and mucosal coating agents can also be applied to manage symptoms. Lidocaine (LC), one of the local anesthetics, is frequently utilized as a topical agent due to its immediate onset and mild duration of action. (Brown & Gupta, 2020; Silva et al., 2017).

Mucoadhesive dosage forms such as tablets, gels and films have been studied in recent years, especially regarding their

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formulation and development strategies (Cevher, Taha, Orlu, & Araman, 2008; Kottke, Majid, Breitkreutz, & Burckhardt, 2020). Mucoadhesive buccal patches may be preferred over conventional solid formulations due to their capability ability to provide an accurate drug dosage, small size, sufficient thickness, flexibility, and patient comfort (Escalona-Rayo et al., 2020; Morales & McConville, 2011). Buccal drug delivery systems are in intimate contact with the buccal mucosa to retain position in the mouth for a specified length of time. This can be achieved using mucoadhesive polymers and is an ideal feature for these systems (Kumar, Naik, Pradhan, Ghosh, & Rath, 2020; Mahdizadeh Barzoki, Emam-Djomej, Mortazavian, Moosavi-Movahedi, & Rafiee Tehrani, 2016). Although there are different synthetic polymers available, natural polymers are frequently utilized in the development of drug delivery systems because of their unique characteristics (Khade, Gadge, & Mahajan, 2020).

Chitosan is a natural polysaccharide that occurs abundantly in nature and is obtained by deacetylation of chitin. Though various natural polymers are available for the development of mucoadhesive drug delivery systems, chitosan is considered to be the most versatile natural polymer of them all. (Escalona-Rayo, Serrano-Castaneda, Lopez-Cervantes, & Escobar-Chavez, 2020; Younes & Rinaudo, 2015).

Chitosan, which is produced commercially from crustaceans, is mostly obtained from shrimp shells. *M. stebbingi*, found in the Aegean and the Mediterranean, can easily be captured and is in great demand both in Turkey and abroad. Unfortunately, there are very few detailed studies on the pharmaceutical use of the shell of this shrimp species in Turkey. One study (Küçükgülmez et al., 2011) evaluates the suitability of chitosan extracted from these shells for pharmaceutical formulations in terms of molecular weight, deacetylation degree, moisture content, water and fat binding capacity, and *in vitro* applicability as a buccal film. The study compares it with commercial chitosan.

Furthermore, the mucoadhesive nature of chitosan is another important property in providing a controlled and predictable drug release profile, making it among the first-choice drugs for controlled release in buccal administration. One reason why it is preferred above other drugs is due to its natural mucoadhesion feature obtained with a strong electrostatic adhesion force between the positively charged chitosan molecules that give chitosan a positive use feature and the negatively charged mucosal surface (Kumar, Vimal, & Kumar, 2016).

Due to its unique and attractive biological properties, including its hydrophilic nature, muco-adhesiveness, biodegradability, and nontoxicity, this cationic biopolymer is widely utilized in pharmaceutical applications and offers well-established polymeric properties (Shariatinia, 2019). Several studies have been conducted in the literature wherein chitosan was evaluated as a mucoadhesive film-forming polymer, and these studies have demonstrated the suitability of chitosan in formulating buccal films for various drugs as a carrier (Radha, Lal, & Devaky, 2022).

Using extracted and commercial chitosan as polymers, this study aimed to show the development of LC-loaded chitosan-based buccal films for the treatment of oral mucositis. *In vitro* characterization and *in vitro* cytotoxicity studies were conducted to evaluate the extracted and commercial chitosan's compatibility in mucositis treatment (Kumria, Al-Dhubiab, Shah, & Nair, 2018).

MATERIALS AND METHODS

Materials

All the pharmaceutical materials used in the study had analytical grades. Chitosan (CS, medium and low molecular weight), lidocaine hydrochloride (LC), propylene glycol (PG), glycerine (G) and tripolyphosphatepenta sodium salt (TPP) were obtained from Sigma-Aldrich (USA). Lactic acid was obtained from Merck (Germany) and pure water from a Millipore system was used for all the formulations.

Methods

Physicochemical Characterization of the Extracted Chitosan

Chitosan (CS) extraction from *M. stebbingi* shells was conducted using a modified method of that outlined by Chang, Tsai, Lee and Fu (1997). This method involves deproteinization and demineralization using sodium hydroxide (NaOH) and hydrochloric acid (HCl). Chitin residue was dried after treatment with hydrogen peroxide. Chitosan was obtained by alkali treatment of chitin with NaOH in distilled water at 120 °C, washed with deionized water to neutral pH, and then dried.

The weight measurements of the raw material and the extracted chitosan were compared to determine the chitosan extraction yield. Samples were dried at 105°C for 24 hours to determine the moisture content, and samples were heated at 530°C for 20 hours to determine the ash content (Küçükgülmez et al., 2011).

The deacetylation degree was determined using potentiometric titration and elemental analysis methods (Küçükgülmez et al., 2011; Tolaimate et al., 2000).

CS solutions in 0.2 M NaCl/0.1 M acetonitrile (AcOH) were produced at various concentrations for molecular weight determination. Using an Ubbelohde capillary viscometer in a water bath maintained at a constant temperature of 25 °C, the efflux times of the solutions were determined in triplicate, indicating the molecular weight (Küçükgülmez et al., 2011; Wang et al., 2006).

Applying a modified method of the one outlined by Wang

and Kinsela (1976), the water binding capacity (WBC) and fat binding capacity (FBC) of chitosan were determined (Wang & Kinsella, 1976).

To evaluate and compare the chemical structure of the extracted chitosan, FTIR spectra of the extracted and commercial chitosan were obtained by the Jasco FTIR-6700, at a frequency range of 4000 – 400 cm^{-1}.

Preparation of Chitosan Films Loaded with Lidocaine Hydrochloride

The films loaded with lidocaine hydrochloride (LC) were prepared by using low (L) and medium (M) molecular weight (MW) of commercial chitosan (CS) and CS extracted (E) from *M. stebbingi* shells with chemical methods. CS (2%, w/w) was dissolved by stirring in distilled water containing 2% lactic acid. Lactic acid was chosen to dissolve chitosan because chitosan lactate can cause more swelling and mucoadhesion compared to CS acetate, and it is also known that drug release is slower in chitosan lactate than in acetate salt (Cafaggi et al., 2005; Şenel et al., 2000). Plasticizers, glycerine, and propylene glycol, in different concentrations, were mixed with the solution (Table 1). After the addition of LC (4%), the solution (80 g) was spread on a glass plate (8 cm x 8 cm) and dried in an incubator at 37ºC. For the formulation of cross-linked films we used a 0.3% TPP solution.

HPLC Assay

A modified HPLC method was used for the determination of LC (Guideline, 2005; Malenovic, Medenica, Ivanovic, Jancic, & Markovic, 2005). Validation studies were performed for the data. HPLC conditions are shown in Table 2 (Demirtürk, Nemutlu, Şahin, & Öner, 2020).

Drug Content Measurements

Film sections (1 cm^2) were taken from the different areas of films (n=3) Calculations were made by measuring the weight of each film section separately. First, the films were dissolved in 10 mL of water, and the HPLC method was used to determine the LC amount of the samples. Drug content was calculated in samples examined by the validated HPLC method (Abouhussein, Nabawari, Shalaby, & Abd El-Bary, 2020).

Scanning Electron Microscopy

The surface morphology of the chitosan films was analyzed by scanning electron microscope (SEM, FEI Quanta FEG 650, US). After the samples were freeze-dried, they were placed on typical sample mounting slips and given a 20 nm thin layer of gold coating using a sputter coater unit.

Film Thickness Uniformity

The thickness uniformity of the films was determined by the digital micrometer (Showa Digimatic, China) by measuring the thickness of film samples (1 cm2) taken from the 5 different sections of the glass plates.

Swelling Degree

The swelling degree of the films was examined by causing them to swell in simulated saliva fluid (SSF) (pH 6.8) at 25 °C. This is based on the principle of leaving a known weight of the film on the media and examining it at certain intervals (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 h). After the swollen films were removed, they were carefully placed on filter paper to remove excess surface water and immediately weighed (n=3) (Abouhussein et al., 2020).

The following formula was used for the water uptake (Eq 1).

$$\text{Swelling Degree (\%)} = \frac{\text{weight of Fwollen Film-Initial Weight of the Film}}{\text{Initial Weight of the Film}} x100 \quad (1)$$

Moisture Content

The amount of moisture present in the films was determined by using the weighting method. A specific size of (1 cm x 1 cm) pre-weighed films was heated to 80°C until it attained a constant weight. The difference in weight gives the degree of moisture content in the films (n=3) (Anwar, Zaman, Raja, Mahmood, & Amjad, 2020).

The moisture content of the formulations was calculated by the following formula (Eq 2):

$$\text{Moisture Content (\%)} = [(\text{Initial Weight-Final Weight}) 100 / \text{Initial Weight}] \quad (2)$$

Texture Profile Analysis

Texture profile analysis was applied to characterize the mechanical properties of the films (TAXT Plus, Stable Micro Systems, United Kingdom). Tensile strength and percentage elongation of the films were evaluated. The test was carried out under conditions of pre-test, 0.1 mm/s and post-test at 0.5 mm/s speed (Alopaeus et al., 2020).

In Vitro Drug Release

In vitro drug release characteristics of the formulations were evaluated by the dialysis bag diffusion method. Simulated saliva fluid (SSF) was used as the dissolution medium (Al-Nemrawi, Alsharif, Alzoubi, & Alkhatib, 2019). Films containing LC were put in dialysis bags and then immersed in the dissolution medium containing 50 mL of SSF at 37 ± 1°C in a water bath. Samples of 1 mL were taken at specified intervals and replaced

Table 1. Codes and Compositions of Fim Formulations.

Formulation Code	Chitosan	Plasticizer	TPP
LG5	Low MW	5% glycerine	0.3%
LPG3	Low MW	3% propylene glycol	0.3%
LG10	Low MW	10% glycerine	0.3%
LPG6	Low MW	6% propylene glycol	0.3%
MG5	Medium MW	5% glycerine	0.3%
MPG3	Medium MW	3% propylene glycol	0.3%
MG10	Medium MW	10% glycerine	0.3%
MPG6	Medium MW	6% propylene glycol	0.3%
EG5	Extracted	5% glycerine	0.3%
EPG3	Extracted	3% propylene glycol	0.3%
EG10	Extracted	10% glycerine	0.3%
EPG6	Extracted	6% propylene glycol	0.3%

Table 2. HPLC conditions.

Device	Shimadzu, LC-2030C Prominence
Stationary Phase	VP-ODS C-18 column
Mobile phase	Water: Acetonitrile (50:50, v/v), pH 2.5
Oven temperature	40 ± 2°C
Flow rate	1 mL.min^{-1}
Injection volume	20 mL
Detection Wavelength	240 nm

with an equal amount of the fresh medium. LC content of the samples was analyzed by the HPLC (n=3).

Evaluation of *In Vitro* Drug Release Kinetics

Data were transferred to the DDSolver program after obtaining the LC release profiles to determine the three most important criteria: adjusted coefficient of determination (R2 adjusted), Akaike information criterion (AIC), and model selection criterion (MSC). The lowest AIC values, maximum R^2 adjusted, and MSC values were used to evaluate different kinetic models (Çevikelli et al., 2024).

Cytotoxicity Evaluation

Cell culture

L929 cell line was purchased from the American Type Culture Collection (ATCC-CCL-1, VA USA) and grown in 10% FBS and 1% penicillin-streptomycin containing Dulbecco's Modified Eagle Medium (DMEM). The cell culture medium was changed every 2-3 days, and subculturing was done when the cells reached 60-70% confluence.

Cytotoxicity assay

Cytotoxic potentials of the formulations were evaluated with MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The tetrazolium ring of MTT dye was metabolized to the purple-colored formazan crystals by mitochondrial active cells (Ghasemi et al., 2021). $1x10^4$/well were seeded into a 96-well plate and incubated overnight for cell attachment. The films were prepared in a cell culture medium as previously described by Yaşayan et al., 2021 for MTT assay. Briefly, both sides of the films were sterilized with UV light for 30 min. Cell culture medium was added to the films (1cm^2/mL) and incubated at 37oC, 5% CO_2. After 24 h incubation, the films were discarded, the cell culture medium was equally added to the final volume of the media, and the medium was used for the cytotoxicity assay. Then, the cells were exposed to the aforementioned cell culture medium at different dilutions (10%-30%-50%-90%-100%) for 24 h. Following 24 h exposure, 20 µL of MTT dye solution (5 mg/mL in 1xPBS) was added to each well and incubated for 3 h at 37 oC. At the end of 3 h incubation, crystals which are formed by viable cells were dissolved in 100 µL of DMSO, and optical density (OD) was read at 590 nm using a microwell plate reader (Epoch, Germany). Cell viability was calculated as a percentage relative to the control group.

Statistical Analysis

Statistical differences were analyzed using SPSS version 21.0 for Windows (SPSS Inc. Illinois, USA). The results were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's test. $p< 0.05$ values were regarded as statistically significant.

RESULTS AND DISCUSSION

Physicochemical Characterization of Extracted Chitosan

M. stebbingi is seen as an economical way to produce CS on an industrial scale due to its high availability and low-cost resources. The reason why *M. stebbingi* shells are preferred is due to their CS yield of approximately 17.5%. Similarities in moisture and ash contents were found between commercial chitosan and extracted CS. The degree of deacetylation, which is an important evaluation parameter for chitosan, was determined over 70% by elemental analysis and potentiometric titration methods

for both extracted and commercial chitosan. Since the molecular weight has a significant impact on the physicochemical and functional properties of CS, it is one of the properties that should be evaluated first. The molecular weight of extracted CS is higher than that of commercial CS (Bao et al., 2018; Sun et al., 2018). The physicochemical characteristics of extracted chitosan are summarized in Table 3.

Table 3. Physicochemical characteristics of extracted chitosan

Parameter	Extracted Chitosan
Yields (%)	17.48 ± 0.64
Moisture (%)	1.33 ± 0.08
Ash (%)	0.61 ± 0.03
Deacetylation degree (%)	95.19 ± 2.56
Molecular weight (kDa)	320-400
Water binding capacity (%)	712.99 ± 11.98
Fat binding capacity (%)	531.15 ± 12.26

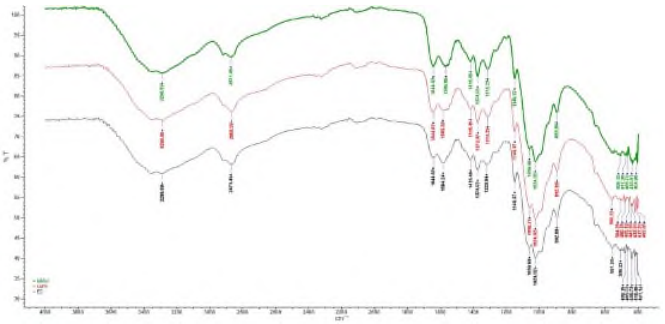

Figure 1. FTIR spectra of the commercial chitosan (HMW, MMW) and extracted chitosan (EC).

As shown in Figure 1, the chemical structure of the extracted CS contains characteristic features of the CS structure, as with the commercial CS. Stretching vibrations of primary NH2 and OH- groups are represented by a broad band around 3750 – 3000 cm^{-1} (max. at 3290 cm^{-1}), while the symmetric and asymmetric stretching vibrations of CH_2 groups of the pyranose ring in the chitosan molecules are represented by a small band between 3000 – 2800 cm-1 (max. at 2871 cm^{-1}) (Gök, Demir, Cevher, Özgümüş, & Pabuccuoğlu, 2019). The amide I bands' characteristic stretching of the CO groups is represented by the band at 1644 cm^{-1}. The small bands (maximum at 1416, 1374, and 1320 cm^{-1}) can be attributed to the bending vibrations of the free methylol groups, whereas the peaks between 1600 and 1500 cm^{-1} (maximum at 1584 cm-1) are the primary NH2 groups' bending vibrations (Gök, 2019). The symmetric and asymmetric stretching vibrations of C-O-C bonds, C-O bonds, and the skeletal vibrations of the chitosan molecule can be detected in bands between 1200 and 900 cm^{-1} (max. at 1149, 1059, and 1024 cm^{-1}), whereas the stretching vibration of the acetyl groups of the chitosan molecules is recorded at 892 cm^{-1} (Gök et al., 2019).

Surface Morphology

At the macroscopic scale, all films were homogeneous and transparent. Surface morphology provides a document for a mixture of CS and plasticizers at different ratios and types (Yeddes et al., 2020). The surfaces of each film were smooth with no apparent pores. However, the SEM micrograph of the films prepared with the extracted CS showed a more regular surface than the formulations prepared with commercial chitosan (Figure 2). Formulations prepared with commercial CS with a medium molecular weight had high surface roughness. A smoother surface was obtained from formulations prepared with low molecular weight commercial CS and extracted. Tensile characteristics may be impacted by the formulation's morphological state, for instance, by crystal formation (Preis, Knop, & Breitkreutz, 2014). Therefore, these perspectives have to be considered during the development of films for drug delivery systems (Kassem, Ismail, Naggar, & Aboulmagd, 2015; Sakloetsakun, Preechagoon, Bernkop-Schnürch, & Pongjanyakul, 2016).

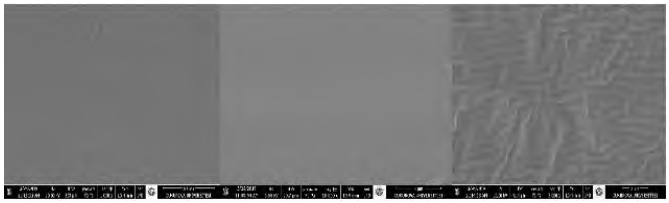

Figure 2. SEM images of films LG5, EG5, MG5, respectively.

Drug Recovery

All films were prepared with 4% w/w drug content according to the dry weight of the polymer, which is about 88.89-93.50% recovery of the total drug used (Table 4).

Thickness Uniformity

The ideal film thickness range for the oral cavity is 0.05 to 1 mm to prevent discomfort during its application. The thicknesses of the films varied between 500 ± 17.89 µm (LPG3) and 1400 ± 23.34 µm (EG10). Except for the EG10 formulation, all film formulations showed proper thickness for their application. Low standard deviations for all formulations were calculated,

Table 4. Experimental results for thickness, drug recovery and swelling degree of chitosan films ((mean±SD, n=3).

Codes of the Formulations	Thickness (µm) (±SD)	Drug Recovery (%)(±SD)	Swelling Degree (%)(±SD)
LG5	850 ± 21.46	88.89 ± 5.57	91.45 ± 4.56
LPG3	500 ± 17.89	90.50 ± 4.45	85.65 ± 5.78
LG10	1000 ± 12.87	91.68 ± 4.87	98.32 ± 4.64
LPG6	700 ± 11.56	92.51 ± 3.78	87.35 ± 2.76
MG5	750 ± 24.76	92.58 ± 4.56	80.21 ± 5.24
MPG3	500 ± 21.34	91.09 ± 4.44	77.41 ± 3.65
MG10	1000 ± 28.40	92.09 ± 2.89	84.76 ± 6.34
MPG6	650 ± 31.35	92.09 ± 5.09	80.23 ± 7.32
EG5	850 ± 23.54	93.50 ± 4.90	137.23 ± 7.86
EPG3	650 ± 31.45	88.54 ± 5.61	111.34 ± 8.35
EG10	1400 ± 23.34	91.51 ± 5.51	121.87 ± 5.98
EPG6	800 ± 13.67	91.56 ± 4.78	102.57 ± 6.67

indicating the thickness uniformity of the films (Jillani et al., 2022).

Swelling Degree

The swelling degree represents the capacity of the films to absorb the surrounding aqueous medium and affects the mucoadhesive property and the drug-release characteristics of the films (Kumria et al., 2018). Swelling of the polymer matrix initiates drug diffusion from the films, and for the hydrophilic polymers, hydration is the leading reason for the adhesion of the polymer to the mucous membrane (Mahdizadeh Barzoki et al., 2016). The high swelling of the films (Figure 3) can be attributed to the hydrophilic nature and the hydrogel formation properties of the chitosan. All of the formulations absorbed SSF medium and showed swelling rapidly after contact with the aqueous medium and maintained physical integrity by remaining intact during the study. The effect of the plasticizer on the swelling degree for chitosan films is also presented in Figure 4. In contrast to CS films with propylene glycol, the swelling degree was increased in those of prepared with glycerine. The lowest percentage swelling degrees were found with the formulations which were prepared by medium molecular weight CS. The highest percentage swelling degrees were calculated for the formulations as 137.23 ± 7.86, 111.34 ± 8.35, 121.87 ± 5.98, and 102.57 ± 6.67, respectively (p<0.05), for the films prepared with extracted CSs. The films plasticized with 5% and 10% glycerine showed higher water content than those of plasticized with 3% and 6% propylene glycol. Then, the extracted CS film obtained a higher swelling degree than films with the other types of CS (p<0.05). The significant hydrodynamic plasticizer water complex is formed by the hydrophilic and hygroscopic properties of plasticizers (Krochta, 2002). The compatibility between the polymers and plasticizers used depends on their similar chemical structures. When the chemical structures of glycerine and propylene glycol are examined, it can be seen that both have lateral hydroxide groups that can form hydrogen bonds with chitosan (Sakwanichol, Sungthongjeen, & Puttipipatkhachorn, 2019). Another factor used to estimate the compatibility of polymers is the fit between solubility parameters. This parameter is the reason why the chitosan aqueous dispersion of glycerine is more compatible than propylene glycol (Calvo et al., 2019).

Figure 3. Photograph of the film upon swelling study.

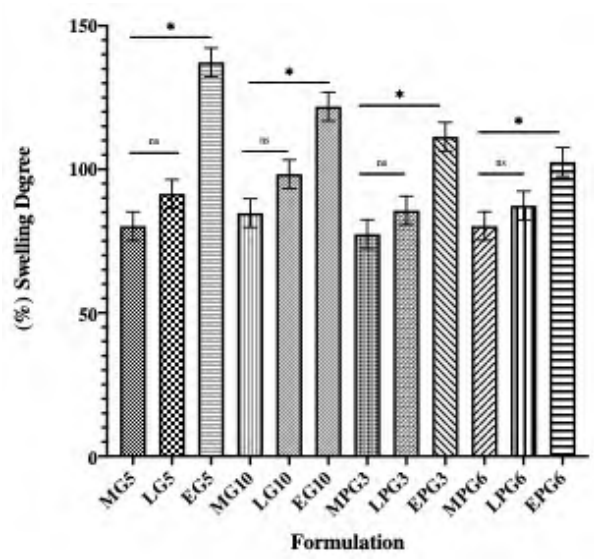

Figure 4. Swelling degrees of chitosan films. Data were shown as mean±standard deviation (SD). *; significant (p<0.05).

Moisture Content

The moisture content of thefilms was determined to investigate integrity in dry conditions. An ideal buccal film should have a moisture content below 5%, increased stability in dry conditions, and a higher moisture absorption capacity, resulting in higher adhesion in the oral cavity (Pilicheva, Uzunova, & Marudova, 2022). Formulations prepared with propylene glycol used as a plasticizer showed a high content of moisture ranging between 14.89% and 23.30%, while formulations containing glycerine showed moisture contents in an acceptable range between 0.98% and 5.47% and were found to be applicable (Figure 5).

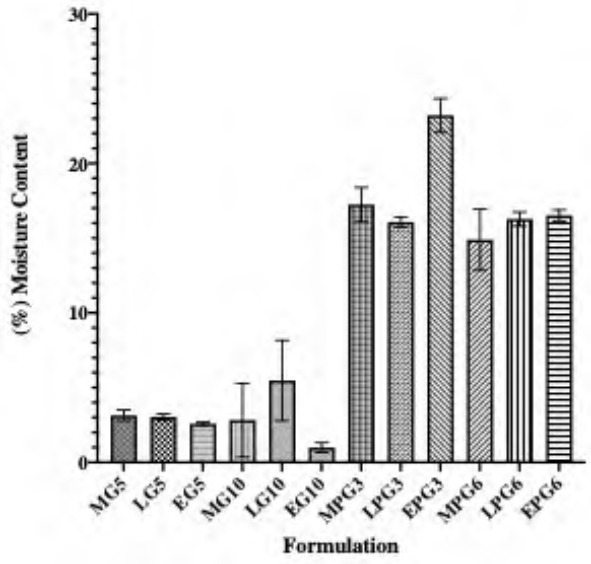

Figure 5. Moisture content of chitosan films. Data were shown as mean±SD.

Texture Profile Analysis

Since the mechanical properties of a film affect its suitability and acceptability, tensile strength and percentage elongation should be monitored for polymeric films. A buccal film should be strong enough to be easily removed from the oral cavity or to be peeled after casting while having enough elasticity to not change drug uniformity during cutting or packing (Abouhussein et al., 2020). Tensile strength determines the strength of the film under the diametric tension, and the percentage elongation represents the stretchability of the film while maintaining physical integrity (Karki et al., 2016).

Texture analyses indicated enough hardness for all films between 1.00 – 4.24 MPa, thus ensuring easy mucosal intra-pocket insertion. The values for tensile strength (TS) and film elongation of the CS film formulations are shown in Table 5. With the addition of plasticizers, films containing glycerine showed reduced tensile strength. This study concluded that films obtained using extracted chitosan with the addition of glycerine as a plasticizer are the most suitable films in terms of mechanical properties. As Figure 6 indicates, increasing the chitosan MW caused an increase in the values of TS or mechanical properties of the films. Due to the increased crosslinking density, the extracted chitosan resulted in the formation of a film with lower extensibility.

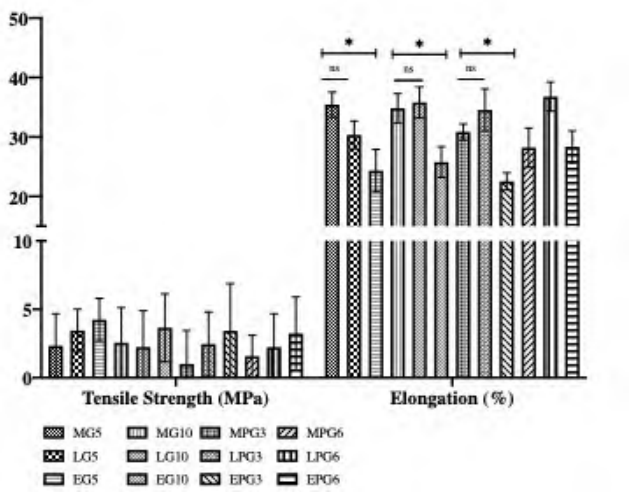

Figure 6. Tensile strength and elongation values of the chitosan film formulations data were shown as mean±SD. *; significant (p<0.05).

The percentage elongation of the films was between 22.54% and 36.78% and increased with the percentage of plasticizer content (Table 5). This finding is consistent with that previously obtained by Calvo et al. (2019), where the highest elongation for chitosan films was acquired when the highest plasticizer ratio was used. In general, we can say that when a high proportion of plasticizers are combined with a polymer such as chitosan, they increase the elongation value, causing a decrease in tensile strength.

In vitro Drug Release Studies

The in vitro drug release from uncross-linked cross-linked and films are shown in Figure 7, and Figure 8, respectively. As shown in both figureS, the increase in the MW of CS was resulted in a decrease in released amount of L. In the presence of TPP, similar drug release pattern was observed. Our aim in this study was to enhance the clinical effect of film formulations using CS. Due to its mucoadhesive properties, the films are expected to remain in the oral cavity and release the drug over an extended period. Uncross-linked films showed LC release between 60.18% and 81.27% at the end of 2 hours and reached a plateau in 6 hours by completing drug release (Figure 7). The cross-linked films released only 22.32% to 37.56% of the LC in 2 hours and maintained drug release for 12 hours. A comparison of LC release from cross-linked films indicates a slower drug release than the uncross-linked films (Figure 8).

Table 5. The values for tensile strength and film elongation of the chitosan film formulations (mean±SD, n=3).

Formulation Code	Chitosan	Plasticizer	TPP
LG5	Low MW	5% glycerine	0.3%
LPG3	Low MW	3% propylene glycol	0.3%
LG10	Low MW	10% glycerine	0.3%
LPG6	Low MW	6% propylene glycol	0.3%
MG5	Medium MW	5% glycerine	0.3%
MPG3	Medium MW	3% propylene glycol	0.3%
MG10	Medium MW	10% glycerine	0.3%
MPG6	Medium MW	6% propylene glycol	0.3%
EG5	Extracted	5% glycerine	0.3%
EPG3	Extracted	3% propylene glycol	0.3%
EG10	Extracted	10% glycerine	0.3%
EPG6	Extracted	6% propylene glycol	0.3%

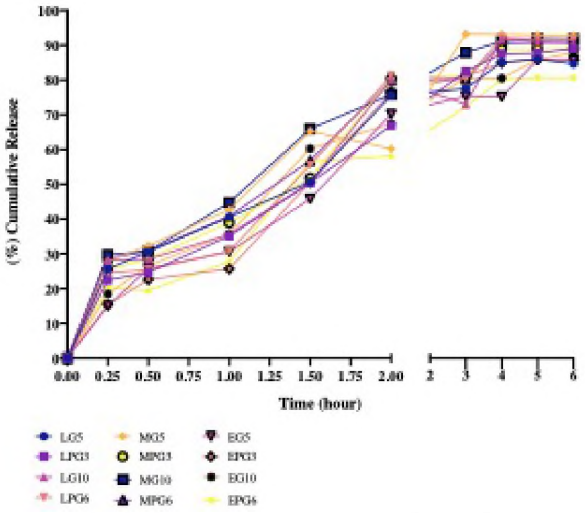

Figure 7. Lidocaine release profile from uncross-linked chitosan films. Data were shown as mean±SD.

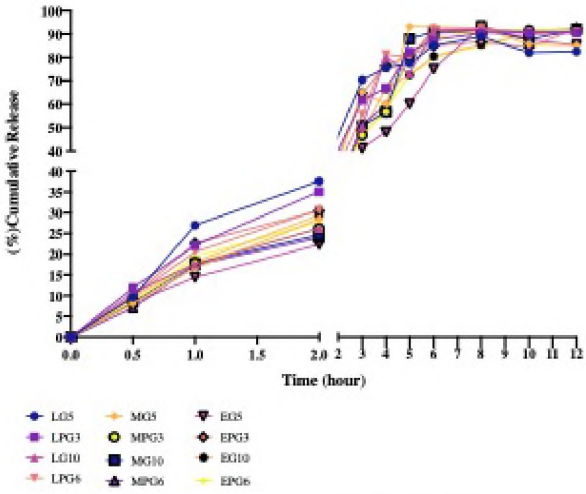

Figure 8. Lidocaine release profile from cross-linked chitosan films. Data were shown as mean±SD.

DD Solver evaluates the goodness of the model fit with statistical parameters such as adjusted coefficient of determination (R^2), Akaike Information Criterion (AIC) and Model Selection Criterion (MSC). When comparing various models, the most accurate fitting is indicated by the highest values of R^2 and MSC, and the lowest value of AIC. Based on the lowest AIC values with the highest R^2 and MSC (Table 6), the first-order model was chosen for formulations (Abdul Rasool, Mohammed, & Salem, 2021).

Cytotoxicity Assay

The cytotoxicity evaluation of the formulations was performed by MTT assay using L929 cells (Yaşayan, Karaca, Akgüner, & Bal Öztürk, 2021). The study was carried out using drug-free formulations containing LC for 24 h. The results are given in Figure 9 and Figure 10 respectively. For drug-free formulations, buccal films prepared with commercial chitosan did not reduce cell viability below 80% up to 50% dilution, while extracted chitosan (EG5) was found to show higher cytotoxic profile in comparison with commercial CH (LG5, MG5). However, extracted chitosan shows a higher cytotoxic profile, and formulation modifications should be performed. Since the therapeutic dose of LC was successfully loaded into films, LC loading to the films decreased the cell viability, and showed dose dependent cytotoxicity in comparison with drug-free formulations, as expected.

Table 6. Evaluation parameters for best fit kinetic model selection.

Formulations	Kinetic Model Parameters (R^2adj, AIC, MSC)					
	Zero-order	First-order	Higuchi	Korsmeyer-Peppas	Hixson-Crowell	Hopfenberg
LG5	0.1468	**0.9222**	0.8777	0.8986	0.8976	0.9110
	78.9675	**57.4136**	61.4868	60.5997	59.8835	59.4185
	-0.0635	**2.3314**	1.8788	1.9774	2.0570	2.1087
LPG3	0.1869	**0.9232**	0.8689	0.8807	0.8841	0.9122
	78.3420	**57.1023**	61.9197	61.8653	60.8069	59.1053
	-0.0153	**2.3446**	1.8093	1.8154	1.9330	2.1221
LG10	-0.0664	**0.9460**	0.8505	0.9062	0.9089	0.9383
	80.1485	**53.2929**	62.4685	59.0731	58.0114	55.3013
	-0.2865	**2.6974**	1.6779	2.0552	2.1731	2.4743
LPG6	0.0705	**0.9224**	0.8687	0.9012	0.8789	0.9113
	78.9665	**56.6143**	61.3528	59.5915	60.6274	58.6188
	-0.1491	**2.3345**	1.8080	2.0037	1.8886	2.1118
MG5	0.0514	**0.8917**	0.8647	0.8999	0.8143	0.8761
	78.0670	**58.5364**	60.5388	58.6243	63.3907	60.5492
	-0.1694	**2.0006**	1.7781	1.9909	1.4613	1.7770
MPG3	0.4371	**0.9628**	0.9294	0.9240	0.9330	0.9575
	75.5380	**51.0812**	56.8490	58.3150	56.3861	53.0852
	0.3524	**3.0698**	2.4290	2.2661	2.4804	2.8472
MG10	0.2755	**0.8982**	0.8781	0.8795	0.8628	0.8836
	77.4976	**59.8390**	61.4597	62.1497	62.5235	61.8418
	0.1000	**2.0621**	1.8820	1.8054	1.7638	1.8396
MPG6	0.3681	**0.9286**	0.8750	0.8648	0.9143	0.9184
	77.7003	**58.0732**	63.1180	64.6214	59.7155	60.0767
	0.2369	**2.4177**	1.8571	1.6901	2.2352	2.1951
EG5	0.4993	**0.9424**	0.9200	0.9097	0.8935	0.9341
	74.1437	**54.6836**	57.6363	59.5247	60.2167	56.6936
	0.4695	**2.6317**	2.3036	2.0938	2.0169	2.4084
EPG3	0.4770	**0.9301**	0.8658	0.8472	0.9040	0.9200
	76.2561	**58.1469**	64.0130	65.9809	60.9982	60.1517
	0.4259	**2.4380**	1.7862	1.5676	2.1212	2.2153
EG10	0.3143	**0.9522**	0.9127	0.9164	0.8932	0.9454
	76.5605	**52,5827**	58.0078	58.4159	59.8291	54.5873
	0.1551	**2.8193**	2.2165	2.1712	2.0142	2.5966
EPG6	0.4411	**0.9219**	0.9083	0.8987	0.8592	0.9108
	74.2172	**56.5014**	57.9493	59.6404	61.8103	58.5040
	0.3596	**2.3280**	2.1671	1.9792	1.7381	2.1055

R^2adj: R^2 adjusted, AIC: Akaike information criterion, MSC: model selection criterion.

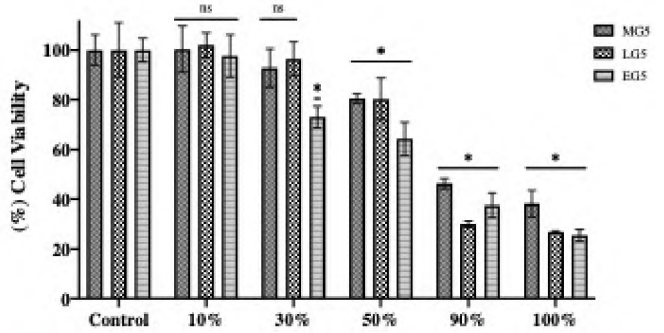

Figure 9. Effects of drug-free MG5, LG5 and EG5 formulations on cell viability. Data were shown as mean±SD. *; significant (p<0.05) versus control group.

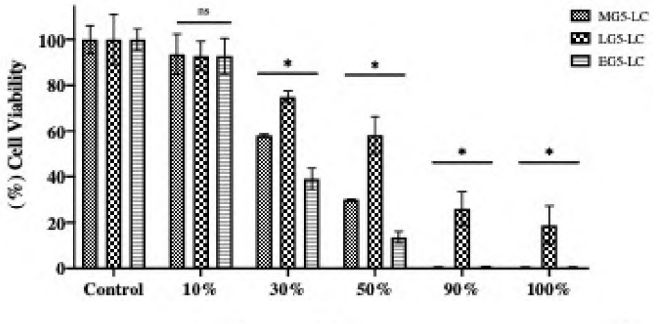

Figure 10. Effects of lidocaine containing MG5, LG5 and EG5 formulations on cell viability. Data were shown as mean±SD. *; significant (p<0.05) versus control group.

CONCLUSION

In this study, films prepared using extracted and commercial CS were investigated by differentiating CS with propylene glycol and glycerine over a wide range of composition ratios. The current work indicates that LH can be successfully loaded to CS-based films while having good mechanical and barrier properties. The addition of glycerine as a plasticizer into the CS film system changed its thermal, mechanical, and swelling properties. The in vitro characterization results show that the most promising film for drug delivery of LC is the one containing glycerine and extracted CS. Based on these observations, extracted CSwas determined to have a positive effect on the physical properties of the polymer films fabricated in this study, yet polymer concentration should be decreased according to the cytotoxicity studies. Commercial CSs showed higher cytocompatibility than the extracted chitosan, according to the MTT studies. In conclusion, lidocaine-loaded CS-based films can be applied as an alternative way to deliver LH for mucositis therapy.

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REFERENCES

Abdul Rasool, B. K., Mohammed, A. A., & Salem, Y. Y. (2021). The optimization of a dimenhydrinate transdermal patch formulation based on the quantitative analysis of in vitro release data by DD-Solver through skin penetration studies. *Scientia Pharmaceutica, 89*(3), 33. https://doi.org/10.3390/scipharm89030033

Abouhussein, D., El Nabarawi, M. A., Shalaby, S. H., & Abd El-Bary, A. (2020). Cetylpyridinium chloride chitosan blended mucoadhesive buccal films for treatment of pediatric oral diseases. *Journal of Drug Delivery Science and Technology, 57*, 101676. https://doi.org/10.1016/j.jddst.2020.101676

Al-Nemrawi, N. K., Alsharif, S. S., Alzoubi, K. H., & Alkhatib, R. Q. (2019). Preparation and characterization of insulin chitosan-nanoparticles loaded in buccal films. *Pharmaceutical Development and Technology, 24*(8), 967-974. https://doi.org/10.1080/10837450.2019.1619183

Alopaeus, J. F., Hellfritzsch, M., Gutowski, T., Scherließ, R., Almeida, A., Sarmento, B., Škalko-Basnet, N., & Tho, I. (2020). Mucoadhesive buccal films based on a graft co-polymer–A mucin-retentive hydrogel scaffold. *European Journal of Pharmaceutical Sciences, 142*, 105142. https://doi.org/10.1016/j.ejps.2019.105142

Anwar, S., Zaman, M., Raja, M. A. G., Mahmood, A., & Amjad, M. W. (2020). Rosuvastatin, Perindopril and Ezetimibe loaded instant release buccal films: De*velopment and in vitro characterization. Journal of Applied Biomedicine*, 18(4). https://doi.org/10.32725/jab.2020.015

Bao, Y., Zhang, H., Luan, Q., Zheng, M., Tang, H., & Huang, F. (2018). Fabrication of cellulose nanowhiskers reinforced chitosan-xylan nanocomposite films with antibacterial and antioxidant activities. *Carbohydrate Polymers, 184*, 66-73. https://doi.org/10.1016/j.carbpol.2017.12.051

Brown, T. J., & Gupta, A. (2020). Management of cancer therapy–associated oral mucositis. *JCO Oncology Practice, 16*(3), 103-109. https://doi.org/10.1200/JOP.19.00652

Cafaggi, S., Leardi, R., Parodi, B., Caviglioli, G., Russo, E., & Bignardi, G. (2005). Preparation and evaluation of a chitosan salt–poloxamer 407 based matrix for buccal drug delivery. *Journal of Controlled Release, 102*(1), 159-169. https://doi.org/10.1016/j.jconrel.2004.09.019

Chang, K. L. B., Tsai, G., Lee, J., & Fu, W. R. (1997). Heterogeneous N-deacetylation of chitin in alkaline solution. *Carbohydrate Research, 303*(3), 327-332. https://doi.org/10.1016/S0008-6215(97)00179-1

Calvo, N. L., Svetaz, L. A., Alvarez, V. A., Quiroga, A. D., Lamas, M. C., & Leonardi, D. (2019). Chitosan-hydroxypropyl methylcellulose tioconazole films: A promising alternative dosage form for the treatment of vaginal candidiasis. *International Journal of Pharmaceutics, 556*, 181-191. https://doi.org/10.1016/j.ijpharm.2018.12.011

Cevher, E., Taha, M., Orlu, M., & Araman, A. (2008). Evaluation of mechanical and mucoadhesive properties of clomiphene citrate gel formulations containing carbomers and their thiolated derivatives. *Drug Delivery, 15*(1), 57-67. https://doi.org/10.1080/10717540701829234

Çevikelli, T., Güven, U. M., & Öztürk, A. A. (2024). Metronidazole Loaded Novel Microemulsion Formulation for Topical Delivery and Characterization With Validated New UPLC Method. *Fabad Journal of Pharmaceutical Sciences, 49*(1), 111-128. https://doi.org/10.55262/fabadeczacilik.1359138

Demirtürk, E., Nemutlu, E., Şahin, S., & Öner, L. (2020). Development and validation of an HPLC method for determination of rofecoxib in bovine serum albumin microspheres. *Turkish Journal of Chemistry, 44*(3), 647-655. https://doi.org/10.3906/kim-1912-45

Elad, S., Yarom, N., Zadik, Y., Kuten-Shorrer, M., & Sonis, S. T. (2022). The broadening scope of oral mucositis and oral ulcerative mucosal toxicities of anticancer therapies.*CA: A Cancer Journal for Clinicians, 72*(1), 57-77. https://doi.org/10.3322/caac.21704

Escalona-Rayo, C. F., Serrano-Castañeda, P., López-Cervantes, M., & Escobar-Chávez, J. J. (2020). Optimization of unidirectional mucoadhesive buccal patches based on chitosan and pluronic® F-127 for metoprolol controlled release: In vitro and ex vivo evaluations. *Journal of Pharmaceutical Innovation*, 15, 556-568.

https://doi.org/10.1007/s12247-019-09401-8

Gök, M. K. (2019). In vitro evaluation of synergistic effect of primary and tertiary amino groups in chitosan used as a non-viral gene carrier system. *European Polymer Journal, 115*, 375-383. https://doi.org/10.1016/j.eurpolymj.2019.03.048

Gök, M. K., Demir, K., Cevher, E., Özgümüş, S., & Pabuccuoğlu, S. (2019). Effect of the linear aliphatic amine functionalization on in vitro transfection efficiency of chitosan nanoparticles. *Carbohydrate Polymers, 207*, 580-587. https://doi.org/10.1016/j.carbpol.2018.12.013

Guideline, I. H. T. (2005). Validation of analytical procedures: text and methodology. *Q2 (R1)*, 1(20), 05.

Hosseinjani, H., Hadjibabaie, M., Gholami, K., Javadi, M., Radfar, M., Jahangard-Rafsanjani, Z., Hosseinjani, E., Shabani, N., Vaezi, M., & Ghavamzadeh, A. (2017). The efficacy of erythropoietin mouthwash in prevention of oral mucositis in patients undergoing autologous hematopoietic SCT: a double-blind, randomized, placebo-controlled trial. *Hematological Oncology, 35*(1), 106-112. https://doi.org/10.1002/hon.2250

Jillani, U., Mudassir, J., Arshad, M. S., Mehta, P., Alyassin, Y., Nazari, K., Yousef, B., Patel, M., Zaman, A., & Sayed, E. (2022). Design and evaluation of agarose based buccal films containing zolmitriptan succinate: Application of physical and chemical enhancement approaches. *Journal of Drug Delivery Science and Technology, 69*, 103041. https://doi.org/10.1016/j.jddst.2021.103041

Karki, S., Kim, H., Na, S.-J., Shin, D., Jo, K., & Lee, J. (2016). Thin films as an emerging platform for drug delivery. *Asian Journal of Pharmaceutical Sciences*, 11(5), 559-574. https://doi.org/10.1016/j.ajps.2016.05.004

Kassem, A. A., Ismail, F. A., Naggar, V. F., & Aboulmagd, E. (2015). Preparation and evaluation of periodontal films based on polyelectrolyte complex formation. *Pharmaceutical Development and Technology, 20*(3), 297-305. https://doi.org/10.3109/10837450.2013.862262

Khade, A., Gadge, G., & Mahajan, U. (2020). An overview on natural polymer based mucoadhesive buccal films for controlled drug delivery. *International Journal of Pharmacy Research & Technology (IJPRT), 10*(1), 48-57. https://doi.org/10.21276/irjps.2019.6.1.7

Kottke, D., Majid, H., Breitkreutz, J., & Burckhardt, B. B. (2020). Development and evaluation of mucoadhesive buccal dosage forms of lidocaine hydrochloride by ex-vivo permeation studies. *International Journal of Pharmaceutics*, 581, 119293. https://doi.org/10.1016/j.ijpharm.2020.119293

Krochta, J. M. (2002). Proteins as raw materials for films and coatings: definitions, current status, and opportunities. *Protein-based Films and Coatings, 1*, 1-40. https://doi.org/10.1201/9781420031980.ch1

Küçükgülmez, A., Celik, M., Yanar, Y., Sen, D., Polat, H., & Kadak, A. E. (2011). Physicochemical characterization of chitosan extracted from Metapenaeus stebbingi shells. *Food Chemistry, 126*(3), 1144-1148. https://doi.org/10.1016/j.foodchem.2010.11.148

Kumar, A., Naik, P. K., Pradhan, D., Ghosh, G., & Rath, G. (2020). Mucoadhesive formulations: Innovations, merits, drawbacks, and future outlook. *Pharmaceutical Development and Technology, 25*(7), 797-814. https://doi.org/10.1080/10837450.2020.1753771

Kumar, A., Vimal, A., & Kumar, A. (2016). Why Chitosan? From properties to perspective of mucosal drug delivery. *International Journal of Biological Macromolecules, 91*, 615-622. https://doi.org/10.1016/j.ijbiomac.2016.05.054

Kumria, R., Al-Dhubiab, B. E., Shah, J., & Nair, A. B. (2018). Formulation and evaluation of chitosan-based buccal bioadhesive films of zolmitriptan. *Journal of Pharmaceutical Innovation, 13*, 133-143. https://doi.org/10.1007/s12247-018-9312-6

Mahdizadeh Barzoki, Z., Emam-Djomeh, Z., Mortazavian, E., Akbar Moosavi-Movahedi, A., & Rafiee Tehrani, M. (2016). Formulation, in vitro evaluation and kinetic analysis of chitosan–gelatin bilayer muco-adhesive buccal patches of insulin nanoparticles. *Journal of Microencapsulation, 33*(7), 613-624. https://doi.org/10.1080/02652048.2016.1234513

Malenovic, A., Medenica, M., Ivanovic, D., Jancic, B., & Markovic, S. (2005). Development and validation of RP–HPLC method for cetrimonium bromide and lidocaine determination. *Il Farmaco, 60*(2), 157-161. https://doi.org/10.1016/j.farmac.2004.11.004

Morales, J. O., & McConville, J. T. (2011). Manufacture and characterization of mucoadhesive buccal films. *European Journal of Pharmaceutics and Biopharmaceutics, 77*(2), 187-199. https://doi.org/10.1016/j.ejpb.2010.11.023

Pilicheva, B., Uzunova, Y., & Marudova, M. (2022). Polyelectrolyte Multilayer Films as a Potential Buccal Platform for Drug Delivery. *Polymers, 14*(4), 734. https://doi.org/10.3390/polym14040734

Preis, M., Knop, K., & Breitkreutz, J. (2014). Mechanical strength test for orodispersible and buccal films. *International Journal of Pharmaceutics, 461*(1-2), 22-29. https://doi.org/10.1016/j.ijpharm.2013.11.033

Pulito, C., Cristaudo, A., Porta, C. L., Zapperi, S., Blandino, G., Morrone, A., & Strano, S. (2020). Oral mucositis: the hidden side of cancer therapy. *Journal of Experimental & Clinical Cancer Research, 39*, 1-15. https://doi.org/10.1186/s13046-020-01715-7

Radha, D., Lal, J. S., & Devaky, K. (2022). Chitosan-based films in drug delivery applications. *Starch-Stärke, 74*(7-8), 2100237. https://doi.org/10.1002/star.202100237

Sakloetsakun, D., Preechagoon, D., Bernkop-Schnürch, A., & Pongjanyakul, T. (2016). Chitosan–gum arabic polyelectrolyte complex films: Physicochemical, mechanical and mucoadhesive properties. *Pharmaceutical Development and Technology, 21*(5), 590-599. https://doi.org/10.3109/10837450.2015.1035727

Sakwanichol, J., Sungthongjeen, S., & Puttipipatkhachorn, S. (2019). Preparation and characterization of chitosan aqueous dispersion as a pharmaceutical film forming material. *Journal of Drug Delivery Science and Technology, 54*, 101230. https://doi.org/10.1016/j.jddst.2019.101230

Shariatinia, Z. (2019). Pharmaceutical applications of chitosan. *Advances in Colloid and Interface Science, 263*, 131-194. https://doi.org/10.1016/j.cis.2018.11.008

Silva, F. C., Marto, J. M., Salgado, A., Machado, P., Silva, A. N., & Almeida, A. J. (2017). Nystatin and lidocaine pastilles for the local treatment of oral mucositis. *Pharmaceutical Development and Technology, 22*(2), 266-274. https://doi.org/10.1080/10837450.2016.1221424

Sun, W., Chen, G., Wang, F., Qin, Y., Wang, Z., Nie, J., & Ma, G. (2018). Polyelectrolyte-complex multilayer membrane with gradient porous structure based on natural polymers for wound care. *Carbohydrate Polymers, 181*, 183-190. https://doi.org/10.1016/j.carbpol.2017.10.068

Şenel, S., İkinci, G., Kaş, S., Yousefi-Rad, A., Sargon, M., & Hıncal, A. (2000). Chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery. *International Journal of Pharmaceutics, 193*(2), 197-203. https://doi.org/10.1016/S0378-5173(99)00334-8

Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer, 41*(7), 2463-2469. https://doi.org/10.1016/S0032-3861(99)00400-0

Wang, J., & Kinsella, J. (1976). Functional properties of novel proteins: Alfalfa leaf protein. *Journal of Food Science, 41*(2), 286-292. https://doi.org/10.1111/j.1365-2621.1976.tb00602.x

Wang, Q. Z., Chen, X. G., Liu, N., Wang, S. X., Liu, C. S., Meng, X. H., & Liu, C. G. (2006). Protonation constants of chitosan with different molecular weight and degree of deacetylation. *Carbohydrate Polymers*, 65(2), 194-201. https://doi.org/10.1016/j.carbpol.2006.01.001

Yaşayan, G., Karaca, G., Akgüner, Z. P., & Bal Öztürk, A. (2021). Chitosan/collagen composite films as wound dressings encapsulating allantoin and lidocaine hydrochloride. *International Journal of Polymeric Materials and Polymeric Biomaterials*, 70(9), 623-635. https://doi.org/10.1080/00914037.2020.1740993

Yeddes, W., Djebali, K., Wannes, W. A., Horchani-Naifer, K., Hammami, M., Younes, I., & Tounsi, M. S. (2020). Gelatin-chitosan-pectin films incorporated with rosemary essential oil: Optimized formulation using mixture design and response surface methodology. *International Journal of Biological Macromolecules, 154*, 92-103. https://doi.org/10.1016/j.ijbiomac.2020.03.092

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs, 13*(3), 1133-1174. https://doi.org/10.3390/md13031133

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Original Article

Comparison of drug–drug interaction checking databases for interactions involving BCR-ABL tyrosine kinase inhibitors

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ABSTRACT

Background and Aims: BCR-ABL tyrosine kinase inhibitors (TKIs) are used for the treatment of chronic myeloid leukemia and are commonly involved in clinically significant drug–drug interactions (DDIs). In this study, we aimed to evaluate the consensus of DDI checking databases for interactions involving BCR-ABL TKIs.

Methods: We checked DDIs of 100 drugs with six BCR-ABL TKIs—dasatinib, imatinib, nilotinib, ponatinib, bosutinib, and asciminib—in two subscription-based databases (UpToDate and Micromedex) and two open-access databases (Drugs.com and Medscape). Databases were compared in terms of severity ratings, literature support ratings, and general interaction mechanism definitions using Fleiss' and Cohen's kappa statistics.

Results: A total of 410 interactions were found. Nilotinib was the most interacted TKI, with 88 interactions. Drugs.com detected the highest number of interactions (n = 355). The overall agreement levels of databases for the severity ratings and general mechanisms were calculated as 0.13 (p = 0) and 0.28 (p = 0), respectively. The Micromedex- UpToDate pair showed the highest agreement level in terms of severity ratings and general mechanism definitions, with kappa values of 0.23 and 0.45, respectively.

Conclusion: The differences among databases for DDIs involving BCR-ABL TKIs were statistically significant. Therefore, healthcare practitioners should check DDIs in multiple databases.

Keywords: Drug-drug interactions, chronic myeloid leukemia, tyrosine kinase inhibitors, TKIs, CML

INTRODUCTION

Chronic myeloid leukemia (CML) is a rare malignancy represented by BCR-ABL1 gene translocation. The Philadelphia chromosome is a cytogenetic feature of CML (Osman, & Deininger, 2021). Tyrosine kinase inhibitors (TKIs) are used to treat CML. Imatinib, dasatinib, and nilotinib are the first-line treatment options (Hsieh, Kirschner & Copland, 2021). Bosutinib, ponatinib, and asciminib are the newer TKI options for the treatment of this disease (Kennedy & Hobbs, 2018; Deeks, 2022).

Drug–drug interactions (DDIs) occur because of pharmacodynamic and pharmacokinetic mechanisms as well as pharmaceutical incompatibilities (Corrie & Hardman, 2011). TKIs are orally administered, target-specific weak bases with bioavailability problems (van Leeuwen, van Gelder, Mathijssen & Jansman, 2014). The increments in gastric pH by acid-suppressive drugs, such as proton pump inhibitors (PPIs), may result in poor therapy response due to the lack of bioavailability of TKIs (van Leeuwen et al., 2017). TKIs are metabolized by the cytochrome P450 enzyme system (CYP450), which puts them into the target of DDIs (van Leeuwen, van Gelder, Mathijssen & Jansman, 2014).

Patients with cancer often require complex treatment schemes, resulting in a higher prevalence of DDI, leading to DDI-related adverse events (Riechelmann & Del Giglio, 2009). A study on patients with cancer who used oral anticancer drug therapy found that 46% of them were exposed to potential DDIs. In this patient group, interactions that affect the nervous system, gastrointestinal tract, and QT interval were common (van Leeuwen et al., 2013).

DDI checking databases help health professionals identify potential DDIs; however, differences in detected DDIs and provided information were noted among DDI checking databases (Suriyapakorn et al., 2019). DDI checking databases show dif-

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ferent levels of scope, consistency, and completeness (Patel& Beckett, 2016). In a study that compared nine DDI checking databases for oral oncolytic-involving DDIs, Lexicomp and Drugs.com showed the highest performance (Marcath et al., 2018).

This study focused on BCR-ABL TKI-involving DDIs and the compatibility of DDI checking databases in terms of severity ratings, literature support ratings, and general interaction mechanism definitions.

METHODS

Drug selection

The following BCR-ABL TKIs were used to treat CML: imatinib, dasatinib, nilotinib, bosutinib, ponatinib, and asciminib (Cancer.org, 2022). Four widely available DDI checking databases were included. According to the DDI sections of the drug information of these TKIs in these databases, 100 drugs were manually chosen. Except for transdermal fentanyl, all chosen drugs can be administered orally (Drugs.com, 2022; UpToDate Interactions, 2022; Medscape, 2022; IBM Micomedex, 2022). The selected drugs are classified according to the Anatomical Therapeutic Chemical Classification/Defined Daily Doses index system in Table 1 (WHO ATC/DDD Index, 2022). Orally administered drugs were chosen because they can be prescribed for acute or chronic diseases in outpatient settings, which could cause difficulties in identifying and monitoring interactions.

DDI Checking Databases

Two subscription-based (UpToDate and Micromedex) and two open-access (Drugs.com and Medscape) DDI checking databases were included. All databases provided information about the severity, mechanism, and management of the interactions. Only UpToDate and Micromedex provided information about the literature support of the interactions. Severity and literature support classifications of interactions are listed in Table 2.

Statistical analysis

The severity ratings, literature support ratings, and proposed mechanisms of these interactions assigned by databases were noted. Summary statistics were used to categorize DDIs according to severity ratings and general mechanisms. Severity ratings and general mechanisms were analyzed using Cohen's kappa and Fleiss' kappa statistics. The literature support ratings of UpToDate and Micromedex were compared using Cohen's kappa formula. The agreement levels of databases were evaluated using the Landis and Koch agreement classification (Landis & Koch, 1977).

RESULTS

After analyzing 100 drugs with six TKIs, 410 DDIs were found. Nilotinib was the most interacted TKI, with 88 (21%) interactions. Dasatinib constituted 20% of DDIs with 83 interactions. The least interacted TKI was asciminib, with 50 interactions (Figure 1).

None of the DDI checking databases detected all interactions. Drugs.com and Micromedex detected the highest and lowest number of interactions, with 355 and 164 interactions, respectively. The distribution of severity ratings differed among databases. The highest number of contraindicated interactions was detected by UpToDate, with 22 interactions. Moderate severity level was the most common severity rating among all databases (Figure 2).

The mechanisms of interactions were categorized according to the literature definitions (Cascorbi, 2012). The number of pharmacokinetic interactions was higher than other mechanism categories among databases. Of the 355 interactions detected by Drugs.com, 239 originated from pharmacokinetic mechanisms. The pharmacodynamic mechanism was the second most common DDI general mechanism. Drugs.com and UpToDate reported the highest pharmacodynamic DDIs, with 110 and 81 interactions, respectively. Some interactions were explained by the combination of pharmacodynamic and pharmacokinetic mechanisms. The highest number of this combination of mechanisms was detected by Medscape, with 30 interactions (Figure 3).

Some interactions with contraindicated severity warnings are listed in Table 3. The combinations of dasatinib and acid-suppressive drugs, such as PPIs and famotidine, were labeled as contraindicated by UpToDate. The proposed mechanism was gastric pH elevation with a result of a decrease in the dasatinib effect (UpToDate Interactions, 2022). Dasatinib, nilotinib, and bosutinib combinations with azole antifungals were listed as contraindicated DDIs. The CYP3A4 inhibition and additive QTc prolongation effects of azole antifungals cause increases in the TKI blood levels and arrhythmia. Additionally, the proposed mechanism for the nilotinib and posaconazole interaction in Medscape was described as CYP3A4 and p-glycoprotein inhibition, which differed from other databases. The combination of nilotinib and sotalol causes QTc prolongation, and this combination comes with a contraindicated warning in the UpToDate and Medscape DDI checking databases (Drugs.com, 2022; UpToDate Interactions, 2022; Medscape, 2022; IBM Solutions, 2022).

Fleiss' kappa statistic was used to evaluate the agreement level of DDI checking databases for categorizing the severity warnings of BCR-ABL TKI-involving interactions. The overall Fleiss kappa values of the four databases in terms of severity ratings and general mechanisms were 0.13 ($p = 0$; standard error [SE], 0.013; 95% confidence interval [CI], 0.10–0.15)

Table 1. Classifications of selected drugs according to the Anatomical Therapeutic Chemical Classification/Defined Daily Doses index system

Anatomical Therapeutic Chemical Classification/Defined Daily Doses drug classes of the selected drugs
N05, psycholeptics: alprazolam, aripiprazole, buspirone, chlorpromazine, clozapine, haloperidol, hydroxyzine, olanzapine, quetiapine, ramelteon, and risperidone
N06, psychoanaleptics: amitriptyline, donepezil, duloxetine, escitalopram, fluoxetine, imipramine, paroxetine, sertraline, and venlafaxine
A02, drugs for acid-related disorders: calcium carbonate, esomeprazole, famotidine, lansoprazole, magnesium carbonate, omeprazole, pantoprazole, rabeprazole, and sodium bicarbonate
J01, antibacterials for systemic use: azithromycin, ciprofloxacin, clarithromycin, doxycycline, levofloxacin, metronidazole, moxifloxacin, and sulfamethoxazole
B01, antithrombotic agents: apixaban, aspirin, clopidogrel, dabigatran, prasugrel, rivaroxaban, and warfarin
N02, analgesics: codeine, fentanyl, morphine, oxycodone, paracetamol, and tramadol
C08, calcium channel blockers: amlodipine, diltiazem, felodipine, nifedipine, and verapamil
C10, lipid-modifying agents: atorvastatin, fluvastatin, gemfibrozil, rosuvastatin, and simvastatin
C07, beta-blocking agents: carvedilol, metoprolol, nebivolol, and sotalol
M01, anti-inflammatory and antirheumatic products: diclofenac, ibuprofen, and naproxen
N03, antiepileptics: carbamazepine, phenobarbital, and phenytoin
A04, antiemetics and antinauseants: aprepitant, granisetron, and ondansetron
J02, antimycotics for systemic use: fluconazole, posaconazole, and voriconazole
G04, urologicals: alfuzosin, silodosine, and solifenacin
A10, drugs used in diabetes: metformin and repaglinide
R03, drugs for obstructive airway diseases: montelukast and theophylline
J04, antimycobacterials: isoniazid and rifampicin,
C01, cardiac therapy: amiodarone and ranolazine
C09, agents acting on the renin–angiotensin system: captopril and losartan
H02, corticosteroids for systemic use: dexamethasone and methylprednisolone
R05, cough and cold preparations: hydrocodone
A12, mineral supplements: potassium bicarbonate
C03, diuretics: spironolactone
M04, antigout preparations: colchicine
J05, antivirals for systemic use: tenofovir
H03, thyroid therapy: levothyroxine
R06, antihistamines for systemic use: cetirizine
A03, drugs for functional gastrointestınal disorders: metoclopramide
G03, sex hormones and modulators of the genital system: megestrol

Table 2. Severity and literature support classifications of databases

Databases	Classifications
	Severity classifications
UpToDate	No interaction between drugs (A), minor (B), moderate (C), major (D), and major (X)
Micromedex	Minor, moderate, major, and contraindicated
Drugs.com	Minor, moderate, major, and major-contraindicated
Medscape	Minor, monitor closely, serious, and contraindicated
	Literature support classifications
UpToDate	Poor, fair, good, and excellent
Micromedex	Fair, good, and excellent

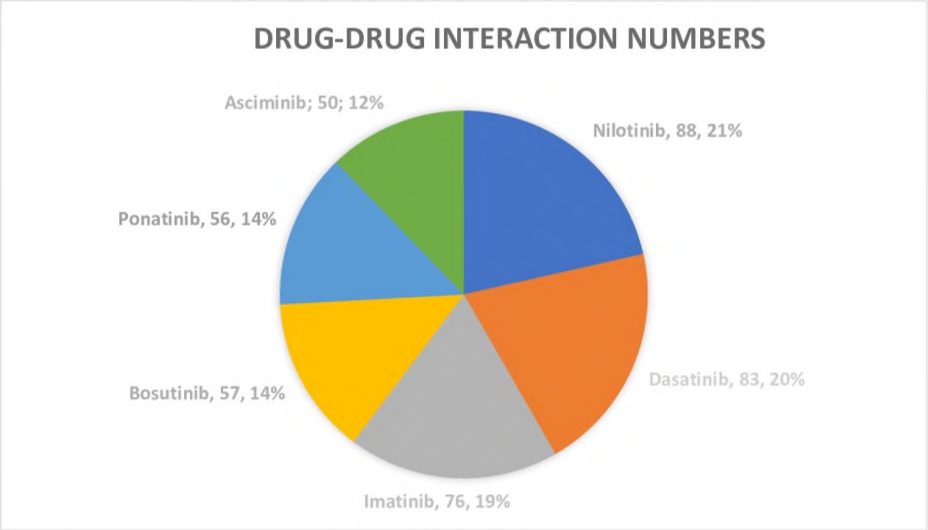

Figure 1. Distribution of drug–drug interactions according to the tyrosine kinase inhibitor type.

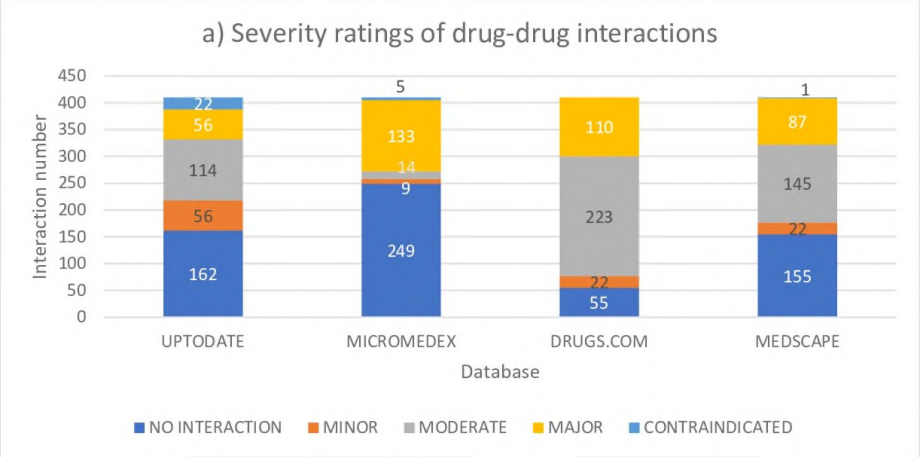

Figure 2. Distribution of the severity ratings and general mechanisms of drug–drug interactions according to the databases.

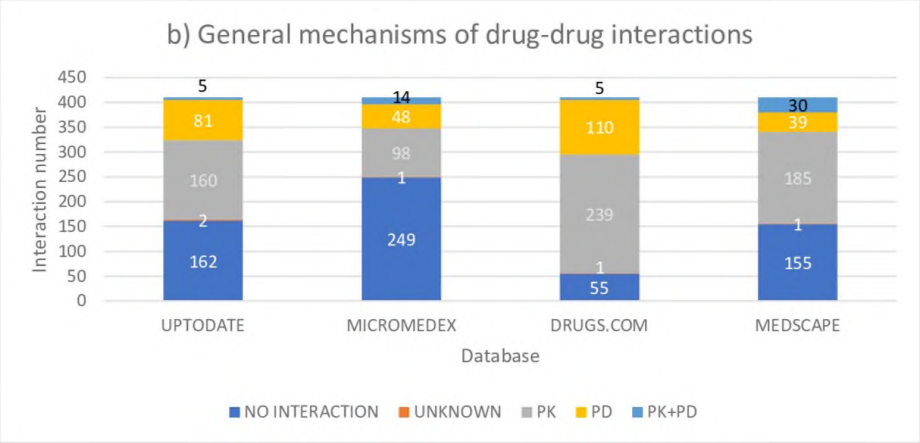

Figure 3. Distribution of the severity ratings and general mechanisms of drug–drug interactions according to the databases.

Table 3. Examples of the contraindicated drug–drug interactions with BCR-ABL tyrosine kinase inhibitors (Drugs.com, 2022; UpToDate Interactions, 2022; Medscape, 2022; IBM Solutions, 2022)

TKI + interacting drug	Databases (severity and LS)	Mechanism	Clinical effect
Dasatinib + PPIs*/famotidine	**U:** Major (X) *LS: Good* **Mi:** Major *LS: Fair* **D:** Major **Me:** Major	(Consensus) Gastric pH ↑	(Consensus) Dasatinib effect ↓
Dasatinib + fluconazole	**U:** Moderate (C) *LS:Fair* **Mi:** Contraind. *LS: Fair* **D:** Moderate **Me:** Monitor closely	CYP3A4 inhibition and QTc prolongation CYP3A4 inhibition CYP3A4 inhibition CYP3A4 inhibition and QTc prolongation	Dasatinib level ↑ and QTTc ↑ Dasatinib level ↑ Dasatinib level ↑ Dasatinib level ↑
Dasatinib + posaconazole	**U:** Major (X) *LS:Fair* **Mi:** Contraind. *LS: Fair* **D:** Major **Me:** Monitor closely	CYP3A4 inhibition and QTc prolongation CYP3A4 inhibition CYP3A4 inhibition CYP3A4 inhibition	Dasatinib level ↑ and QTc ↑ Dasatinib level ↑ Dasatinib level ↑ Dasatinib level ↑
Nilotinib + fluconazole	**U:**Moderate (C) *LS: Fair* **Mi**:Contraind. *LS: Fair* **D:** Major **Me:** Serious	QTc prolongation by a QT-prolonging moderate CYP3A4 inhibitor drug CYP3A4 inhibition and QTc prolongation QTc prolongation CYP3A4 inhibition and QTc prolongation	QTc ↑ Nilotinib level ↑ and QTc ↑ QTc ↑ Nilotinib level ↑ and QTc ↑
Nilotinib + posaconazole	**U:** Major (X) *LS: Fair* **Mi:** Contraind. *LS :Fair* **D:** Major **Me:** Monitor closely	CYP3A4 inhibition CYP3A4 inhibition QTc prolongation CYP3A4 and p-glycoprotein inhibition	Nilotinib level ↑ Nilotinib level ↑ QTc ↑ Nilotinib level ↑
Bosutinib + posaconazole/voriconazole	**U:** Major (X) *LS: Good* **Mi:** Major *LS: Fair* **D:** Major **Me:** Serious	(Consensus) CYP3A4 inhibition	(Consensus) Bosutinib level↑
Bosutinib + clarithromycin	**U:** Major (X) *LS: Good* **Mi:** Major *LS: Fair* **D:** Major **Me:** Serious	CYP3A4 inhibition and QTc prolongation CYP3A4 inhibition CYP3A4 inhibition CYP3A4 inhibition	Bosutinib level ↑ and QTc ↑ Bosutinib level ↑ Bosutinib level ↑ Bosutinib level ↑
Nilotinib + sotalol	**U:** Major (X) *LS: Fair* **Mi:** Major *LS: Fair* **D:** Major **Me:** Contraind.	(Consensus) QTc prolongation	(Consensus) QTc ↑
Nilotinib + colchicine	**U:** Major (D) *LS: Good* **Mi:** Contraind. *LS: Fair* **D:** Major	CYP3A4 and p-glycoprotein inhibition CYP3A4 and p-glycoprotein inhibition P-glycoprotein inhibition	Colchicine level ↑ Colchicine level ↑ Colchicine level ↑
Nilotinib + Amiodarone	**U:** Major (X) *LS: Fair* **Mi:** Major *LS: Fair* **D:** Major **Me:** Serious	QTc prolongation QTc prolongation and CYP3A, CYP2C8, and p-glycoprotein inhibition QTc prolongation QTc prolongation and CYP3A4 and p-glycoprotein inhibition	QTc ↑ QTc ↑ and ↑ levels of both drugs QTc ↑ QTc ↑ and ↑ levels of both drugs

Abbreviations: TKI, tyrosine kinase inhibitor; LS, literature support ratings; U, UpToDate; Mi, Micromedex; D, Drugs.com; Me, Medscape; CYP3A4, cytochrome P450 3A4 enzyme; PPI, proton pump inhibitors; Contraind, contraindicated.
*Lansoprazole, pantoprazole, omeprazole, esomeprazole, and rabeprazole.

Table 4. Kappa values of databases for the severity ratings, literature support ratings, and general mechanisms of drug–drug interactions in binary combinations

Database pairs	Kappa value	Agreement level	Standard error	95% CI
Severity rating agreements				
Micromedex–UpToDate	0.23	Fair	0.025	0.179–0.278
Micromedex–Medscape	0.22	Fair	0.027	0.170–0.277
Micromedex–Drugs.com	0.19	Slight	0.022	0.142–0.227
UpToDate–Drugs.com	0.11	Slight	0.027	0.066–0.172
Drugs.com–Medscape	0.1	Slight	0.031	0.038–0.158
UpToDate–Medscape	0.09	Slight	0.031	0.030–0.115
Literature support rating agreements				
Micromedex–UpToDate	0.25	Fair	0.032	0.183–0.307
General mechanism agreements				
Micromedex–UpToDate	0.45	Moderate	0.035	0.382–0.519
Micromedex–Medscape	0.37	Fair	0.036	0.302–0.423
UpToDate–Drugs.com	0.36	Fair	0.033	0.291–0.421
Micromedex–Drugs.com	0.25	Fair	0.026	0.198–0.301
UpToDate–Medscape	0.22	Fair	0.038	0.143–0.292
Drugs.com–Medscape	0.16	Slight	0.031	0.096–0.218

Abbreviation: CI, confidence interval.

and 0.28 (p = 0; SE, 0.014; 95% CI, 0.25–0.30). According to the agreement level classification, the kappa level of severity ratings indicated that the databases showed a slight agreement level. The kappa value for the general mechanisms of interactions, that is, 0.28, indicated a fair agreement level among databases.

Databases were compared in binary combinations to identify the severity and literature support agreement levels of database pairs. UpToDate and Micromedex showed a fair agreement level in terms of severity and literature support ratings, with kappa values of 0.23 and 0.25, respectively. The other database pairs showed slight agreement in terms of severity classifications. The lowest agreement level among database pairs was identified in the UpToDate–Medscape databases, with a kappa value of 0.09. The Micromedex–UpToDate database pair showed the highest agreement level among database pairs, with a kappa value of 0.45 in terms of general mechanism agreements. The Drugs.com–Medscape database pair showed the lowest agreement level, with a kappa value of 0.16, and the other database pairs showed fair agreement levels (Table 4).

DISCUSSION

This study demonstrated that databases had a slight agreement level regarding BCR-ABL TKI-involving DDIs in terms of severity ratings. Only two database pairs—Micromedex–UpToDate and Micromedex–Medscape—showed a fair agreement level, and the other database pairs slightly agreed on the severity ratings of interactions. Micromedex and UpToDate showed a fair agreement level in terms of literature support ratings.

The proposed mechanisms attracted to DDIs were also commonly different among databases. The overall kappa value for the general mechanism compatibility of the databases was 0.28, with a fair agreement level. The highest agreement level was observed between Micromedex and UpToDate, with a kappa value of 0.45, which indicated a moderate agreement level. Drugs.com and Medscape, two open-access databases, were slightly compatible in terms of the general mechanism explanation of DDIs, which was the lowest agreement level among database pairs.

In a study that compared DDI checking databases for bipolar medication-involving DDIs, databases showed a slight agreement level in terms of severity ratings (Monteith, Glenn, Gitlin M & Bauer, 2020). In another study that evaluated the sensitivity of five databases on oral oncolytic-involving DDIs, the databases showed a significant difference, and Lexi-Interact and Drugs.com had the highest sensitivity (95%) (Bossaer & Thomas, 2017).

Polypharmacy has increased among elderly patients in recent decades (Haider, Johnell, Thorslund & Fastbom, 2007). Comorbidities and polypharmacy are related to the number of DDIs among these patients (Hohl, Dankoff, Colacone & Afilalo, 2001). DDIs are related to hospital visits and hospitalizations. DDI checking databases help health practitioners to check interactions between multiple drugs (Vonbach, Dubied, Krähenbühl & Beer, 2008). However, the information provided by these databases about interactions and their ability to detect interactions is variable (Reis & Cassiani, 2010). In our study, none of the databases detected all interactions, and the information provided by the databases differed. Drugs.com detected

355 interactions, which made it the most detecting database in our study.

CML is a hematological disorder with a median age of 55 years that presents with a mutation resulting in an active BCR-ABL1 tyrosine kinase. Before the discovery of BCR-ABL TKIs, the treatment options for CML were busulfan and hydroxyurea, with major cytotoxic complications (An et al., 2010). After the introduction of TKIs for the treatment of CML, patients' life expectancies reached near the normal range (Luskin & DeAngelo, 2018). TKIs are often involved in DDIs with different mechanisms (van Leeuwen et al., 2014).

Because of the older age of patients with CML, comorbidities are common, with an incidence of 55.5% (Saydam et al., 2022). Drugs used to treat comorbidities and TKIs may interact and change the TKI efficacy (Luskin & DeAngelo, 2018). Gastric pH-changing drugs, CYP3A4 inhibition or induction, drugs with a changing effect on p-glycoprotein and other transport activities, and combination with QTc prolongations change the effect of TKIs (van Leeuwen et al., 2014). In a study that evaluated the TKI-related DDIs in 105 patients with CML, 159 DDIs were detected (Osorio et al., 2018).

Some interactions in our study with contraindicated warnings were listed. All databases listed interactions with dasatinib and acid-suppressive drugs. There was a consensus on the proposed mechanism for this interaction, which was explained by the decrease in the dasatinib effect due to the gastric pH elevation. UpToDate rated this group of interactions with a good documentation level, whereas Micromedex considered the literature support level as fair (Drugs.com, 2022; UpToDate Interactions, 2022; Medscape, 2022; IBM Micromedex, 2022).

The nilotinib–posaconazole interaction was found in all databases. UpToDate and Micromedex showed a contraindicated warning with a CYP3A4 inhibition-related mechanism (UpToDate Interactions, 2022; IBM Micromedex, 2022). Medscape also reported an increase in the nilotinib blood levels due to p-glycoprotein inhibition (Medscape, 2022). On the other hand, the proposed mechanism of this interaction was shown as QTc prolongation due to the combining of two drugs with a QTc prolongation effect in Drugs.com with a major severity warning (Drugs.com, 2022).

CONCLUSION

CML is a hematological malignancy treated with BCR-ABL TKIs. These drugs are often involved in DDIs because of their chemical structures and metabolic pathways. Databases are important tools for detecting and understanding DDIs; however, significant differences in severity ratings, literature support levels, and the proposed mechanisms are noted among databases. In our study, databases, at most, showed slight agreement in terms of severity ratings and fair agreement in terms of general mechanisms. The significant differences among databases are concerning, and these disparities should be resolved in the future to provide better healthcare to patients with CML. We recommend using multiple DDI databases to evaluate BCR-ABL TKI-involving DDIs.

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REFERENCES

An, X., Tiwari, A. K., Sun, Y., Ding, P. R., Ashby, C. R., Jr, & Chen, Z. S. (2010). BCR-ABL tyrosine kinase inhibitors in the treatment of Philadelphia chromosome positive chronic myeloid leukemia: a review. *Leukemia Research, 34*(10), 1255–1268.

Bossaer, J. B., & Thomas, C. M. (2017). Drug Interaction Database Sensitivity With Oral Antineoplastics: An Exploratory Analysis. *Journal of Oncology Practice, 13*(3), e217–e222.

Cancer.org. (2022, Nov 1). Targeted Therapies for Chronic Myeloid Leukemia. Retrieved from https://www.cancer.org/cancer/chronic-myeloid-leukemia/treating/targeted-therapies.html

Cascorbi I. (2012). Drug interactions–principles, examples and clinical consequences. *Deutsches Arzteblatt International, 109*(33-34), 546–556.

Corrie, K., Hardman, J. G. (2011). Mechanisms of drug interactions: pharmacodynamics and pharmacokinetics. *Anaesthesia and Intensive Care Medicine, 12*(4), 156-159.

Deeks E. D. (2022). Asciminib: First Approval. *Drugs, 82*(2), 219–226.

Drugs.com. (2022, Sep 7). Drug Interactions Checker. Retrieved from https://www.drugs.com/drug_interactions.html

Haider, S. I., Johnell, K., Thorslund, M., & Fastbom, J. (2007). Trends in polypharmacy and potential drug-drug interactions across educational groups in elderly patients in Sweden for the period 1992 - 2002. *International Journal of Clinical Pharmacology and Therapeutics, 45*(12), 643–653.

Hohl, C. M., Dankoff, J., Colacone, A., & Afilalo, M. (2001). Polypharmacy, adverse drug-related events, and potential adverse drug in-

teractions in elderly patients presenting to an emergency department. *Annals of Emergency Medicine, 38*(6), 666–671.

Hsieh, Y. C., Kirschner, K., & Copland, M. (2021). Improving outcomes in chronic myeloid leukemia through harnessing the immunological landscape. *Leukemia, 35*(5), 1229–1242.

IBM Micromedex. (2022, Sep 7). Drug Interactions. Retrieved from https://www.micromedexsolutions.com/home/dispatch

Kennedy, J. A., & Hobbs, G. (2018). Tyrosine Kinase Inhibitors in the Treatment of Chronic-Phase CML: Strategies for Frontline Decision-making. *Current Hematologic Malignancy Reports, 13*(3), 202–211.

Landis, J. R., & Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics, 33*(1), 159–174.

Luskin, M. R., & DeAngelo, D. J. (2018). How to treat chronic myeloid leukemia (CML) in older adults. *Journal of Geriatric Oncology, 9*(4), 291–295.

Marcath, L. A., Xi, J., Hoylman, E. K., Kidwell, K. M., Kraft, S. L., & Hertz, D. L. (2018). Comparison of Nine Tools for Screening Drug-Drug Interactions of Oral Oncolytics. *Journal of Oncology Practice, 14*(6), e368–e374.

Medscape. (2022, Sep 7). Drug Interaction Checker. Retrieved from https://reference.medscape.com/drug-interactionchecker

World Health Organization. (2022, Sep 1). Collaborating Centre for Drug Statistics Methodology. ATC/DDD Index 2022 Retrieved from https://www.whocc.no/atc_ddd_index/

Monteith, S., Glenn, T., Gitlin, M., & Bauer, M. (2020). Potential Drug interactions with Drugs used for Bipolar Disorder: A Comparison of 6 Drug Interaction Database Programs. *Pharmacopsychiatry, 53*(5), 220–227.

Osman, A. E. G., & Deininger, M. W. (2021). Chronic Myeloid Leukemia: Modern therapies, current challenges and future directions. *Blood Reviews, 49*, 100825.

Osorio, S., Escudero-Vilaplana, V., Gómez-Centurión, I., Pérez-López, R., Ayala, R., Vall-Llovera, F., . . . CML Spanish Group (GELMC) (2018). Drug-to-drug interactions of tyrosine kinase inhibitors in chronic myeloid leukemia patients. Is it a real problem?. *Annals of Hematology, 97*(11), 2089–2098.

Patel, R. I., & Beckett, R. D. (2016). Evaluation of resources for analyzing drug interactions. *Journal of the Medical Library Association : JMLA, 104*(4), 290–295.

Reis, A. M., & Cassiani, S. H. (2010). Evaluation of three brands of drug interaction software for use in intensive care units. *Pharmacy World & Science : PWS*, 32(6), 822–828.

Riechelmann, R. P., & Del Giglio, A. (2009). Drug interactions in oncology: how common are they?. *Annals of Oncology, 20*(12), 1907–1912.

Saydam, G., Ali, R., Demir, A. M., Eskazan, A. E., Guvenc, B., Haznedaroglu, I. C., . . . Ilhan, O. (2022). The effect of comorbidities on the choice of tyrosine kinase inhibitors in patients with chronic myeloid leukemia. *International Journal of Hematologic Oncology, 1*1(1), IJH38.

Suriyapakorn, B., Chairat, P., Boonyoprakarn, S., Rojanarattanangkul, P., Pisetcheep, W., Hunsakunachai, N., . . . Khemawoot, P. (2019). Comparison of potential drug-drug interactions with metabolic syndrome medications detected by two databases. *PloS One, 14*(11), e0225239.

Uptodate. (2022, Sep 7). Lexicomp Drug Interactions. Retrieved from https://www.uptodate.com/drug-interactions/?source=responsive_home#di-druglist

van Leeuwen, R. W., van Gelder, T., Mathijssen, R. H., & Jansman, F. G. (2014). Drug-drug interactions with tyrosine-kinase inhibitors: a clinical perspective. The Lancet. *Oncology, 15*(8), e315–e326.

van Leeuwen, R. W., Brundel, D. H., Neef, C., van Gelder, T., Mathijssen, R. H., Burger, D. M., & Jansman, F. G. (2013). Prevalence of potential drug-drug interactions in cancer patients treated with oral anticancer drugs. *British Journal of Cancer, 108*(5), 1071–1078.

van Leeuwen, R. W. F., Jansman, F. G. A., Hunfeld, N. G., Peric, R., Reyners, A. K. L., Imholz, A. L. T., . . . Mathijssen, R. H. J. (2017). Tyrosine Kinase Inhibitors and Proton Pump Inhibitors: An Evaluation of Treatment Options. *Clinical Pharmacokinetics, 56*(7), 683–688.

Vonbach, P., Dubied, A., Krähenbühl, S., & Beer, J. H. (2008). Evaluation of frequently used drug interaction screening programs. *Pharmacy World & Science : PWS, 30*(4), 367–374.

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Istanbul J Pharm, 54 (1): 40–48
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Original Article

The therapeutic approach to fibrocystic breast disease in the MCF-10A cell culture model: Striking efficacy of polyphenols

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ABSTRACT

Background and Aims: Standard treatment regimens for fibrocystic breast disease (FBD) do not provide a permanent cure and have undesirable side effects. This study aims to investigate the therapeutic potential of different honey and propolis, as well as some important polyphenols, on breast epithelial cells (MCF-10A).

Materials and Methods: The effects of five honey, two propolis extracts and seven polyphenol samples on the cell viability were assessed the WST-1 assay. Content analysis of the propolis samples was performed using high performance liquid chromatography (HPLC).

Results: Chestnut and cedar honey had antiproliferative effects on MCF-10A cells at all doses (1-10 µg/mL), as well as pine honey at the highest dose. However, multifloral honey had no similar effect. Chinese propolis had significant antiproliferative effects on MCF-10A cells at doses of 50-250 µg/mL and on the human periodontal ligament (hPDL) control cells at a dose of 5 µg/mL. Türkiye propolis only had an antiproliferative effect on MCF-10A cells at the highest dose ($p = 0.0013$). Higher levels of ferulic acid, kaempferol, caffeic acid, pinocembrin and quercetin were detected in Türkiye propolis, while Chinese propolis was rich in pinostrobin. Ferulic acid, pinostrobin and galangin showed antiproliferative properties on MCF-10A cells ($p < 0.0001$), whereas the remaining four polyphenols had no significant effect on cell viability ($p > 0.05$).

Conclusion: The findings of the study highlight the antiproliferative effects of pinostrobin, ferulic acid and galangin on MCF-10A cells and has also confirmed the antiproliferative effects of honey and propolis samples to be due to their polyphenolic properties. Therefore, this study suggests that polyphenolic substances may have both preventive and therapeutic potential in FBD.

Keywords: Fibrocystic breast, honey, MCF-10A, polyphenol, propolis

INTRODUCTION

Fibrocystic breast disease (FBD) is the most common type of benign breast disease, occurring in 30% to 60% of women worldwide, often in the 30-50 age group (Gopalani et al., 2020). The term fibrocystic describes benign breast diseases including a variety of non-malignant lesions such as nipple discharge, trauma, mastalgia, and benign tumors. While these benign lesions are not associated with an increased risk of malignancy, they are associated with up to a 50% risk of developing breast cancer under certain histopathological and clinical conditions. Some sex steroid hormones such as estrogen and progesterone have effects on this disease's progression, evaluation and treatment (McMullen, Zoumberos, & Kleer, 2019; Tu et al., 2019). FBD has clinical findings and symptoms such as a palpable mass, skin dimpling, thickening, pain and nipple discharge (Vorherr, 1986). Although fibrocyst formation is associated with high estrogen levels, more research is needed to elucidate the exact pathophysiology of FBD (Vorherr, 1986; Brkić et al., 2018). Cystic mastitis, cystic hyperplasia and adenosis were reported in histological examinations (Greenblatt, Samaras, Vasquez, Nezhat, 1982).

Various treatment methods are applied to cure FBD or alleviate its symptoms, especially breast pain (Alipour, et al., 2021; Sasaki et al., 2018). While mechanical support of the breast, vitamin supplements, dietary restrictions, hormonal manage-

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ment, non-steroidal anti-inflammatory drugs, danazol and tamoxifen have frequently been used for treatment, no completely efficient and noncontroversial option is found regarding FBD, and surgical intervention is recommended for unresponsive and recurrent cases (Brkic et al., 2018; Alipour et al., 2021; Irgebay et al., 2017; Yadav, Sharma, Singh, Gupta, 2020). Studies on progressive cases of FDB have reported the use of danazol at doses of 200 and 400 mg/day, despite its serious side effects, eases the symptoms of the disease (Yadav et al., 2020; Gateley, Miers, Mansel, Hughes, 1992). Additionally, afimoxifen gel, which is a tamoxifen metabolite, has promising effects on FBD without serious side effects (Mansel et al., 2007). The effects of different herbal products on treating FBD have also been investigated as an alternative to existing therapeutics that do not provide a permanent cure and that have undesirable side effects. Among the herbal supplements recommended for treatment, evening primrose oil, flaxseed oil and vitamin E have been reported to relieve symptoms without side effects (Gateley et al., 1992; Godazandeh, Ala, Motlaq, Sahebnasagh & Bazi, 2021).

Apart from being a nutrient, honey produced by *Apis mellifera* (honeybee) has also been used as a herbal medicine, along with other bee products such as propolis, pollen, and royal jelly, to treat various diseases throughout history (Lusby, Coombes, & Wilkinson, 2005). The amount and different types of phenolic substances that give honey its antioxidant properties vary according to the flora used (Jaganathan, & Mandal, 2009; Mandal & Mandal., 2011). Propolis is a natural product honeybees make from various plants' leaves, stems and buds to repair and protect their hives. Propolis contains phenolic acids, tannins, polysaccharides, terpenes, aromatic acids, aldehydes and many other chemicals, and has important pharmacological properties (Silici, & Kutluca, 2005). These chemicals, called phytochemicals, are produced as secondary metabolites in plants and turned into bioactive compounds with beneficial health effects when consumed as nutrients (Naeem & Ugur., 2020). Phenolic compounds are characterized as natural sources of the antioxidant requirement of metabolism. They show their antioxidant activity by binding free radicals or chelating with metals (Verma, Hucl, & Chibbar, 2009). Based on the chemical structures, phenolic compounds are generally classified as flavonoids (e.g., anthocyanidins, flavones and flavonols, flavanones, flavanols and isoflavones), phenolic acids, stilbenes and lignans (Khan, & Dangles, 2014).

Previous studies have reported the effects of honey and propolis on malignant diseases of the mammary gland may vary depending on their polyphenol content (Seyhan et al., 2017; Seyhan et al., 2019). When considering the individual effects of polyphenolic substances on breast cancer cells, different effects of these polyphenols have been reported (Yang et al., 2014; Omene et al., 2013; Hung., 2004; Abbas Momtazi-Borojeni, Behbahani, Sadeghi-Aliabadi, 2013; Serafim et al., 2011). Although bee products and polyphenolic compounds have been studied extensively in malignant cell lines, there is a limited number of in vitro research focusing on FBD in the literature.

Therefore, this study aims to investigate the potential antiproliferative effects of honey and propolis samples, whose therapeutic effects have been shown on malignant breast cell lines in previous studies, on MCF-10A cells modeling FBD and on human periodontal ligament fibroblast (hPDL) control cells, together with the bioactive polyphenols found in the samples' contents.

MATERIALS AND METHODS

Sample Preparation

Honey and propolis samples were obtained from Altıparmak Gıda Inc. (Istanbul, Türkiye) and stored at +4°C. These samples were produced for consumption and verified to be free of biological or heavy metal contamination through detailed chemical analysis. Polyphenols (ferulic acid, kaempferol, caffeic acid, pinocembrin, pinostrobin, galangin and quercetin) were purchased from Merck (Darmstadt, Germany) and stored at +4°C. All samples were dissolved in 60% ethanol (60% ethanol; 40% water) and stored at -20°C.

HPLC Analysis

The amounts of the phenolic compounds in propolis were determined by high performance liquid chromatography (HPLC, Waters 600 controller and Waters 996 PDA detector). Prior to analysis, propolis extracts were prepared by dissolving 0.1 g of propolis in 25 mL of 60% ethanol and introduced into the HPLC system. A C18 column was used for the analysis of phenolic compounds. As mobile phases, 0.1% trifluoroacetic acid (TFA) prepared in distilled water and 0.1% TFA prepared in acetonitrile were used. During the analysis, the flow rate was set at 1 mL/min, and the phenolic compounds in propolis were measured at wavelengths of 280 nm, 312 nm, and 360 nm (Bino et al., 2005; Ahn, Kumazawa, Hamasaka, Bang, & Nakayama, 2004).

Cell Culture

MCF-10A breast epithelial cells, a model of fibrocystic cell disease, were purchased from ATCC (ATCC, Rockville, MD). These MCF-10A cells were maintained in a suitable growth medium DMEM F12 containing 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin-streptomycin (all obtain from Gibco, Thermo Fisher Scientific, Waltham, MA, USA) , and a bullet kit in humidified air at 37ºC and 5% CO_2. Normal hPDL cells were purchased from the Lonza Group (Basel, Switzerland). The hPDL cells were cultured using the Stromal Cell Growth Medium BulletKit and ReagentPack Subculture Reagents (Lonza, Basel, Switzerland) following the

manufacturer-recommended protocol at 37ºC in humidified air with 5% CO_2. The study used hPDL as a control cell line.

WST-1 Analysis

All cell proliferation analyses of this study were performed using the Cell Proliferation Reagent water-soluble tetrazolium salt-1 (WST-1; Roche, Manheim, Germany). WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt), a tetrazolium salt, reacts with mitochondrial dehydrogenases in the mitochondria of living cells to form a yellow formazan crystal. The yellow colour is correlated with the presence of living cells and this change in formazan formation can be measured spectrophotometrically at a wavelength of 450nm. Briefly, after the number of cells were counted on the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA), 1×10^4 cells were seeded in each well of a 96-well plate (Sarstedt, Nümbrecht, Germany) in 3 replicates. The cells were allowed to adhere for 24 h, and the medium was aspirated. Then, intended doses of samples (for honey samples 1, 2.5, 5, 7.5, 10 µg/mL doses; for propolis samples 2.5, 5, 50, 100, 250 µg/mL doses; for phenolic substances 2.5, 5, 7.5, 10, 15, 20, 22.5, 25, 30, 37.5 µg/mL) and medium alone as negative control were applied within a fresh medium with 3% FBS (Seyhan et al., 2017; Seyhan et al., 2019). After 46 h of incubation, 10 µL WST-1 was applied to each well in the dark. 2 h later, the absorbances of the wells at 450 nm (with the reference wavelength at 600nm) were measured using Multiscan enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific, Massachusetts, USA).

In the experiments, all doses were performed as triple biological replicates. Untreated cells were used as control group (negative) (DMEMF12 for MCF10A cells and Bulletkit hPDL cells) and the values of the groups are given as mean±standard deviation (X±SD). Viability of control cells was set to 100%. Cell viability is given by the following formula:

Viability (%) = OD of treated group / OD of negative control group ×100.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Prism Software, San Diego, CA, USA). The equality of means of independent groups was tested by one-way analysis of variance (ANOVA), and Dunnet's test was used as the post-hoc test following ANOVA to calculate the difference between the mean of each dose group with the mean of the control group. Values of $p < 0.05$ are considered significant.

RESULTS

HPLC Analysis of the Propolis Samples

The amounts of important phenolic substances in the Chinese propolis and Türkiye propolis were measured using the HPLC analysis. Accordingly, Türkiye propolis had higher ferulic acid (3.12 mg/g vs. 1.03 mg/g), kaempferol (1.13 mg/g vs. 0.15 mg/g), caffeic acid (7.98 mg/g vs. 0.21 mg/g), pinocembrin (9.84 mg/g vs. 0.15 mg/g) and quercetin (0.73 mg/g vs. 0.14 mg/g) levels compared to the Chinese propolis. However, the pinostrobin level in the Chinese propolis was higher than in the Türkiye propolis (17.99 mg/g vs. 10.85 mg/g). Galangin content was similar in both propolis samples (13.90 mg/g vs. 13.80 mg/g; Figure 1).

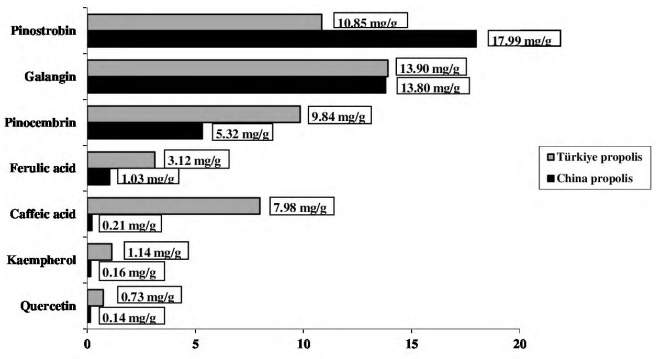


Figure 1. HPLC analysis in China and Türkiye propolis samples.

Cell Viability and Proliferation

The effects of chestnut, pine, cedar, multifloral and artificial honey on the viability of MCF-10A cell lines were investigated at doses of 1-10 µg/mL (Figure 2). Accordingly, both chestnut and cedar honey were observed to have an antiproliferative effect on the MCF-10A cell line at all doses (1-10 µg/mL, $p < 0.0001$) and pine honey to have the same effect at its highest dose (10 µg/mL, $p < 0.0001$). Multifloral and artificial honey had no cytotoxic effects on MCF-10A cell lines (Table 1).

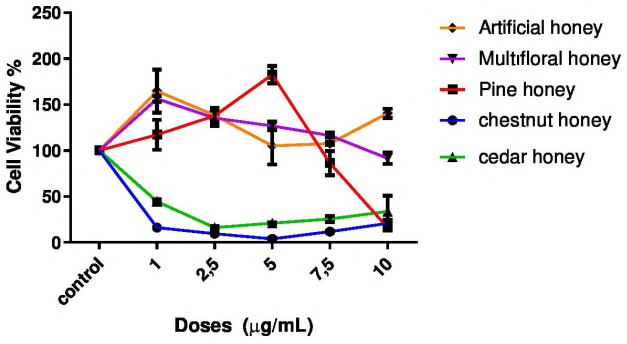


Figure 2. 48th-hour cell viability data of chestnut, pine, cedar, multifloral, and artificial honey samples in MCF-10A cell line.

Table 1. *The effects of Chestnut, Pine, Cedar, Multifloral, and Artificial honey on the MCF-10A cell line using the WST-1 Assay*

Doses (µg/mL)	Chestnut Honey	Pine Honey	Cedar Honey	Multifloral Honey	Artificial Honey
	X ± *SD*	X ± *SD*	X ± *SD*	X ± *SD*	X ± *SD*
1	16.16 ± 1.01 *	117.20 ± 16.20	44.52 ± 2.15 *	156.26 ± 3.31 *	164.64 ± 23.45
2.5	9.56 ± 0.92 *	137.87 ± 7.47 **	16.14 ± 1.90 *	135.22 ± 8.44 *	138.38 ± 7.70 ****
5	3.78 ± 1.54 *	182.65 ± 9.44 *	21.01 ± 0.53 *	126.82 ± 4.66 *	105.02 ± 20.15
7.5	11.73 ± 0.75 *	86.31 ± 13.10	25.59 ± 3.04 *	116.33 ± 2.07 **	107.66 ± 1.31
10	20.66 ± 4.04 *	16.25 ± 1.78 *	33.57 ± 17.25 *	91.48 ± 6.17	140.50 ± 5.04 ****

Data are expressed as percentage of absorbance values compared to negative control wells (mean standard deviation, X ± SD). Control cell viability was set at 100%. For each dose, comparisons were made with medium-only controls. *, p<0.0001; **, p< 0.01; ***, p< 0.001; ****, p< 0.05.

The antiproliferative effects of propolis originating from Türkiye and China on the MCF-10A and hPDL cells were analyzed using the WST-1 test at doses of 1-250 µg/mL (Figure 3). The effects of Chinese propolis on MCF-10A cells at high doses (50, 100, 250 µg/mL) were antiproliferative (p < 0.001). Chinese propolis also had a significant antiproliferative effect on the hPDL cell line at moderate-to-high doses (5–100 µg/mL). Meanwhile, the Türkiye propolis had no cytotoxic effect on either cell line up to 100 µg/mL (p>0.05). It had an antiproliferative effect in the MCF-10A cells, but only at the highest dose (250 µg/mL; p = 0.0013; Table 2).

Antiproliferative effects of ferulic acid (20-30 µg/mL), galangin (15-30 µg/mL), and pinostrobin (5-30 µg/mL) were observed on the MCF-10A cell line at 48 h (p < 0.0001). Kaempferol was also found to have a moderate cytotoxic effect on the MCF-10A cells at high doses (for 20 µg/mL, p < 0.05, for 30 µg/mL, p = 0.0004). Meanwhile, pinocembrin, caffeic acid and quercetin had no significant effects on the MCF-10A cell line (p > 0.05; Figure 4).

DISCUSSION

FBD is the most common benign breast disease in women, and in some cases requiring a differential diagnosis from breast cancer. This study investigated the antiproliferative effects of multifloral, cedar, pine, chestnut and artificial honey samples, as well as two propolis samples (Chinese and Türkiye) and seven polyphenols with known cytotoxic effects, on MCF-10A cells modeling FBD.

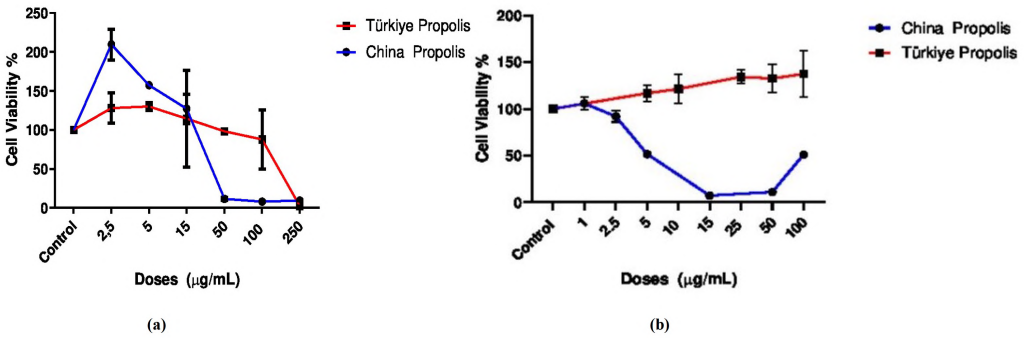
Different results have been obtained from studies that examined the effects of bee products on viability in MCF-10A cells based on the type and geographical origin of the product. In a study in which MCF-10A cells were treated with 1-10% doses of honey produced by Asian giant bees (*Apis dorsata*) from Tualang trees in Malaysian, no significant antiproliferative effect was reported even after 72 hours of incubation (Fauzi, Norazmi, & Yaacob, 2011). Similarly, Manuka honey was reported to cause no significant loss of viability in MCF-10A cells at low concentrations (0.6% and 1.25%) after 72 hours of incubation and to have a cytotoxic effect at high concentrations (2.5% and 5%; Aryappalli et al., 2017). While the present study observed no cytotoxic effect for multifloral honey originating from the Southeastern Anatolia region of Türkiye origin on MCF-10A cells, tree honey (cedar, pine, and especially chestnut) had significant antiproliferative effects on these cells (Figure 2). Previous study has determined the total phenolic contents and antioxidant capacities of the honey samples included in this study to rank from highest to lowest as chestnut, cedar, pine and multifloral honey (Seyhan et al., 2017). Taken together, the cytotoxic effects of honey samples on MCF-10A cells can be said to parallel their total phenolic content and antioxidant activities.

Studies investigating the effects of propolis on the MCF-10A cell line have mostly focused on the flavonoids and other bioactive components in the contents of propolis. Mohamed et al. (2020) reported that IC50 values for propolis extracts produced by *Tetrigona apicalis* bees on the Malay Peninsula regarding MCF-10A cells at 24 h, 48 h and 72 h to be 49.55 µg/mL, 56.05 µg/mL and 72.10 µg/mL, respectively. The Chinese propolis in

Table 2. *The effects of Chinese Propolis and Türkiye Propolis in MCF-10A and hPDL cell line Using the WST-1 Assay*

MCF-10A			
Chinese propolis		**Türkiye propolis (from Kartal)**	
Doses (µg/mL)	**X ± *SD***	**Doses (µg/mL)**	**X ± *SD***
2.5	209.639 ± 19.666 *	**2.5**	128.061 ± 19.453
5	157.37 ± 0.654 **	**5**	130.208 ± 4.951
15	127.1 ± 18.806	**15**	114.508 ± 62.133
50	11.5613 ± 1.877 *	**50**	98.2276 ± 1.131
100	7.99492 ± 0.454 *	**100**	87.8683 ± 38.127
250	9.50964 ± 0.571 *	**250**	1.777± 0.148 **
hPDL			
Chinese propolis		**Türkiye propolis (from Kartal)**	
Doses (µg/mL)	**X ± *SD***	**Doses (µg/mL)**	**X ± *SD***
1	105.925 ± 6.889	**5**	116.933 ± 8.638
2.5	92.098 ± 5.989	**10**	121.476 ± 15.942
5	51.789 ± 2.352 ***	**25**	134.334 ± 7.313 ****
15	7.196 ± 0.245 ***	**50**	132.540 ± 15.176
50	11.121 ± 0.303 ***	**100**	137.541 ± 24.515 ****
100	51.242 ± 0.623 ***		

Data are expressed as percentage of absorbance values compared to negative control wells (mean standard deviation, X ± SD). Control cell viability was set at 100%. For each dose, comparisons were made withmedium-only controls.
*, p< 0.001; **, p< 0.01; ***, p< 0.0001; ****, p< 0.05.



(a) (b)

Figure 3. 48th-hour cell viability data of Türkiye (Istanbul/Kartal) and China propolis samples in MCF-10A (a) and hPDL (b) cells.

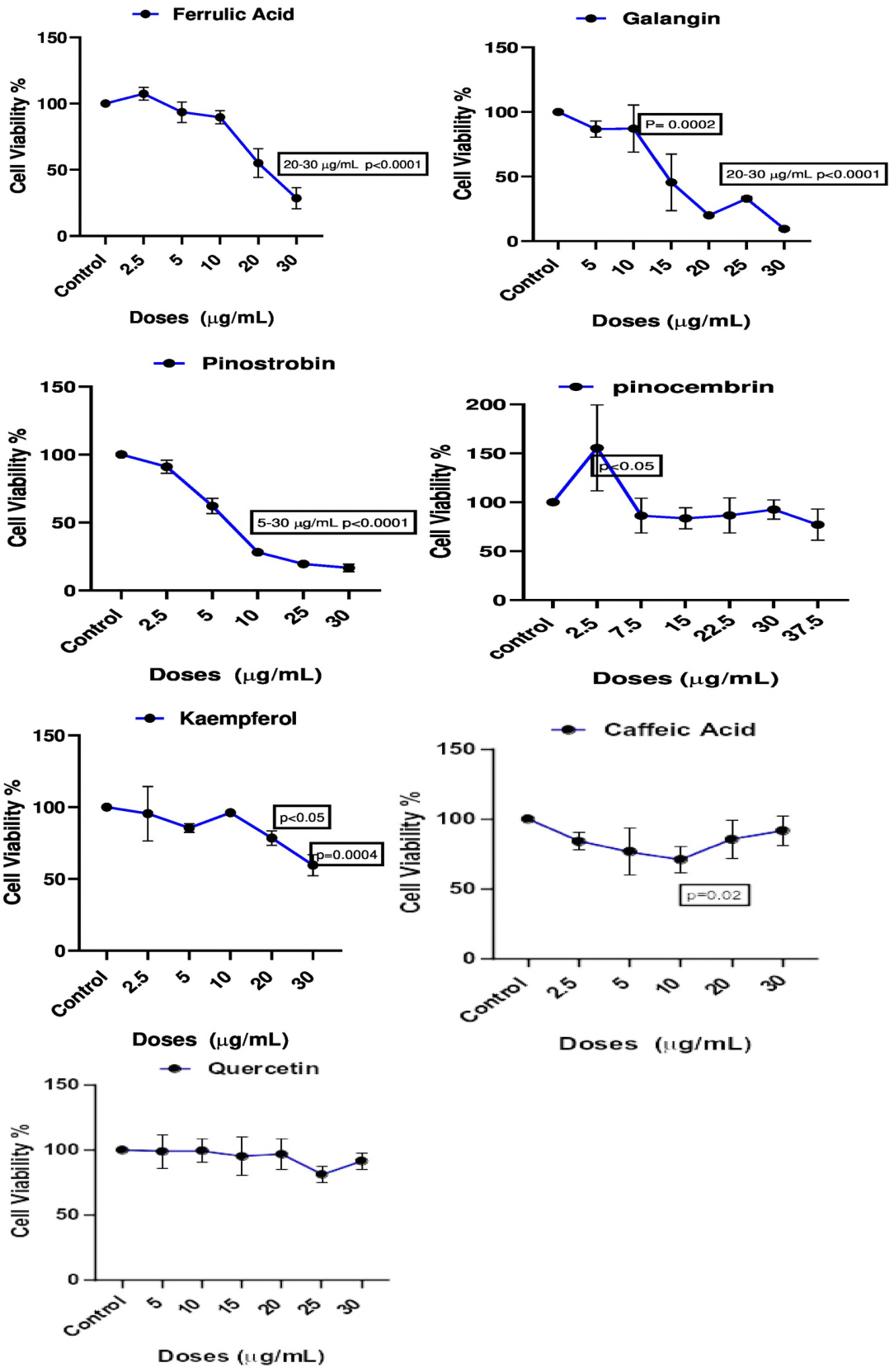


Figure 4. 48th hour cell viability data of ferrulic acid, galangin, pinostrobin, pinocembrin, kaempferol, caffeic acid quercetin samples in MCF-10A cell line.

the current study showed antiproliferative effects on MCF-10A cells at high doses (50-250 µg/mL) and on the hPDL control cell line at medium and high doses (5–100 µg/mL). Additionally, the Türkiye propolis had no significant effect on cell viability in both the MCF-10A and hPDL cell lines at the 1-100 µg/mL doses but it had an antiproliferative effect only at the 250 µg/mL dose on the MCF-10A cells (Table 2, Figure 3). These results indicate that propolis samples from distant regions affect the viability of the cells differently.

When considering how the Türkiye propolis showed no cytotoxic effects in the control hPDL cell line, this propolis type can be suggested as being protective of normal cells. A previous study examined the different cytotoxic effects of propolis extracts from different geographical origins on breast cancer cell lines (Seyhan et al., 2019) and found that, while the cytotoxic effect of Chinese propolis extract was observed starting from 15 µg/mL on MCF-10A cells ($p < 0.01$), the Türkiye propolis was only found to be cytotoxic at high doses (250-500 µg/mL, $p < 0.01$). Previous studies have also shown the cytotoxic effects of honey (chestnut, cedar and pine) and propolis (Chinese and Türkiye) samples to be apoptotic in breast cancer cell lines and MCF-10A cells (Seyhan et al., 2017; Seyhan et al., 2019).

The present study performed polyphenolic content analyses of propolis samples using HPLC to determine whether the different effects of Türkiye and Chinese propolis on cell viability in MCF-10A cell lines depend on the polyphenolic compounds in their contents. The results from the HPLC analysis revealed the Türkiye propolis to be rich in ferulic acid, kaempferol, caffeic acid, and pinocembrin and the Chinese propolis to be rich in pinostrobin.

Meanwhile, only a limited number of studies have so far investigated the effects of these polyphenolic substances on MCF-10A cell viability. Song, Yan, Zhou & Zhen, (2017) reported galangin at 5.4 and 10.8 µg/mL doses and at 24 h to have no significant effect on viability in MCF-10A cells. Still, the current study showed the antiproliferative effects of galangin treatment on these cells at 48 h and a dose range of 10-20 µg/mL ($p = 0.0002$; Figure 4). This observed difference may stem from the application of galangin to the cells at different doses and/or the evaluation of its effects over different time periods. Therefore, the effects of galangin on MCF-10A cells need to be evaluated over wider dose and time ranges to obtain more precise results.

In a recent study examining the effects of kaempferol on the MCF-10 cell line at 72 hours and at a dose range of 0.003–14.31 µg/mL, it was reported that the IC50 value of this polyphenolic substance was 10.6 µg/mL (37 µM) (Pham, Sakoff, Vuong, Bowyer, & Scarlett, 2018). On the other hand, Der Medizin and Abutayeh (2014) suggested that kaempferol (0.05–100 µg/mL) and quercetin (0.05–100 µg/mL) show no cytotoxic effect on MCF-10A cells at 24 h. The current study confirmed that kaempferol has a mild antiproliferative effect on MCF-10A cells only at high doses (for 20 µg/mL, $p < 0.05$; for 30 µg/mL, $p = 0.0004$). However, this study found no remarkable effect of quercetin ($p > 0.05$; Figure 4).

A recent study reported cell proliferation not to be affected when MCF-10A cells are cultured with pinostrobin at doses of 0.003 µg/mL-5.40 µg/mL for 24 h (Jones & Gehler., 2020). In contrast, this study showed that pinostrobin at doses of 5–30 µg/mL over 48 h had a very strong antiproliferative effect on these cells ($p < 0.0001$; Figure 4). Therefore, this study suggests that the effect of pinostrobin on MCF-10A cells should be investigated over wider dose and time intervals. Furthermore, pinocembrin was also previously reported to have no effect on MCF-10A cell viability (doses of 0.26 to 12.81 µg/mL over 72 h; Aiello et al., 2017). Consistent with this finding, no significant effect of pinocembrin at doses of 7.5-37.5 µg/mL (48 h) on MCF-10 cell viability was observed in the present study, but a mild cytotoxic effect was present at the low dose (2.5 µg/mL) (Figure 4).

To the best of the authors' knowledge, this study is also the first in the literature to investigate the effects of ferulic acid and caffeic acid on MCF-10A cells. Ferulic acid showed a significant antiproliferative effect on the cells at high doses (20–30 µg/mL, $p < 0.0001$), whereas caffeic acid did not. When evaluating the Türkiye and Chinese propolis extracts alongside their polyphenol contents and effects the Türkiye propolis is rich in ferulic acid and kaempferol, which showed antiproliferative effects only at high doses, and caffeic acid, quercetin and pinocembrin polyphenols, which did not affect viability in the MCF-10A cell line at the given doses. Therefore, a part of the cytotoxic effect of Türkiye propolis on the MCF-10A cells can be attributed to its ferulic acid content. Meanwhile, the Chinese propolis was found to be rich in pinostrobin, which had a strong antiproliferative effect even at low doses. Therefore, the cytotoxic effect of China propolis can be associated with its pinostrobin content.

Individual analyses of the polyphenolic content of honey samples could not be performed. Therefore, this study was unable to evaluate the effects of honey samples on the viability of MCF-10A cells in terms of their polyphenol content. However, the study did obtain findings that tree honey (cedar, pine and especially chestnut) may be effective against FBD. We aim to reach more precise results in future studies evaluating the effects of tree and multifloral honey samples regarding content analysis, as well as the apoptotic effects of polyphenolic substances.

CONCLUSION

In conclusion, the study's findings highlight the potential effects of pinostrobin, ferulic acid and galangin on the fibrocystic breast disease model and investigating their combined effects may

be rewarding in terms of discovering new prophylactic and complementary therapies with no serious side effects.

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REFERENCES

Abbas Momtazi-borojeni, A., Behbahani, M., & Sadeghi-Aliabadi, H. (2013). Antiproliferative activity and apoptosis induction of crude extract and fractions of Avicennia marina. *Iranian Journal of Basic Medical Sciences, 16*(11), 1203.

Abutayeh, A. (2014). Die Wirkung der kommerziell erworbenen Flavonoide (Quercetin, Kaempferol und Chrysin) auf die malignen Mammakarzinomzellen (MCF7 und BT20) und die benignen veränderten Mammazellen (MCF10a und MCF12a)(Östrogenrezeptor positiv und negativ) in vitro (Doctoral dissertation, Universitätsmedizin Rostock).

Ahn, M. R., Kumazawa, S., Hamasaka, T., Bang , K. S., & Nakayama, T. (2004). Antioxidant activity and constituents of propolis collected in various areas of Korea. *Journal of Agricultural and Food Chemistry, 52*(24), 7286–7292. https://doi.org/10.1021/jf048726s.

Aiello, F., Armentano, B., Polera, N., Carullo, G., Loizzo, M. R., Bonesi, M., ... & Tundis, R. (2017). From vegetable waste to new agents for potential health applications: Antioxidant properties and effects of extracts, fractions and pinocembrin from *Glycyrrhiza glabra L.* aerial parts on viability of five human cancer cell lines. *Journal of Agricultural and Food Chemistry, 65*(36), 7944-7954. https://doi.org/10.1021/acs.jafc.7b03045

Alipour, S., Rastad, H., Saberi, A., Faiz, F., Maleki-Hajiagha, A., & Abedi, M. (2021). Metformin in the management of fibrocystic breast disease: a placebo-controlled randomized clinical trial. *journal of Faculty of Pharmacy, Tehran University of Medical Sciences, 29*(2), 389–396. https://doi.org/10.1007/s40199-021-00424-6

Aryappalli, P., Al-Qubaisi, S. S., Attoub, S., George, J. A., Arafat, K., Ramadi, K. B., ... & Al-Ramadi, B. K. (2017). The IL-6/STAT3 signaling pathway is an early target of manuka honey-induced suppression of human breast cancer cells. *Frontiers in Oncology, 7*, 167. https://doi.org/10.3389/fonc.2017.00167

Bino, R. J., De Vos, C. R., Lieberman, M., Hall, R. D., Bovy, A., Jonker, H. H., & Levin, I. (2005). The light-hyperresponsive high pigment-2dg mutation of tomato: alterations in the fruit metabolome. *New Phytologist, 166*(2), 427-438. https://doi.org/10.1111/j.1469-8137.2005.01362.x

Brkic, M., Vujovic, S., Ivovic, M., Gajic, M. T., Marina, L., Ivanisevic, M. F., & Franic, D. (2018). The role of E2/P ratio in the etiology of fibrocystic breast disease, mastalgia and mastodynia. *Acta Clinica Croatica, 57*(4), 756. https://doi: 10.20471/acc.2018.57.04.18

Fauzi, A. N., Norazmi, M. N., & Yaacob, N. S. (2011). Tualang honey induces apoptosis and disrupts the mitochondrial membrane potential of human breast and cervical cancer cell lines. *Food and Chemical Toxicology, 49*(4), 871-878. https://doi.org/10.1016/j.fct.2010.12.010

Gateley, C. A., Maddox, P. R., Pritchard, G. A., Sheridan, W., Harrison, B. J., Pye, J. K., ... & Mansel, R. E. (1992). Plasma fatty acid profiles in benign breast disorders. *Journal of British Surgery, 79*(5), 407-409. https://doi.org/10.1002/bjs.1800790511

Gateley, C. A., Miers, M., Mansel, R. E., & Hughes, L. E. (1992). Drug treatments for mastalgia: 17 years experience in the Cardiff Mastalgia Clinic. *Journal of the Royal Society of Medicine, 85*(1), 12-15. https://doi.org/10.1177/014107689208500105

Godazandeh, G., Ala, S., Motlaq, T. M., Sahebnasagh, A., & Bazi, A. (2021). The comparison of the effect of flaxseed oil and vitamin E on mastalgia and nodularity of breast fibrocystic: a randomized double-blind clinical trial. *Journal of Pharmaceutical Health Care and Sciences, 7*(1), 1-8. https://doi.org/10.1186/s40780-020-00186-4

Gopalani, S. V., Janitz, A. E., Martinez, S. A., Gutman, P., Khan, S., & Campbell, J. E. (2020). Trends in Cancer Incidence among American Indians and Alaska natives and non-hispanic whites in the United States, 1999–2015. *Epidemiology, 31*(2), 205-213. https://doi.org/10.1097/EDE.0000000000001140

Greenblatt, R. B., Samaras C, Vasquez, J. M., Nezhat C. (1982). Fibrocystic disease of the breast. *Clinical Obstetrics and Gynecology, 25*(2), 365-71.

Hung, H. (2004). Inhibition of estrogen receptor alpha expression and function in MCF-7 cells by kaempferol. *Journal of Cellular Physiology, 198*(2), 197-208. https://doi.org/10.1002/jcp.10398

Irgebay, Z., Yeszhan, B., Sen, B., Tuleukhanov, S., Brooks, A. D., Sensenig, R., & Orynbayeva, Z. (2017). Danazol alters mitochondria metabolism of fibrocystic breast Mcf10A cells. *Breast (Edinburgh, Scotland), 35*, 55–62. https://doi.org/10.1016/j.breast.2017.06.007

Jaganathan, S. K., & Mandal, M. (2009). Antiproliferative effects of honey and of its polyphenols: A review. *Journal of Biomedicine and Biotechnology*, 2009, 830616. https://doi.org/10.1155/2009/830616

Jones, A. A., & Gehler, S. (2020). Acacetin and pinostrobin inhibit malignant breast epithelial cell adhesion and focal adhesion formation to attenuate cell migration. *Integrative Cancer Therapies, 19*, 1534735420918945. https://doi.org/10.1177/1534735420918945

Khan, M. K., & Dangles, O. (2014). A comprehensive review on flavanones, the major citrus polyphenols. *Journal of Food Composition and Analysis, 33*(1), 85-104. https://doi.org/10.1016/j.jfca.2013.11.004

Lusby, P. E., Coombes, A. L., & Wilkinson, J. M. (2005). Bactericidal activity of different honeys against pathogenic bacteria. *Archives of Medical Research, 36*(5), 464-467. https://doi.org/10.1016/j.arcmed.2005.03.038

Mandal, M. D., & Mandal, S. (2011). Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine, 1*(2), 154-160. https://doi.org/10.1016/S2221-1691(11)60016-6

Mansel, R., Goyal, A., Nestour, E. L., Masini-Etévé, V., O'Connell, K., & Afimoxifene (4-OHT) Breast Pain Research Group. (2007). A phase II trial of Afimoxifene (4-hydroxytamoxifen gel) for cyclical mastalgia in premenopausal women. *Breast Cancer Research and Treatment, 106*, 389-397. https://doi.org/10.1007/s10549-007-9507-x

McMullen, E. R., Zoumberos, N. A., & Kleer, C. G. (2019). Metaplastic breast carcinoma: update on histopathology and molecular alterations. *Archives of Pathology & Laboratory Medicine, 143*(12), 1492-1496. https://doi.org/10.5858/arpa.2019-0396-RA

Mohamed, W. A. S., Ismail, N. Z., Omar, E. A., Abdul Samad, N., Adam, S. K., & Mohamad, S. (2020). GC-MS evaluation, antioxidant content, and cytotoxic activity of propolis extract from Peninsular Malaysian stingless bees, Tetrigona apicalis. *Evidence-Based Complementary and Alternative Medicine, 2020*. https://doi.org/10.1155/2020/8895262

Omene, C., Kalac, M., Wu, J., Marchi, E., Frenkel, K., & O'Connor, O. A. (2013). Propolis and its active component, caffeic acid phenethyl ester (CAPE), modulate breast cancer therapeutic targets via an epigenetically mediated mechanism of action. *Journal of Cancer Science & Therapy, 5*(10), 334.

Pham, H. N. T., Sakoff, J. A., Vuong, Q. V., Bowyer, M. C., & Scarlett, C. J. (2018). Comparative cytotoxic activity between kaempferol and gallic acid against various cancer cell lines. *Data in brief, 21*, 1033-1036. https://doi.org/10.1016/j.dib.2018.10.121

Sasaki, J., Geletzke, A., Kass, R. B., Klimberg, V. S., Copeland III, E. M., & Bland, K. I. (2018). Etiology and management of benign breast disease. *The Breast*, 79-92. https://doi.org/10.1016/B978-0-323-35955-9.00005-2

Serafim, T. L., Carvalho, F. S., Marques, M. P., Calheiros, R., Silva, T., Garrido, J., ... & Oliveira, P. J. (2011). Lipophilic caffeic and ferulic acid derivatives presenting cytotoxicity against human breast cancer cells. *Chemical Research in Toxicology, 24*(5), 763-774. https://doi.org/10.1021/tx200126r

Seyhan, M. F., Yılmaz, E., Timirci-Kahraman, Ö., Saygılı, N., Kısakesen, H. İ., Eronat, A. P., ... & Öztürk, O. (2017). Anatolian honey is not only sweet but can also protect from breast cancer: Elixir for women from artemis to present. *IUBMB life, 69*(9), 677-688. https://doi.org/10.1002/iub.1652

Seyhan, M. F., Yılmaz, E., Timirci-Kahraman, Ö., Saygılı, N., Kısakesen, H. İ., Gazioğlu, S., ... & Öztürk, O. (2019). Different propolis samples, phenolic content, and breast cancer cell lines: Variable cytotoxicity ranging from ineffective to potent. *IUBMB life, 71*(5), 619-631. https://doi.org/10.1002/iub.1996

Silici, S., & Kutluca, S. (2005). Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. *Journal of Ethnopharmacology, 99*(1), 69-73. https://doi.org/10.1016/j.jep.2005.01.046

Song, W., Yan, C. Y., Zhou, Q. Q., & Zhen, L. L. (2017). Galangin potentiates human breast cancer to apoptosis induced by TRAIL through activating AMPK. *Biomedicine & Pharmacotherapy, 89*, 845-856. https://doi.org/10.1016/j.biopha.2017.01.062

Tu, C., Ren, X., He, J., Zhang, C., Chen, R., Wang, W., & Li, Z. (2019). The value of LncRNA BCAR4 as a prognostic biomarker on clinical outcomes in human cancers. *Journal of Cancer, 10*(24), 5992. https://doi.org/10.7150/jca.35113

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. *Food Chemistry, 116*(4), 947-954.

Vorherr, H. (1986). Fibrocystic breast disease: Pathophysiology, pathomorphology, clinical picture, and management. *American Journal of Obstetrics and Gynecology 154*(1):161–179. https://doi.org/10.1016/0002-9378(86)90421-7.

Yadav, P., Sharma, A., Singh, L., & Gupta, R. (2020). Management of fibrocystic breast disease: A comprehensive review. *Journal of Advanced Scientific Research, 11*(04), 30-37.

Yang, B., Huang, J., Xiang, T., Yin, X., Luo, X., Huang, J., ... & Ren, G. (2014). Chrysin inhibits metastatic potential of human triple-negative breast cancer cells by modulating matrix metalloproteinase-10, epithelial to mesenchymal transition, and PI3K/Akt signaling pathway. *Journal of Applied Toxicology, 34*(1), 105-112. https://doi.org/10.1002/jat.2941

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Original Article

Investigating the antifungal, antioxidant, and antibacterial activities of *Ocimum basilicum* L. and *Mentha piperita* L. essential oils and their synergistic potentials with antibiotics

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ABSTRACT

Background and Aims: This research focuses on assessing the antioxidant, antifungal and antibacterial properties of *Mentha piperita* Lamiaceae and *Ocimum basilicum* Lamiaceae essential oils and their potential synergistic effects with various antibiotics.

Methods: The study identifies the chemical composition of *M. piperita* and *O. basilicum* essential oils by employing gas chromatography-mass spectrometry (GC-MS), cupric reducing antioxidant capacity (CUPRAC), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) methods to identify antioxidant activity. The study also uses the minimal inhibitory concentration (MIC) method for the antibacterial and antifungal activity tests.

Results: The main constituents of *M. piperita* are menthol (51.89%), L-menthone (17.81%), L-menthol (10.17%), and menthyl acetate 6.29%. The main constituents of *O. basilicum* are 65.51% estragole, 18.51% L-linalool, 2.69% bisabolene, and 2.66% trans-4-methoxycinnamaldehyde. With regard to the DPPH method, IC50 values of 0.028 and 0.019 were found for *M. piperita* and *O. basilicum*, respectively, based on the inhibition values. The results for the CUPRAC method indicate *O. basilicum* to show more antioxidant activity than *M. piperita*. According to the MICs, the essential oils are effective against bacteria at 1:4-1:16 dilutions, while the MIC values for the oil mixture (1:1) are significantly lower at a dilution of up to 1:2048. When combining the oils combined with the antibiotics (i.e., tobramycin and ceftazidime), they provide a synergistic activity against *Staphylococcus epidermidis, Escherichia coli*, and *Klebsiella pneumoniae*. The antifungal activity tests reveal no sufficient activity against the mold *Aspergillus niger*, while a limited effect was observed against the yeast *Candida albicans*.

Conclusion: The results show that the studied essential oils, especially their mixture at a 1:1 ratio, could be a good treatment option either alone or as a drug adjuvant due to their antibacterial and antioxidant properties.

Keywords: Antibacterial, antioxidant, essential oil, *Mentha piperita* L., *Ocimum basilicum* L., antibiotics

INTRODUCTION

The widespread use of antibiotics has triggered resistance in sensitive bacteria, leading to the ineffectiveness of antibiotics (Liu et al., 2017; Stanojevic et al., 2017). To overcome the increased antibiotic resistance in pathogenic bacterial strains, more effective antimicrobial agents must be developed. Plants are prevalent sources for new antibacterial agents, and many essential oils have been found to be effective against microorganisms (Jalal, El Atki, Lyoussi, & Abdellaoui, 2015; Marwa, Fikri-Benbrahim, Qu-Yahia, & Farah, 2017). *Mentha piperita* L. and *Ocimum basilicum* L., which belong to the Lamiaceae family, are among these essential oils and have antiviral (Saharkhiz et al., 2012), antibacterial (Liu et al., 2017, Stanojevic et al., 2017), antifungal (Al-Maskri et al., 2011; Tullio, Roana, Scalas, & Mandras et al., 2019), and antioxidant (Al-Maskri et al., 2011, Aşkın & Kaynarca, 2020; Kizil, Hasimi, Tolan, Kilinc, & Yüksel, 2010) properties. Sometimes, different combinations of essential oils are able to provide higher efficacy against bacteria, resulting in lower doses and reduced toxic side effects (Gutierrez, Barry-Ryan, & Bourke, 2008; Clemente, Aznar, Silva, & Nerín, 2016).

Fungal infections are also a prevailing problem, and their treatment has become difficult due to resistant strains (Limon, Skalski, & Underhill, 2017; Hay, 2006). Plants with high an-

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tifungal activity can reduce the resistance of these strains and can also be used as alternative treatment agents (Stanojevic et al., 2017). Nonetheless, antifungal activity has not been a commonly used method or research topic (Letessier, Svoboda, & Walters, 2001). Antioxidants are essential chemicals that stop reactive free radicals from initiating and escalating oxidative processes (Ismail, Marjan, & Foong, 2004). High phenolic plants can also be shown as powerful antioxidants (Akyuz, Şahin, Islamoglu, Kolayli, & Sandra, 2014).

Antibiotic resistance has become a worldwide public health concern due to the ongoing appearance of new bacterial strains that are resistant to antibiotics, reduced the effectiveness of antibiotics and necessitated the use of more costly therapies when infections become untreatable by initial antimicrobials (Langeveld, Veldhuizen, & Burt, 2014). One of the most effective strategies to fight antibiotic resistance is to combine antibiotics with natural substances such as essential oils. The objectives of this combination are to reduce microbial toxicity and antibiotic resistance while producing synergistic antibacterial activities (Ju et al., 2020). However, limited data are still found regarding the antibacterial activities of *M. piperita* and *O. basilicum* alone or as a mixture of them combined with antibiotics against multidrug-resistant bacteria.

This study first obtained the chemical constituents of *M. piperita* and *O. basilicum* essential oils using gas chromatography and established their antioxidant characteristics. Subsequently, the study identified these oils' antimicrobial properties against common infectious bacteria and fungi both on their own as well as mixed together. Investigating potential synergistic antibacterial interactions between the oils and certain antibiotics (such as ciprofloxacin, tobramycin, ceftazidime, and meropenem) is another goal of the study.

MATERIALS AND METHODS

Chemicals

Essential oils

M. piperita and *O. basilicum* essential oils were supplied from the commercial market. For the antioxidant activity assays, readily available essential oils of *M. piperita* and *O. basilicum* were diluted to 1:10 using Polysorbate 80. The other parts of the study used the essential oils undiluted.

Reagents

For the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, 1 mM:50 mL of DPPH radical solution was prepared according to Torres-Martínez et al. (2018). Due to the DPPH solution's light sensitivity, aluminum foil was used as a cover, and the solution was kept at +4°C in a dark environment. A fresh solution was prepared daily for the experiments. For the cupric ion reducing antioxidant capacity (CUPRAC) method, 10^{-2} M copper (II) chloride ($CuCl_2$), 7.5 x 10^{-3} M neocuprine (Nc), and 1 M ammonium acetate (NH_4Ac) solutions were prepared according to Apak, Güçlü, Özyürek, & Karademir, (2004). Due to the Nc solution's light sensitivity, it was also stored wrapped in aluminum foil. A 10^{-4} M gallic acid solution was also prepared.

Microbial Strains

The American Type Culture Collection (ATCC) standard strains of gram-positive bacteria *Staphylococcus aureus* (ATCC 29213) and *S. epidermidis* (ATCC 12228) and gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 4352), and *Proteus mirabilis* (ATCC 14153) were used for identifying the antibacterial activities. The yeast *Candida albicans* (ATCC 10231) and a spore suspension of the mold *Aspergillus niger* (ATCC 16404) were used in the antifungal tests.

Culture Media

Cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories) and Roswell Park Memorial Institute (RPMI) -1640 medium (Sigma) buffered to pH 7.0 were used for the antimicrobial activity tests for the bacteria and fungi, respectively.

Antimicrobial agents

The antibiotics tobramycin, ceftazidime, ciprofloxacin, and meropenem and the antifungal fluconazole were obtained from Sigma-Aldrich (St Louis, MO, USA). Excluding the meropenem, 1280 µg/mL stock solutions were prepared in accordance with the manufacturer's instructions and kept at –80ºC. The meropenem solutions were prepared daily.

Gas Chromatography

The characterization of the essential oils was accomplished by Thermo Scientific GC TRACE 1300 using an MS detector TSQ 8000 Evo and a Thermo Scientific Tr-5MS chromatographic column (length = 30 m, inner diameter (ID) = 0.25 mm, film thickness = 25 µm). The carrier gas the study used was helium (flow rate = 1.0 mL/min). The MS conditions were: ionization voltage = 70 eV; emission current = 40 mA; acquisition and scan range = 35–450 amu, and sampling rate = 1.0 scan/s. The inlet temperature = 250°C, and the oven temperature was programmed to remain at 80°C for four minutes, rise to 220°C over 30 min, and finally rise to 320°C over five minutes. The split ratio was 100:1, the injection volume was 1 µL, and the interface temperature was 320°C.

The composition of the selected essential oils was determined based on their retention time (RT) by comparing their mass spectral fragmentation patterns with the ones existing in the MS library (i.e., flavor2.hp, Wiley9, mainlib, replib, nist_ri). The GC peak area without correction factor was the basis for calculating the constituents' relative concentrations (%).

Antioxidant Activity

The antioxidant activities of the essential oils were assessed by employing the DPPH and CUPRAC methods.

DPPH Antioxidant Assay

The study adhered to Torres-Martínez et al.'s (2018) method. To check the absorbance values, five tubes were prepared for each essential oil sample. 0.6 mL of the DPPH radical solution was placed into each tube, and 0.02, 0.04, 0.06, 0.08, and 0.1 mL of essential oils were added to the respective tubes. These mixtures were then filled to 6 mL with methanol. The incubation time was 30 minutes in a dark environment at room temperature. For the blank solution, 5.4 mL of methanol was added to 0.6 mL of the DPPH radical solution and incubated for 30 minutes in a dark environment at room temperature. At the end of the 30 minutes, the optic densities of both the samples and the blank solution were taken at 517 nm.

For the DPPH experiment, the inhibition value should be calculated to understand the antioxidant values. Inhibition values of *M. piperita* and *O. basilicum* essential oils were calculated using Equation 1, where A_{DPPH} = DPPH absorbance value of the blank and $A_{extract}$ = the absorbance value of the sample.

$$\%\text{inhibition} = [(A_{DPPH} - A_{extract})/A_{DPPH}]x100 \quad (1)$$

The IC50 value is a concentration of the antioxidant substance that inhibits 50% of the DPPH radical in the environment. It is indirectly proportional to the antioxidant activity, which means that smaller values have higher antioxidant activity (Molyneux, 2004). For the IC50 values, the sample volumes that provide 50% inhibition of the radical are calculated from the acquired graph formulas (y=ax+b), where a is the slope and b is the y-intercept (Equation 2).

$$50\% = a \times (\text{sample volume}) + b \quad (2)$$

CUPRAC Antioxidant Assay

Based on Apak et al. (2004), the CUPRAC method was applied with respect to antioxidant activity. To check the absorbance values, five tubes were prepared for each essential oil sample. 1 mL of each of the prepared solutions was added to each tube, with a total of 3 mL of solution being obtained: 1 mL of Nc, 1 mL of $CuCl_2$, and 1 mL of NH_4Ac. Consequently, different volumes of the essential oils were added to 0.2, 0.4, 0.6, 0.8 and 1 mL tubes, respectively. These mixtures were then filled to 4.1 mL with distilled water. The incubation time was 30 minutes in a dark environment at room temperature. The same procedure was applied to the gallic acid to be used for the comparison. For the blank solution, 1 mL of each of the prepared solutions was added, and a total of 3 mL of solution was obtained: 1 mL of Nc, 1 mL of $CuCl_2$, and 1 mL of NH4Ac. The solution was filled to 4.1 mL by adding 1.1 mL of distilled water, then it was incubated in a dark environment at room temperature for 30 minutes. Following the incubation, the samples were taken, and the absorbance values of both the samples and the blank solution were examined at a wavelength of 450 nm in the UV-VIS spectrophotometer. The gallic solution was used to compare the results from the *M. piperita* and *O. basilicum* essential oils regarding the CUPRAC method.

Determining the antimicrobial activities

The antibacterial and antifungal activities of *M. piperita* and *O. basilicum* were tested alone as well as a 1:1 mixture of the oils using the microbroth dilution technique The minimum inhibitory concentration (MIC) values were then determined according to the Clinical and Laboratory Standards Institute (CLSI, 2006, 2000). Two-fold serial dilutions of the oils were prepared in CAMHB for the bacteria and the RPMI-1640 medium for the fungi in 96 U-shaped microtiter plates. Each well was inoculated with 50 µL of fresh broth cultures, which yielded 5×10^5 cfu/mL for the bacteria and 5×10^3 cfu/mL for fungi. The plates were covered with plastic bags to avoid drying and incubated for 18-24 hrs. at 37°C for the bacteria, for 48 hrs. at 35°C for the C. *albicans*, and for 48-72 hrs. at 25^{o}C for *A. niger*. The MIC values are the lowest concentrations of essential oils that inhibit the visible growth of microorganisms. The reference antibiotic and antifungals were ciprofloxacin and fluconazole, respectively.

Determining the combined effects of essential oils and antibiotics

To determine the antibacterial activities of oils in combination with the antibiotics, the antibiotics' MIC values were tested both alone and in combination with *M. piperita, O. basilicum*, and a 1:1 mixture of the two oils. Two-fold dilutions of the antibiotics between 64-0.062 µg/mL in CAMHB were prepared, and the oils were added to the corresponding wells of the plates to give a final concentration of 5% for the pure oils. To do this, the antibiotics tobramycin, ceftazidime, ciprofloxacin, and meropenem were used against the bacteria that are sensitive to the essential oils in accordance with their MIC values (CLSI, 2006; Andrews, 2001).

RESULTS

Gas chromatography results

Figure 1 presents the GC-MS chromatogram of *M. piperita* and *O. basilicum*, and Table 1 provides the compositions of the essential oils. The most represented compounds for *M. piperita* are monoterpenes, which constitute 86.61 % of this oil's total components. Based on the results, menthol can be stated as the most represented component (51.89%), followed by L-menthone (17.81%), L-menthol (19.17%), and

menthyl-acetate (6.29%). The most represented compounds for *O. basilicum* are phenylpropenes at 65.51% and monoterpenes at 18.51% of the total oil composition. For *O. basilicum*, estragole is the most represented component (65.51%), followed by L-linalool (18.51%), bisabolene (2.69%), and trans-4-methyxcinnamaldehyde (2.66%).

Antioxidant activity results

The DPPH and CUPRAC methods were employed to identify antioxidant activity. Inhibition values were calculated according to the acquired absorbance values (Table 2). According to the results obtained from the DPPH method, the highest and lowest inhibition values were determined as 54.38 and 9.48 for *M. piperita* and as 40.48 and 13.30 for *O. basilicum*. The CUPRAC method was employed to establish the effects of the concentration of antioxidant compounds in the diluted *O. basilicum* and *M. piperita* essential oils on inhibiting the DPPH radical and the effect from antioxidant compounds being absorbed in diluted oils and Gallic acid (Figures 2-3). The IC50 value was calculated as 0.019 for *O. basilicum*, and 0.028 for *M. piperita*. The results indicate *M. piperita* and *O. basilicum* to indeed show antioxidant activity. When comparing the two oils, *O. basilicum* shows higher antioxidant activity than the *M. piperita* essential oil.

Antimicrobial activity results

The *in vitro* antimicrobial activities of the studied essential oils against bacteria and fungi were evaluated using the CLSI criteria, with Table 3 summarizing the MIC values. For the standardization of the study, the MIC values of ciprofloxacin and fluconazole were also determined against bacteria and fungi, respectively, and the results were found to be within the quality control limits reported by the CLSI (2014). In the antifungal activity assays, neither *M. piperita* and *O. basilicum* individually nor their mixture showed any activity against the mold *A. niger*, while they showed limited activity against the yeast *C. albicans* (Table 3). Hence, no significant antifungal activity was detected from the essential oils.

When identifying their antibacterial activity, while the *O. basilicum* essential oil showed higher activity against *E. coli* and *K. pneumonia* at respective dilutions of 1:32 and 1:16, the *M. piperita* essential oil was more effective against *S. epidermidis*, *E. coli*, and *K. pneumonia* at the respective dilutions of 1:8, 1:8, and 1:16. Similarly, the mixture of essential oils (1:1) was also effective, especially against the same bacteria; interestingly, this mixture increased the activities up to a dilution of 1:2048.

Results from the combination of essential oils and antibiotics

Table 4 summarizes the MIC values of antibiotics combined with the essential oils individually and as an oil mixture against the more sensitive bacteria *S. epidermidis, E. coli*, and *K. pneumoniae*. According to these results, the studied essential oils have synergistic effects against *S. epidermidis, E. coli*, and *K. pneumoniae* when combined with ceftazidime, while the tobramycin or ciprofloxacin combinations showed synergistic activity against E. coli and *K. pneumoniae*. These results indicate that combining essential oils with the antibiotics increase their inhibitory effects against the selected bacteria.

DISCUSSION

This study has investigated the antioxidant, antifungal and antibacterial activities of *M. piperita* and *O. basilicum* essential oils individually and as a 1:1 mixture. Furthermore, their combinations with selected antibiotics were also examined not only to advance the understanding of essential oil properties but to also highlight potential synergies with antibiotics for developing more effective strategies.

In line with prior research, the analysis results of the selected essential oils obtained through the DPPH and CUPRAC methods have identified antioxidant activity. The fact that the DPPH method lacks a standard value made comparing the antioxidant activities difficult (Deng, Cheng, & Yang, 2011). Likewise, due to not having a fixed value, the found values cannot be verified with a standard analysis. The DPPH radical is sensitive to light, oxygen in air, and pH. For this reason, various results are obtained from each iteration. Thus, the results cannot be compared with those from distinct studies (Sharma & Bhat, 2009). However, based on the absorbance and inhibition values found in the current and previous studies (Aşkın & Kaynarca, 2020; Kizil et al., 2010), both *M. piperita* and *O. basilicum* essential oils are concluded to have antioxidant activity, and this activity is expected to increase as the oil content increases. This study prepared samples at different concentrations and compared the results with respect to these concentrations. The inhibition values (Table 2) and the increase in IC50 values display the antioxidant activity of the studied oils. When comparing the IC50 values for the two oils, the antioxidant activity of the *O. basilicum* essential oil was higher than that of *M. piperita*, based on the IC50 value for the *O. basilicum* essential oil being lower. When considering the lack of a standard result using the DPPH method, the decision was made to observe antioxidant activity using the CUPRAC method. Total antioxidant capacity (TAC) was calculated using the measured absorbance values of the oils at different concentrations (Figure 3). Accordingly, antioxidant activity was observed for both essential oils using the DPPH and CUPRAC methods.

Previous studies have identified the *M. piperita* and *O.*

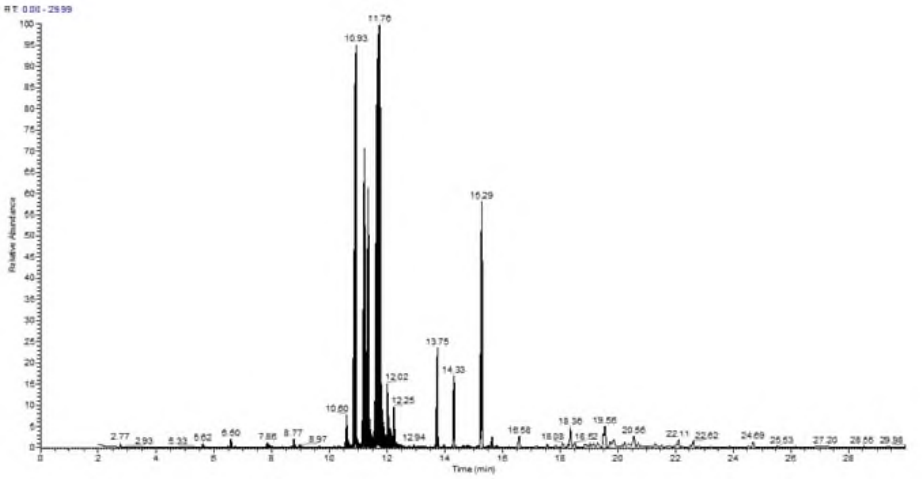

Figure 1a. GC-MS chromatogram of *M. piperita* L.

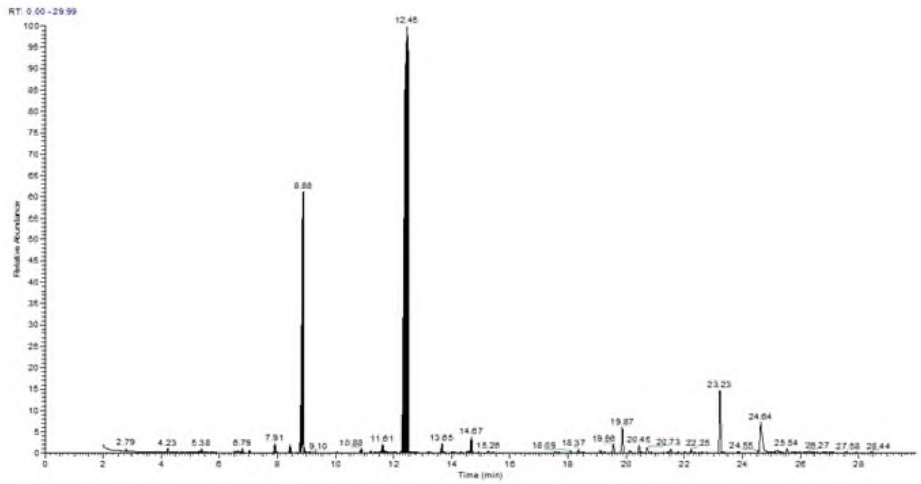

Figure 1b. GC-MS chromatogram of *O. basilicum* L.

basilicum essential oils to show antibacterial (Liu et al., 2017; Stanojevic et al., 2017) and antifungal (Al-Maskri et al., 2011; Tullio et al., 2019) activities against several microorganisms. When considering the antifungal activity assays, while limited activity occurred against *C. albicans*, no effect was found on *A. niger*. Although the antibacterial activity results are similar to those from previous studies, several reasons may exist for the lack of antifungal activity compared to other studies (Stanojevic et al., 2017; Al-Maskri et al., 2011). For example, the differences in antifungal activities might occur as a result of the diversities of fungi and/or due to the filamentous structure of *A. niger*, with essential oils perhaps being unable to interact with the cell components. The quality, purity, production method, and trademark of the studied oils might also be other possible reasons for the lack of antifungal activity. On the other hand, this study's antibacterial activity results support other researchers' findings. While the *M. piperita* and *O. basilicum* essential oils individually show moderate antibacterial activity against *S. epidermidis*, *E. coli*, and *K. pneumoniae*, they had quite a valuable effect when combined together. Therefore, combining these two oils is considered to have a possible synergistic effect on the studied bacteria, with the mixture of *M. piperita* and *O. basilicum* essential oils perhaps being an alternative treatment against antibiotic resistant infectious agents.

The antimicrobial activities of the essential oils may have several mechanisms, and these mechanisms may affect various biochemical and structural functions such as cytoplasm,

Table 1. *The compositions of the essential oils identified by GC-MS analysis*

Mentha piperita			*Ocimum basilicum*		
Compound Name	**RT (min)**	**% Area**	**Compound Name**	**RT (min)**	**% Area**
2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	5.07	0.00	3-Pyrrolidinecarboxylic acid, 5-oxo-1-(2-pyridinylmethyl)-	5.11	0.00
(E)-4-cyanopent-3-en-1-ol	5.33	0.01	2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	5.38	0.05
Acetyl bromide (CAS)	5.62	0.01	(E)-4-cyanopent-3-en-1-ol	5.57	0.00
2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	6.60	0.03	Acetyl bromide (CAS)	5.85	0.00
dl-Limonene	6.60	0.23	Benzenamine (CAS)	6.79	0.05
2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	6.99	0.00	3-Pyrrolidinecarboxylic acid, 5-oxo-1-(2-pyridinylmethyl)-	7.03	0.02
2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	7.46	0.00	Linalool Oxide (2)	7.91	0.38
1-Octanol (CAS)	7.86	0.27	2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	7.91	0.06
2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	7.87	0.03	Ethanone, 1-(methylenecyclopropyl)-	7.91	0.02
L-Linalool	8.77	0.39	Ethanone, 1-(methylenecyclopropyl)-	8.43	0.02
Propanoic acid, 2-(phenylmethoxy)-	10.60	0.03	Trans-Linalool Oxide	8.43	0.34
Propanoic acid, 2-(phenylmethoxy)-	10.93	0.11	2-[(Phenylamino)carnonyl)cyclohexanecarboxylic acid	8.43	0.06
L-Menthone	10.93	17.81	1H-Pyrrole, 2-methyl-	8.65	0.01
Propanoic acid, 2-(phenylmethoxy)-	11.22	0.05	Hex-2-yn-4-one, 2-methyl-	8.88	0.86
Ethanone, 1-(methylenecyclopropyl)-	11.35	3.23	L-Linalool	8.88	18.52
Propanoic acid, 2-(phenylmethoxy)-	11.75	0.27	Diaminomaleonitrile	8.89	0.66
Menthol	11.76	51.89	Ethanone, 1-(methylenecyclopropyl)-	10.88	0.01
L-menthol	11.76	10.18	Cyclopentene, 1-(1-methylethyl)-	10.88	0.01
Bromoacetic acid, 2-tetrahydrofurylmethyl ester	12.52	0.01	Ethanone, 1-(methylenecyclopropyl)-	11.58	0.02
Bromonitromethane	13.74	0.09	L-(-)-Menthol	11.61	0.81
Pulegone	13.75	2.20	1-Heptyn-6-one	11.61	0.15
Phosphorocyanidous difluoride	14.32	0.20	Hex-2-yn-4-one, 2-methyl-	12.36	0.04
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)- (CAS)	14.33	1.61	Estragole	12.45	65.51
Phosphorocyanidous difluoride	15.28	1.36	Propanoic acid, 2-(phenylmethoxy)-, methyl ester	12.50	3.80
Menthyl acetate	15.29	6.30	N-Formyldithiocarbamic acid	12.52	0.41
Cyclohexanol, 3-methyl-2-(1-methylethyl)-, acetate, (1α,2α,3α)-	15.64	0.28	Bromonitromethane	13.65	0.03
Phosphorocyanidous difluoride	15.64	0.05	Z-Citral	13.65	0.35
Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	16.58	0.25	Bromine azide	14.67	0.02
β-Bourbonene	18.36	0.66	(E,E)-3,7-Dimethyl-1-(methoxymethoxy)-1,6-octadien-3-ol	14.67	0.64
beta-Caryophyllene	19.56	0.77	Phosphorocyanidous difluoride	15.26	0.01
Germacrene D	19.86	0.43	trans-Caryophyllene	19.56	0.36
γ-Cadinene (CAS)	20.56	0.72	Trans-A-Bergamotene	19.87	1.11
β-Cubebene	22.11	0.23	trans-β-Farnesene	20.45	0.33
β-cadinene	22.62	0.14	Bisabolene	23.23	2.70
(-)-Caryophyllene oxide	24.69	0.16	trans-4-Methoxycinnamaldehyde	24.64	2.66

Table 2. *Inhibition values of the essential oils in different amounts (DPPH).*

Volume	**Inhibition %**	
	Mentha piperita L.	***Ocimum basilicum L.***
0.02mL	9.475	13.301
0.04mL	17.669	15.502
0.06mL	23.431	18.469
0.08mL	41.485	25.837
0.1mL	54.383	40.478

enzyme system, and protein structure. These oils are able to change the permeability of membrane proteins as well as their functions and can also adhere to the bacterial cell wall and interact with the proteins, disrupting their regular functions (Johnson-Henry, Hagen, Gordonpour, Tompkins, & Sherman, 2007). These effects are also thought to be higher against Gram-positive bacteria compared to Gram-negative bacteria (Nazzaro, Fratianni, Martino, Coppola, & De Feo, 2013). This may be a result of their very different cell wall structures. The two-layer cell wall structure of the Gram-negative bacteria possible does not easily permit the penetration of drugs, antibiotics, phenolic compounds (such as thymol, carvacrol, and eugenol), and essential oils; meanwhile, the Gram-positive bacteria has an uncomplicated cell wall (Trombetta et al., 2005). Slightly dif-

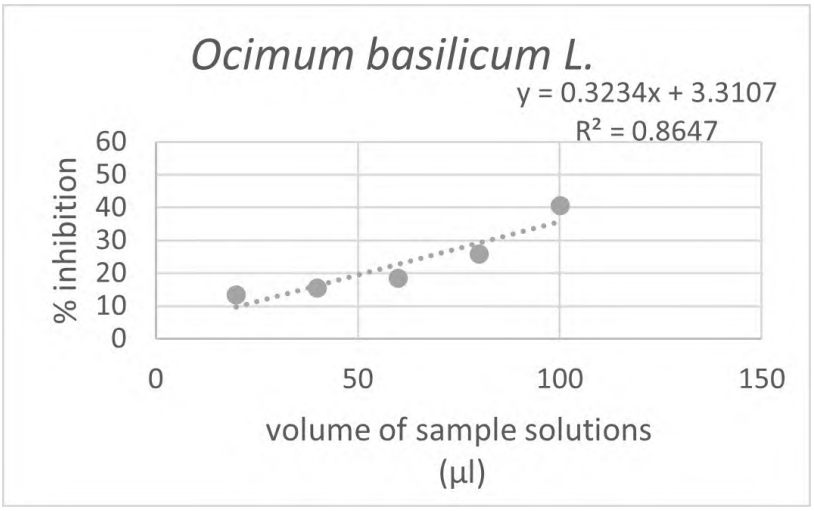

Figure 2a. The effect of the concentration of antioxidant compounds in diluted *Ocimum basilicum* L. essential oils on the inhibition of DPPH radical.

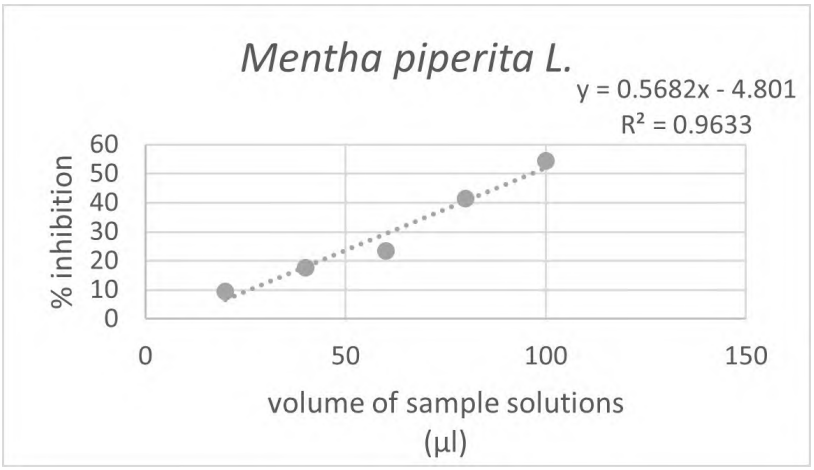

Figure 2b. The effect of the concentration of antioxidant compounds in diluted *Mentha piperita* L. essential oils on the inhibition of DPPH radical.

Table 3. *MIC values (dilution ratios) of the studied essential oils against various microorganisms.*

Samples	Microorganisms							
	Bacteria						Fungi	
	S.a	S.e	P.a	E.c	K.p	P.m	C.a	A.n
Ocimum basilicum L.	1:4	1:4	1:4	1:32	1:16	1:4	1:2	-
Mentha piperita L.	1:4	1:8	1:4	1:8	1:16	1:4	1:2	-
Mixture of them (1:1)	1:2	1:1024	1:2	1:256	1:2048	1:8	1:4	-

*MICs are given for the dilutions of the pure essential oils, **S.a = S.aureus; S.e = S.epidermidis; P.m = P. mirabilis; E.c = E.coli; K.p = K.pneumoniae; P.a = P.aeruginosa; C.a = C.albicans; A.n = A. niger

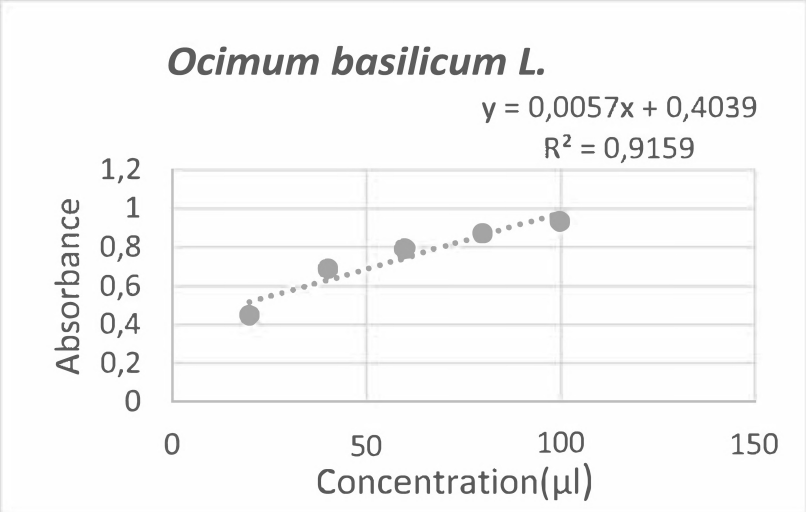

Figure 3a. The effect of substances on absorbance at different concentrations for *O. basilicum* L. (CUPRAC method).

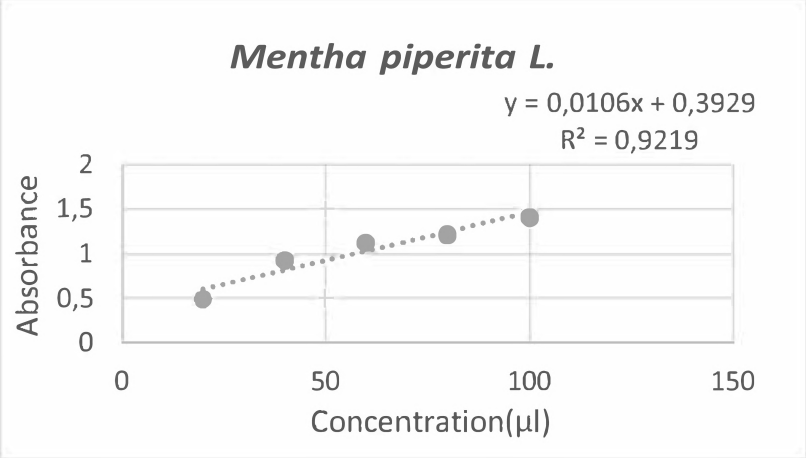

Figure 3b. The effect of substances on absorbance at different concentrations for *M. piperita* L. (CUPRAC method).

ferent from most other studies, the *M. piperita* and *O. basilicum* essential oils in this study affected both Gram-positive and Gram-negative bacteria. In addition, when comparing the antibacterial activities of *M. piperita* and *O. basilicum* essential oils with each other, *O. basilicum* was observed to show higher activity than *M. piperita*.

The antibacterial and antioxidant activities of essential oils generally come from their active terpene molecules (Poonkodi, 2016; Ouakouak, Chohra, & Denane, 2015), such as thyme, eugenol, and linalool, which are constituents of *M. piperita* and *O. basilicum* (Cox-Georgian, Ramadoss, Dona, & Basu, 2019). On the other hand, menthol as a phenolic monoterpene also shows antimicrobial affects (Saharkhiz et al., 2012), and the antibacterial activities of *M. piperita* may be due to its menthol composition (İşcan, Kirimer, Kürkcüoğlu, Başer, & Demirci, 2002). Aside from these, the linalool component was also shown to have antioxidant, antibacterial, and antifungal effects (Hussain, Anwar, Sherazi, & Przybylski, 2008); therefore, the activities of *O. basilicum* may be due to linalool. When determining the chemical compositions of the essential oils using gas chromatography, this study found monoterpenes to make up 86.61% of the total components in the *M. piperita* essential oil. However, phenylpropanes make up 65.51% and monoterpenes 18.51% of *O. basilicum*'s overall oil composition. These results support the fact that these essential oils have antioxidant and antimicrobial properties.

Previous studies have identified combining antibiotics and essential oils to have potential synergistic effects (Fadli et al., 2012; Rosato, Vitali, Laurentis, Armenise, & Milillo, 2007) against some resistant bacteria. For instance, a study done with cinnamon essential oil concluded that essential oils could be used as an alternative therapeutic application (El Atki et al.,

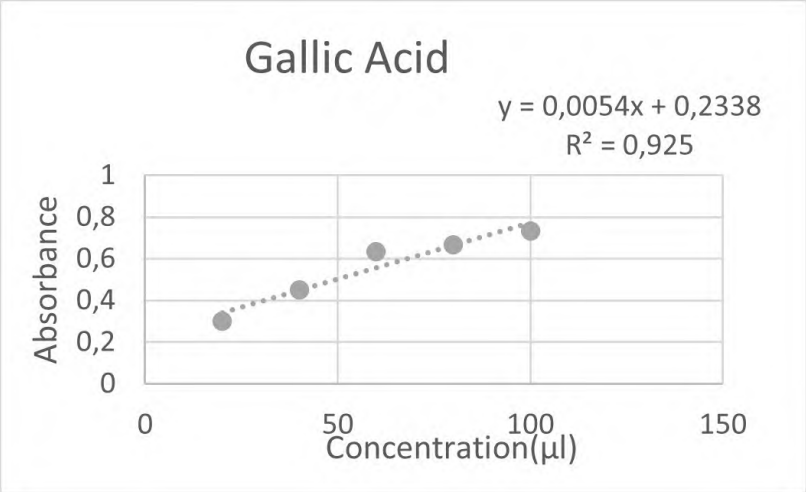

Figure 3c. The effect of substances on absorbance at different concentrations for Gallic acid (CUPRAC method)

Table 4. *MIC values (µg/mL) of antibiotics with essential oils against various microorganisms*

Samples	Microorganisms		
	S. epidermidis	***E. coli***	***K. pneumoniae***
Ciprofloxacin	0.062	0.25	0.25
Ciprofloxacin + *Ocimum basilicum L.*	≤ 0.062	**0.062**	**0.062**
Ciprofloxacin + *Mentha piperita L.*	≤ 0.062	**0.062**	**0.062**
Ciprofloxacin + Oil Mixture	≤ 0.062	**0.062**	**0.062**
Tobramycin	0.125	0.5	0.25
Tobramycin + *Ocimum basilicum L.*	**≤ 0.062**	**0.062**	**0.062**
Tobramycin + *Mentha piperita L.*	**≤ 0.062**	**0.062**	**0.062**
Tobramycin + Oil Mixture	**≤ 0.062**	**0.062**	**0.062**
Ceftazidime	0.5	0.25	1
Ceftazidime + *Ocimum basilicum L.*	**0.062**	**0.062**	**0.062**
Ceftazidime + *Mentha piperita L.*	**0.062**	**0.062**	**0.062**
Ceftazidime + Oil Mixture	**0.062**	**0.062**	**0.062**
Meropenem	0.125	0.125	0.062
Meropenem + *Ocimum basilicum L.*	**≤ 0.062**	**≤ 0.062**	≤ 0.062
Meropenem + *Mentha piperita L.*	**≤ 0.062**	**≤ 0.062**	≤ 0.062
Meropenem + Oil Mixture	**≤ 0.062**	**≤ 0.062**	≤ 0.062

2019), and another study established the *M. piperita* essential oil as having a synergistic effect with certain antibiotics (Talei, Mohammadi, Bahmani, & Kopaei, 2017). Even though ciprofloxacin combined with the oils did not significantly inhibit *S. epidermidis* in this study, they did lower the MIC values and increase the antibacterial activities of other antibiotics,

with ceftazidime in particular having a higher inhibitory effect against *S. epidermidis*. While the MIC value of ceftazidime against *S. epidermidis* was 0.5 µg/mL, it was 0.062 µg/mL when combined with the oils. Similarly, the *M. piperita* and *O. basilicum* essential oils increased the inhibitory effects of all studied antibiotics against *E. coli*, with the oils showing more effective results when used with tobramycin in particular, increasing the inhibitory effect of antibiotics by lowering the MIC values from 0.5 to 0.062 µg/mL. Against *K. pneumoniae*, however, the antibiotic-essential oil combinations were unable to change the meropenem MICs but significantly increased the ceftazidime antibiotic activity. On its own, ceftazidime's MIC was 1 µg/mL against K. pneumonia, but this decreased to 0.062 µg/mL when combined with the essential oils.

Even though the MIC determination is still the gold standard for testing the antimicrobial activities of compounds, some molecules have enhancer activities, and synergistic interactions with some antibiotics. While the microbroth checkerboard method has high throughput and is the basic technique for determining antimicrobial combinations, determining MIC alongside the presence of a fixed concentration enhancer, similar to this study, can also be preferred as a fast and simpler preliminary screening test. If a MIC value decreases four-fold through the combination, that combination can be said to create a synergistic effect (Rand, Houck, Brown, & Bennett, 1993). Therefore, the *M. piperita* and *O. basilicum* essential oils can be said to have synergistic interaction with antibiotics against the studied bacteria.

To the best of this study's knowledge, no such study has been done before with the mixture of the two studied essential oils. While these oils did not have significant effects on all the bacteria studied herein, they did show a synergistic effect with several of the antibiotics. For example, the mixture of ciprofloxacin and the 1:1 oil mixture created a synergistic effect, reducing the MIC values of the antibiotics against *E. coli* and *K. pneumoniae*; however, no clear reduction was observed for *S. epidermidis*. Similarly, the synergistic effect of meropenem and the combined oils increased the inhibitory effect of the antibiotic against *S. epidermidis* and *E. coli*; however, the expected decrease against *K. pneumoniae* MIC values was unobservable. Also, when tobramycin or ceftazidime are combined with *M. piperita* or O. basilicum, each combination can provide much more effective antibacterial activity. According to these results, the mixture of *M. piperita* and *O. basilicum* essential oils, whether alone or as a 1:1 combination with antibiotics, gives promising, natural, and environmentally friendly alternative antibacterial and antioxidant treatment strategies for clinics and the pharmaceutical industry.

CONCLUSION

This present study has determined the *M. piperita* and *O. basilicum* essential oils to exhibit antioxidant activities. These oils also have antibacterial effects, with the mixture of these two oils increasing this effect. Meanwhile, the oils showed no significant antifungal activity. Furthermore, the combination of these two essential oils with certain antibiotics also showed synergistic effects against specific bacteria. In conclusion, although experiments need to be conducted on more types and greater numbers of microorganisms, the *M. piperita* and *O. basilicum* essential oils, especially at a 1:1 mixture, could provide a good treatment option individually or as a drug adjuvant with their antibacterial and antioxidant activities.

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REFERENCES

Akyuz, E., Şahin, H., Islamoglu, F., Kolayli, S., & Sandra, P. (2014). Evaluation of phenolic compounds in Tilia rubra subsp. caucasica by HPLC-UV and HPLC-UV-MS/MS. *International journal of food properties, 17*(2), 331-343.

Al-Maskri, A. Y., Hanif, M. A., Al-Maskari, M. Y., Abraham, A. S., Al-sabahi, J. N., & Al-Mantheri, O. (2011). Essential oil from Ocimum basilicum (Omani Basil): a desert crop. *Natural product communications, 6*(10), 1934578X1100601020.

Andrews J. M. (2001). Determination of minimum inhibitory concentrations. *The Journal of antimicrobial chemotherapy, 48*(48-71).

Apak, R., Güçlü, K., Özyürek, M., & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of agricultural and food chemistry, 52*(26), 7970-7981.

Aşkın, B., & Kaynarca, G. B. (2020). Determination of Antioxidant Properties and Composition of Rosemary and Thyme Essential Oils. *Turkish Journal of Agriculture-Food Science and Technology, 8*(10), 2105-2112.

Clemente, I., Aznar, M., Silva, F., & Nerín, C. (2016). Antimicrobial properties and mode of action of mustard and cinnamon essential oils and their combination against foodborne bacteria. *Innovative Food Science & Emerging Technologies, 36*, 26-33.

Clinical and Laboratory Standards Institute (CLSI). (2014). Performance standards for antimicrobial susceptibility testing; 2th informational supplement. M100-S24. Wayne, PA: CLSI.

Clinical and Laboratory Standards Institute (CLSI). (2000). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard M27-A NCCLS, Wayne, PA; CLSI..

Clinical and Laboratory Standards Institute (CLSI). (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard M7-A5. Wayne, PA: CLSI.

Cox-Georgian, D., Ramadoss, N., Dona, C., & Basu, C. (2019). Therapeutic and medicinal uses of terpenes. In *Medicinal Plants* (pp. 333-359). Springer, Cham.

Deng, J., Cheng, W., & Yang, G. (2011). A novel antioxidant activity index (AAU) for natural products using the DPPH assay. *Food Chemistry, 125*(4), 1430-1435.

El Atki, Y., Aouam, I., El Kamari, F., Taroq, A., Nayme, K., Timinouni, M., ... & Abdellaoui, A. (2019). Antibacterial activity of cinnamon essential oils and their synergistic potential with antibiotics. *Journal of Advanced Pharmaceutical Technology & Research, 10*(2), 63.

Fadli, M., Saad, A., Sayadi, S., Chevalier, J., Mezrioui, N. E., Pagès, J. M., & Hassani, L. (2012). Antibacterial activity of Thymus maroccanus and Thymus broussonetii essential oils against nosocomial infection–bacteria and their synergistic potential with antibiotics. *Phytomedicine, 19*(5), 464-471.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology, 124*(1), 91-97.

Hay, R. J. (2006). Fungal infections. *Clinics in Dermatology, 24*(3), 201-212.

Hussain, A. I., Anwar, F., Sherazi, S. T. H., & Przybylski, R. (2008). Chemical composition, antioxidant and antimicrobial activities of basil (Ocimum basilicum) essential oils depends on seasonal variations. *Food Chemistry, 108*(3), 986-995.

Ismail, A., Marjan, Z. M., & Foong, C. W. (2004). Total antioxidant activity and phenolic content in selected vegetables. *Food chemistry, 87*(4), 581-586.

İşcan, G., Kirimer, N., Kürkcüoğlu, M., Başer, H. C., & Demirci, F. (2002). Antimicrobial screening of Mentha piperita essential oils. *Journal of Agricultural and Food Chemistry, 50*(14), 3943-3946.

Jalal, Z., El Atki, Y., Lyoussi, B., & Abdellaoui, A. (2015). Phytochemistry of the essential oil of Melissa officinalis L. growing wild in Morocco: Preventive approach against nosocomial infections. *Asian Pacific Journal of Tropical Biomedicine, 5*(6), 458-461.

Johnson-Henry, K. C., Hagen, K. E., Gordonpour, M., Tompkins, T. A., & Sherman, P. M. (2007). Surface-layer protein extracts from Lactobacillus helveticus inhibit enterohaemorrhagic Escherichia coli O157: H7 adhesion to epithelial cells. *Cellular Microbiology, 9*(2), 356-367.

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Qian, H., & Yao, W. (2022) Synergistic interactions of plant essential oils with antimicrobial agents: a new antimicrobial therapy, *Critical Reviews in Food Science and Nutrition*, 62:7, 1740-1751.

Kizil, S., Hasimi, N., Tolan, V., Kilinc, E., & Yuksel, U. (2010). Mineral content, essential oil components and biological activity of two mentha species (M. piperita L., M. spicata L.). *Turkish Journal of Field Crops, 15*(2), 148-153.

Langeveld, W.T., Veldhuizen E.J.A., & Burt S.A. (2014). Synergy between essential oil components and antibiotics: a review, *Critical Reviews in Microbiology, 40*:1, 76-94.

Letessier, M. P., Svoboda, K. P., & Walters, D. R. (2001). Antifungal activity of the essential oil of hyssop (Hyssopus officinalis). *Journal of Phytopathology, 149*(11-12), 673-678.

Limon, J. J., Skalski, J. H., & Underhill, D. M. (2017). Commensal fungi in health and disease. *Cell host & microbe, 22*(2), 156-165.

Liu, Q., Meng, X., Li, Y., Zhao, C. N., Tang, G. Y., & Li, H. B. (2017). Antibacterial and antifungal activities of spices. *International Journal of Molecular Sciences, 18*(6), 1283.

Marwa, C., Fikri-Benbrahim, K., Ou-Yahia, D., & Farah, A. (2017). African peppermint (Mentha piperita) from Morocco: Chemical composition and antimicrobial properties of essential oil. *Journal of Advanced Pharmaceutical Technology & Research 8*(3), 86.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity.*Songklanakarin Journal of Science and Technology 26*(2), 211-219.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., & De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals, 6*(12), 1451-1474.

Ouakouak, H., Chohra, M., & Denane, M. (2015). Chemical composition, antioxidant activities of the essential oil of Mentha pulegium L, South East of Algeria. *International Letters of Natural Sciences, 39*.

Poonkodi, K. A. T. H. I. R. V. E. L. (2016). Chemical composition of essential oil of Ocimum basilicum L.(Basil) and its biological activities-an overview. *Journal of Critical Reviews, 3*(3), 56-62.

Rand, K. H., Houck, H. J., Brown, P., & Bennett, D. (1993). Reproducibility of the microdilution checkerboard method for antibiotic synergy. *Antimicrobial Agents and Chemotherapy, 37*(3), 613-615.

Rosato, A., Vitali, C., De Laurentis, N., Armenise, D., & Milillo, M. A. (2007). Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. *Phytomedicine, 14*(11), 727-732.

Saharkhiz, M. J., Motamedi, M., Zomorodian, K., Pakshir, K., Miri, R., & Hemyari, K. (2012). Chemical composition, antifungal and antibiofilm activities of the essential oil of Mentha piperita L. International *Scholarly Research Notices, 2012.*

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*(4), 1202-1205.

Stanojevic, L. P., Marjanovic-Balaban, Z. R., Kalaba, V. D., Stanojevic, J. S., Cvetkovic, D. J., & Cakic, M. D. (2017). Chemical composition, antioxidant and antimicrobial activity of basil (Ocimum basilicum L.) essential oil. *Journal of Essential Oil Bearing Plants, 20*(6), 1557-1569.

Talei, G. R., Mohammadi, M., Bahmani, M., & Kopaei, M. R. (2017). Synergistic effect of Carum copticum and Mentha piperita essential oils with ciprofloxacin, vancomycin, and gentamicin on Gram-negative and Gram-positive bacteria. *International Journal of Pharmaceutical Investigation, 7*(2), 82.

Torres-Martínez, R., García-Rodríguez, Y. M., Ríos-Chávez, P., Saavedra-Molina, A., López-Meza, J. E., Ochoa-Zarzosa, A., & Garciglia, R. S. (2017). Antioxidant activity of the essential oil and its major terpenes of Satureja macrostema (Moc. and Sessé ex Benth.) Briq. *Pharmacognosy Magazine, 13*(Suppl 4), S875.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., ... & Bisignano, G. (2005). Mechanisms of an-

tibacterial action of three monoterpenes.*Antimicrobial Agents and Chemotherapy, 49*(6), 2474-2478.

Tullio, V., Roana, J., Scalas, D., & Mandras, N. (2019). Evaluation of the antifungal activity of Mentha x piperita (Lamiaceae) of Pancalieri (Turin, Italy) essential oil and its synergistic interaction with azoles. *Molecules, 24*(17), 3148.

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Original Article

Antimicrobial activities of *Scorzonera ketzkhowelii Sosn.* ex Grossh. (Asteraceae) and determination of natural compounds by LC-HRMS analysis

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ABSTRACT

Background and Aims: *Scorzonera ketzkhowelii* Sosn. ex Grossh. (Asteraceae) was reported as a new species in the Flora of Turkey in 2010. However, there have been no prior biological or chemical investigations conducted.

Methods: This study aims to investigate the antimicrobial activities of the petroleum ether, dichloromethane, ethyl acetate, and n-butanol fractions derived from *S. ketzkhowelii*, and determination of secondary metabolites by Liquid Chromatography with High Resolution Mass Spectrometry (LC-HRMS) analysis on the most active antimicrobial fractions.

Results: Notably, all of the fractions demonstrated antimicrobial activity against selected microorganisms, with the dichloromethane fraction of the aerial part exhibiting a higher inhibition of microbial growth compared to the other extracts. The findings of our study revealed the presence of several phenolic compounds in the dichloromethane fractions from both the aerial and subaerial parts, including 3,4-dihydroxybenzaldehyde, salicylic acid, dihydrocaffeic acid, caffeic acid, caffeic acid phenethyl ester, chlorogenic acid, quercetin, hyperoside, naringenin, apigenin, hispidulin, hispidulin 7-glucoside, chrycin, emodin, and carnosic acid. Furthermore, dichloromethane fraction of the aerial parts contained additional phenolic compounds such as homogentisic acid, verbascoside, (+)-trans taxifolin, apigenin 7-glucoside, luteolin, orientin, and chrysoeriol.

Conclusion: The plant's demonstrated antimicrobial attributes and the diverse range of phenolic compounds it contains present it as a promising subject for continued research and potential applications within the realms of medicine and pharmaceuticals. Further exploration of its bioactivity and potential health benefits may reveal novel avenues for its practical utilization.

Keywords: *Scorzonera ketzkhowelii*, Asteraceae, antimicrobial activity, LC-HRMS, phenolic compounds

INTRODUCTION

The genus *Scorzonera* L. is a member of the Asteraceae family and encompasses around 160 species worldwide. Within the Flora of Turkey, this genus is characterized by the presence of 52 species, totalling 59 taxa (Coşkunçelebi, Makbul, Gültepe, Okur, & Güzel, 2015). Among the people of Turkey, plants from the *Scorzonera* genus are commonly referred to as 'Teke sakalı' but are also known by various other names such as Kök sakızı, Dağ sakızı, Angıt otu, Nerebent, Tekercik, Çetotu, Parım, Purrık, and İskorçina (Makbul, 2012).

Scorzonera is primarily consumed as a food source, with chewing gum derived from the latex of its roots. Moreover, it has been utilized in traditional medicine for an extensive period for the purpose of treating cardiovascular diseases, renal disorders, stomach pain, infertility, and serving as an analgesic, wound healer, galactagog, and anthelmintic (Altundağ & Öztürk, 2011; Göç & Mat, 2019; Nadiroğlu, Behçet, & Çakılcıoğlu, 2019; Tuzlacı, 2016). Extensive research has been conducted to explore its activity and chemical composition. Numerous secondary metabolites, including triterpenes, sterols, sesquiterpenes, sesquiterpene lactones, lignans, neolignans, phenolic acids, flavonoids, coumarins, dihydroisocoumarins, and stilbene derivatives, have been identified, and their struc-

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tures have been elucidated. These plants have demonstrated a diverse array of bioactivities, encompassing anti-Alzheimer, anti-inflammatory, antinociceptive, antioxidant, antidiabetic, antibacterial, antifungal, anti-leishmaniasis, anti-tyrosinase, anti-HIV, hepatoprotective, and wound healing properties (Bahadır Acıkara et al., 2012; Granica & Zidorn, 2015; Şahin, Boğa, & Sarı, 2022; Aynur Sarı, 2012; Zidorn, Ellmerer-Müller, & Stuppner, 2000).

Scorzonera ketzkhowelii was documented as a novel species in the Flora of Turkey in the year 2010. (Hamzaoğlu, Aksoy, Martin, Pınar, & Çölgeçen, 2010). However, there have been no prior biological or chemical investigations conducted on S. *ketzkhowelii*.

As antibiotic resistance continues to rise, there is an escalating demand for the exploration of novel antimicrobial agents. Given the promising potential of plants in this context, it is imperative to investigate their antimicrobial properties. This study seeks to evaluate the antimicrobial activities of petroleum ether, dichloromethane, ethyl acetate, and n-butanol fractions extracted from both the aerial and subaerial parts of *S. ketzkhowelii*. Additionally, the objective is to elucidate the chemical composition of the most potent fractions exhibiting antimicrobial activity through analysis using Liquid Chromatography with High Resolution Mass Spectrometry (LC-HRMS).

MATERIAL AND METHODS

Plant Material and Extraction

Specimens of *Scorzonera ketzkhovelii* were gathered from Yusufeli/Artvin, Turkey, situated at an elevation of 2122 m, during July 2019. A voucher specimen has been archived under the reference number ISTE 115803 at Istanbul University.

The aerial and subaerial components of *S. ketzkhowelii* were subjected to air-drying while safeguarding them from direct sunlight and 100 grams of each part were subjected to ethanol extraction via the percolation technique. The ethanol extract obtained was concentrated at 45°C through the use of a rotary evaporator. The resultant dried ethanol extracts (aerial part 10,95 g; subaerial part 11,76 g) were then reconstituted in an ethanol/water mixture (1:2) and successively subjected to extraction with petroleum ether, dichloromethane, ethyl acetate, and n-butanol solvents. All fractions underwent investigation for antimicrobial activity, with dichloromethane extracts utilized for LC-HRMS analysis.

Antimicrobial Activity

The Minimum Inhibitory Concentration (MIC) values of the fractions were validated against reference strains using the microdilution method in accordance with the criteria established by the Clinical Laboratory Standards Institute (CLSI). (CLSI, 2000, 2006).

Three Gram-positive standard test bacteria: (*Enterococcus feacalis* ATCC 29212), (*Staphylococcus epidermidis* ATCC 12228), (*Staphylococcus aureus* ATCC 29213); four Gram-negative standard test bacteria: (*Escherichia coli* ATCC 25922), (*Klebsiella pneumoniae* ATCC 4352), (*Proteus mirabilis* ATCC 14153), (*Pseudomonas aeruginosa* ATCC 27853); three yeasts (*Candida albicans* ATCC 10231), (*Candida parapsilosis* ATCC 22019), (*Candida tropicalis* ATCC 750) were used for testing the activity. All microorganisms were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

The fractions derived from the subaerial and aerial parts ethanol extracts of *S. ketzkhovelii*, namely petroleum ether, dichloromethane, ethyl acetate, and n-butanol, were formulated at a concentration of 10,000 mg/L in DMSO (Dimethyl sulfoxide) solvent. Subsequently, serial twofold dilutions spanning from 5000 mg/L to 2.4 mg/L were prepared in the growth medium.

The inoculum for each bacterium was prepared from a 4–6 hour broth culture, while each yeast strain was cultured for 24 hours. The inocula were adjusted to a turbidity equivalent to a 0.5 McFarland standard, then diluted in Mueller-Hinton broth (Difco, Detroit, USA) to achieve a final concentration of 5×10^5 colony-forming units per milliliter (cfu/ml) for bacteria. For yeast, the inocula were diluted in RPMI-1640 medium (Sigma-Aldrich) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid; Sigma-Aldrich, Steinheim, Germany) to a pH of 7.0, resulting in a final concentration of 0.5–2.5 $\times 10^3$ cfu/ml in the test tray. The trays were covered and placed in plastic bags to prevent evaporation. Incubation occurred at 35°C for 18–24hours for trays containing Mueller-Hinton and at 35°C for 46–50 hours for those containing RPMI-1640 medium.

The MIC was characterized as the minimum concentration of the fractions that resulted in total inhibition of observable growth. Reference materials such as Cefuroxime, Ceftazidime, Clotrimazole, Ampicillin, and Amphotericin B were used, and all experiments were conducted in triplicate.

Preparation of Samples for LC-HRMS Analysis

To prepare the dichloromethane fractions of *S. ketzkhowelii*, 50 mg of dried material from both the aerial and subaerial parts were dissolved in methanol in a volumetric flask. The flask was then placed in an ultrasonic bath to achieve a clear solution. Subsequently, a solution of dihydrocapsaicin, employed as an internal standard, was introduced.

Instruments and Chromatographic Conditions of LC-HRMS

LC-HRMS experiments were conducted using a Thermo ORBITRAP Q-EXACTIVE mass spectrometry system based in

Bremen, Germany, equipped with a Troyasil C18 HS column (150 mm x 3 mm, 5 µm particle size, İstanbul, Turkey). The mobile phases A and B consisted of 1% formic acid-water and 1% formic acid-methanol, respectively. The gradient program was as follows: 0-1.00 min with 50% A and 50% B, 1.01-6.00 min with 100% B, and finally, 6.01-15 min with 50% A and 50% B. The flow rate of the mobile phase was set at 0.35 mL/min, the injection volume at 10 µL, and the column temperature at 220°C.

Compound identification was achieved by comparing the retention times of standard compounds (with a purity range of 95-99%, as specified in the chemicals section) and HRMS data from the Bezmialem Vakıf University Drug Application and Research Center Library. Dihydrocapsaicin (purity 95%) served as an internal standard in LC-HRMS measurements to mitigate repeatability issues arising from external factors such as ionization repeatability in mass spectrometry measurements.

RESULTS AND DISCUSSION

In previous investigations involving *Scorzonera* species, assessments of antimicrobial activity were conducted on fractions derived from ethanol/methanol crude extracts (Şahin, Sarı, Özsoy, Özbek Çelik, & Koyuncu, 2020a, 2020b; A. Sarı, Şahin, Özsoy, & Özbek Çelik, 2019). Consistent with these earlier protocols, the present study adopted a comparable approach, subjecting the fractions to similar analyses. The ethanol extract of the plant was obtained using the percolation method to obtain a spectrum of apolar-polar compounds. These compounds were subsequently segregated into sub-fractions using four solvents with varying polarities (petroleum ether, dichloromethane, ethyl acetate, n-butanol). The examination aimed to elucidate the correlation between the polarity of the fractions and their antimicrobial activities.

Table 1 presents the antimicrobial activity of all the fractions. The results demonstrate the effectiveness of the fractions in inhibiting the growth of the selected microorganisms *in vitro*, as evidenced by minimum inhibitory concentration (MIC) values falling within the range of 625 mg/L to 39 mg/L.

The current study revealed that both the petroleum ether and dichloromethane fractions obtained from aerial parts displayed antimicrobial activity against both gram-positive and gram-negative bacteria. The dichloromethane fraction, in particular, exhibited a more potent antibacterial effect against a broader spectrum of bacteria. All fractions, except for the petroleum ether fraction derived from aerial parts, displayed antimicrobial activity against *Candida parapsilosis.* Furthermore, the petroleum ether fraction from aerial parts demonstrated an anticandidal effect at 39 mg/L against *Candida tropicalis*.

These observations align with earlier research findings. In the investigation of antimicrobial activity carried out on n-hexane, chloroform, ethyl acetate, and water fractions derived from the methanol extract of *Scorzonera aucheriana* DC.'s aerial parts, it was observed that the chloroform fraction exhibited potent antimicrobial activity against all tested microorganisms (Erik, Yalçın, Coşkunçelebi, Alpay Karaoğlu, & Yaylı, 2022). Similarly, a study on *Scorzonera sandrasica* Hartvig & Strid revealed that the chloroform extract displayed robust inhibitory activity against bacteria belonging to *Stenotrophomonas maltophilia* and *Staphylococcus aureus* species (Ugur, Sarac, Ceylan, Duru, & Beyatli, 2010). In the antimicrobial study of *Scorzonera undulata* Vahl, the petroleum ether fraction demonstrated significant antimicrobial efficacy specifically against *S. aureus*, with a minimum inhibitory concentration (MIC) of 500 mcg/mL (Ben Abdelkader et al., 2010). Conversely, *S. pygmaea* Sibth. & Sm. and *S. hieraciifolia* Hayek species exhibited notably weak antimicrobial effects in studies conducted by (Şahin et al., 2020a) and (A. Sarı et al., 2019).

Aerial dichloromethane fraction showed higher antimicrobial activity compared to other extracts. For this reason, the chemical content of the aerial dichloromethane fraction was investigated in comparison with the content of the subaerial dichloromethane extract. Detected compounds in R2 (Dichloromethane fraction of the subaerial parts of *Scorzonera ketzkhowelii*) and H2 (Dichloromethane fraction of the aerial parts of *Scorzonera ketzkhowelii*) by LC- HRMS were given in Table 2, molecule drawings were given in Table 3, and LC-HRMS chromatograms were given in Figure 1-2.

As a result of LC-HRMS analysis, detected phenolic compounds in both dichloromethane fractions of the aerial and subaerial parts were: 3,4-dihydroxybenzaldehyde, salicylic acid, dihydrocaffeic acid, caffeic acid, caffeic acid phenethyl ester, chlorogenic acid, quercetin, hyperoside, naringenin, apigenin, hispidulin, hispidulin 7-glucoside, chrycin, emodin and carnosic acid. Chlorogenic acid was the major compound in both fractions.

In addition to these compounds, homogentisic acid, verbascoside, (+)-trans taxifolin, apigenin 7-glucoside, luteolin, orientin and chrysoeriol phenolic compounds were also detected in the H2.

Furthermore, the H2 fraction contained quantities exceeding 1 g/kg of chlorogenic acid, quercetin, hyperoside, caffeic acid, salicylic acid, naringenin, hispudilin, hispudilin 7-glucoside, and luteolin compounds. Similarly, the R2 fractions exhibited concentrations of chlorogenic acid, naringenin, and caffeic acid exceeding 1 g/kg.

Phenolic compounds derived from plants, including phenolic acids, flavonoids, stilbenes, and tannins, have the capability to hinder the growth and functions of numerous microorganisms, encompassing food-related pathogens and clinically significant bacteria, fungi, and protozoa (Ávila, Smânia, Monache, & Smânia, 2008; Batovska et al., 2009; Nielsen, Boesen, Larsen, Schønning, & Kromann, 2004). Due to the structural and chemical diversity among these various molecules, they can mani-

Table 1. Antimicrobial activity results of *Scorzonera ketzkhowelii* fractions (MIC mg/L)

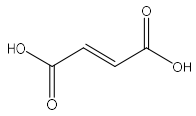
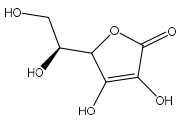
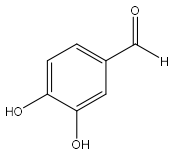
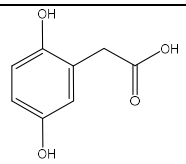
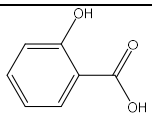
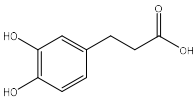
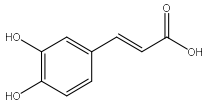
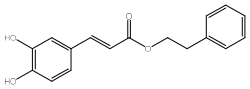
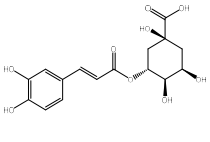
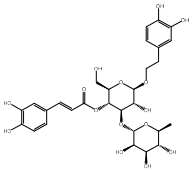
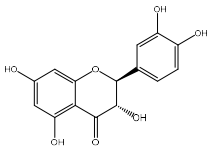
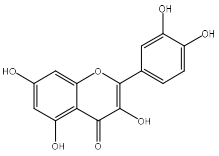
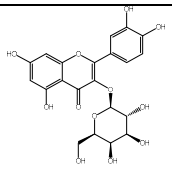
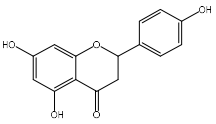
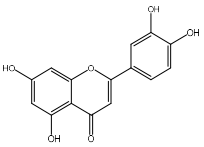
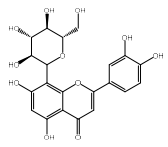
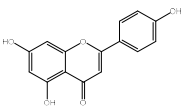
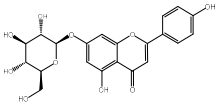
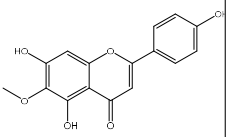
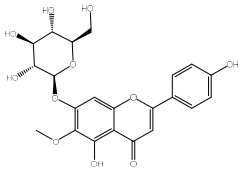
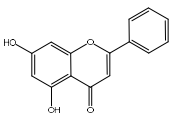
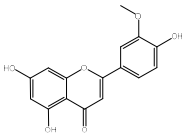
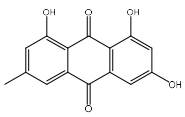
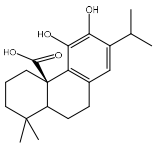
	P. aeruginosa ATCC 27853	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 4352	*P. mirabilis* ATCC 14153	*E. faecalis* ATCC 29212	*S. epidermitidis* ATCC 12228	*S. aureus* ATCC 29213	*C. albicans* ATCC 10231	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750
H1	625	-	-	-	312.5	625	312.5	-	-	39.06
H2	-	312.5	625	625	625	625	312.5	-	312.5	-
H3	-	-	-	-	-	-	-	-	312.5	-
H4	-	-	-	-	-	-	-	-	312.5	-
R1	-	-	-	-	-	-	-	-	312.5	-
R2	-	-	-	-	-	-	-	-	312.5	-
R3	-	-	-	-	-	-	-	-	312.5	-
R4	-	-	-	-	-	-	-	-	312.5	-
Standarts	Seftazidim pentahidrat	Sefuroksim Na	Sefuroksim Na	Sefuroksim Na	Ampisilin Na	Sefuroksim-Na	Sefuroksim-Na	Klotrimazol	Amfoterisin B	Amfoterisin B
	2.4	4.9	4.9	2.4	8.0	9.8	1.2	4.9	0.5	1

H1: Petroleum ether fraction of aerial part, H2: Dichloromethane fraction of aerial part, H3: Ethyl acetate fraction of aerial part, H4: n-Butanol fraction of aerial part R1: Petroleum ether fraction of subaerial part R2: Dichloromethane fraction of subaerial part, R3: Ethyl acetate fraction of subaerial part, R4: n-Butanol fraction of subaerial part. The antimicrobial activity results of fractions are expressed as the MIC (Minimum Inhibitory Concentration) determined by the microdilution method.

Table 2. Detected compounds in R2 and H2 by LC- HRMS

	Compounds	R2	mg/kg	H2	mg/kg	U (%)
1	Ascorbic acid	3.38	124.64	5.68	215.11	3.94
2	Chlorogenic acid	640.51	23591.49	208.53	7899.02	3.58
3	Fumaric acid	143.99	5303.54	196.93	7459.28	2.88
4	Verbascoside	-	<LOD	0.09	3.33	2.93
5	Orientin	-	<LOD	5.86	222.01	3.67
6	Caffeic acid	36.69	1351.53	123.60	4681.97	3.74
7	(+)-*trans* taxifolin	-	<LOD	0.82	31.06	3.35
8	Hyperoside	0.88	32.23	169.24	6410.49	3.46
9	Apigenin 7-glucoside	-	<LOD	0.85	32.31	3.59
10	Quercetin	0.37	13.70	184.32	6981.78	2.95
11	Salicylic acid	8.59	316.21	117.04	4433.45	1.89
12	Naringenin	45.99	1694.03	71.70	2715.91	4.20
13	Luteolin	-	<LOD	69.07	2616.36	3.42
14	Apigenin	2.86	105.27	17.96	680.30	2.87
15	Hispidulin	0.24	8.69	80.43	3046.48	3.41
16	Caffeic acid phenethyl ester	0.01	0.48	0.02	0.61	3.13
17	Chrysin	1.49	54.95	1.74	65.98	3.24
18	Emodin	0.00	0.07	0.01	0.23	4.27
19	Homogentisic acid	-	<LOD	7.18	272.12	4.35
20	3,4-Dihydroxybenzaldehyde	0.49	17.97	2.20	83.37	3.79
21	Hispidulin 7-glucoside	1.76	64.64	71.39	2704.20	4.57
22	Carnosic acid	0.04	1.51	0.18	6.89	2.58
23	Dihydrocaffeic acid	0.39	14.48	0.46	17.35	0.86
24	Chrysoeriol	-	<LOD	20.32	769.55	2.08

Table 3. Detected compounds in *S. ketzkhowelii* Grossh. dichloromethane fraction by LC- HRMS

 **Fumaric acid**	 **Ascorbic acid**	 **3,4-Dihydroxybenzaldehyde**	 **Homogentisic acid**
 **Salicylic acid**	 **Dihydrocaffeic acid**	 **Caffeic acid**	 **Caffeic acid phenethyl ester**
 **Chlorogenic acid**	 **Verbascoside**	 **(+)-*trans* taxifolin**	 **Quercetin**
 **Hyperoside**	 **Naringenin**	 **Luteolin**	 **Orientin**
 **Apigenin**	 **Apigenin 7-glucoside**	 **Hispidulin**	 **Hispidulin 7-glucoside**
 **Chrysin**	 **Chrysoeriol**	 **Emodin**	 **Carnosic acid**

fest a range of antimicrobial effects, such as permeabilizing and destabilizing the cell membrane or inhibiting extracellular enzymes (Suresh Babu et al., 2005).

Indeed, it was observed that the H2 fraction contained a higher concentration of phenolic compounds compared to the R2 fraction. Therefore, it is logical to expect it to exhibit potent antimicrobial activity. However, the question arises as to why, despite the anticipation of a higher phenolic content in the ethyl acetate fraction compared to the dichloromethane fraction, the ethyl acetate fractions did not demonstrate antimicrobial activity on the same scale. One possible explanation could be the presence of terpenic compounds in the dichloromethane fraction, which are known to exhibit strong antimicrobial activity, in addition to phenolic compounds (Erik et al., 2022; Aynur Sarı, Özbek, & Özgökçe, 2009; Sweidan et al., 2020). Furthermore, it is worth noting that the phenolic compounds in the ethyl acetate fraction are primarily present in the form of sugar-bound glycosides, whereas those in the dichloromethane fraction are predominantly in their aglycone form. Literature indicates that apolar phenolics, aglycone forms are reported to be more potent in terms of antimicrobial activity, thus supporting the consistency of our results with existing literature and lending further credence to it (Miklasińska-Majdanik, Kępa, Wojtyczka, Idzik, & Wąsik, 2018; Takó et al., 2020).

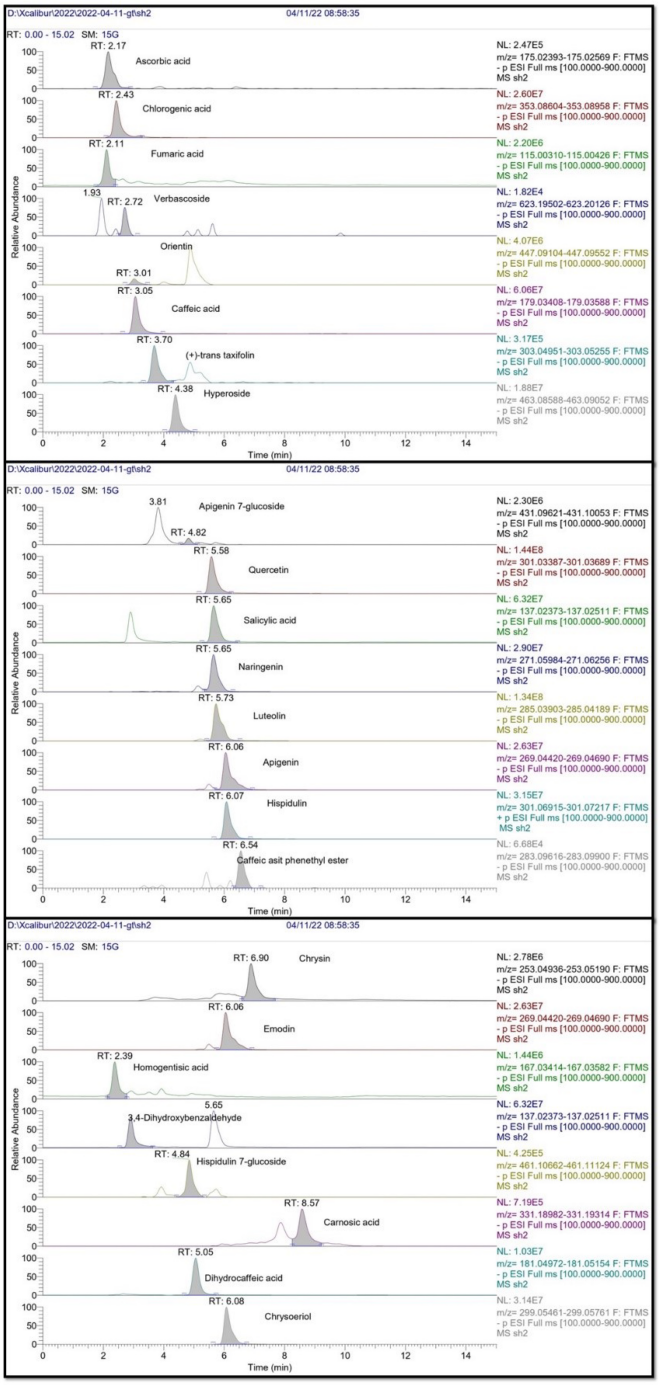


Figure 1. LC-HRMS chromatogram of H2 (Dichloromethane fraction of the aerial part)

CONCLUSION

In this study, which was carried out for the first time on the *Scorzonera ketzkhowelii* species, the antimicrobial activities of petroleum ether, dichloromethane, ethyl acetate and n-butanol fractions obtained from the ethanol extract of the plant were revealed. As a result of the study, dichloromethane fractions of the plant exhibited strong antimicrobial activity. Based on this, the phenolic chemical contents of these dichloromethane

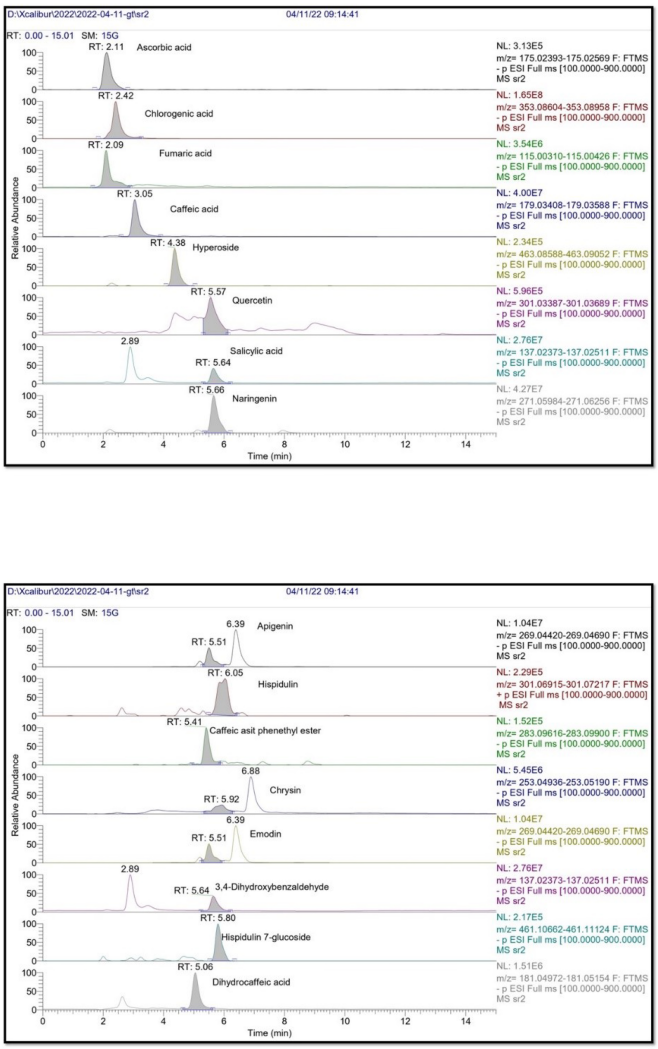


Figure 2. LC-HRMS chromatogram of R2 (Dichloromethane fraction of the subaerial part)

fractions were determined by the LC-HRMS method and their relationship with antimicrobial activity was explained. Studies have demonstrated that phenolic compounds derived from natural origins display robust antimicrobial activity against a range of clinically significant pathogens linked to microbial infections. Moreover, they enhance the susceptibility of multi-drug resistant strains to bactericidal or bacteriostatic antibiotics.

The antimicrobial activity study results of the *Scorzonera ketzkhowelii* species confirm each other with previous studies showing that phenolic compounds obtained from natural sources exhibit potent antimicrobial activity against a number of clinically important pathogens associated with microbial infections. Moreover, in this study, it is thought that the dichloromethane fraction exhibits stronger antimicrobial activity than the ethylacetate fraction, and that the nonpolar phenolic compounds found in the plant may exhibit stronger antimicrobial activity than the polar phenolic compounds, and that the antimicrobial effect of the phenolic compounds with other, es-

pecially terpenic compounds, found in the dichloromethane fraction may be strengthened with a synergistic effect.

These findings not only provide valuable insights into the antimicrobial potential of *Scorzonera ketzkhowelii* but also expand our knowledge of its chemical composition. The plant's antimicrobial properties and the presence of diverse phenolic compounds make it a promising candidate for further research and potential applications in the field of medicine and pharmaceuticals. Further investigations into its bioactivity and potential health benefits may uncover new avenues for its utilization.

This is the first chemical composition analysis and antimicrobial activity report for the *Scorzonera ketzkhowelii*. This plant could be evaluated for further phytochemical and other biological activity search.

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REFERENCES

Altundağ, E., & Öztürk, M. (2011). Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. *Procedia - Social and Behavioral Sciences*, 19(June), 756–777. Retrieved from https://doi.org/10.1016/j.sbspro.2011.05.195

Ávila, H. P., Smânia, E. de F. A., Monache, F. D., & Smânia, A. (2008). Structure–activity relationship of antibacterial chalcones. *Bioorganic & Medicinal Chemistry*, 16(22), 9790–9794. Retrieved from https://doi.org/10.1016/j.bmc.2008.09.064

Bahadır Acıkara, Ö., Çitoğlu, G. S., Dall'Acqua, S., Šmejkal, K., Cvačka, J., & Žemlička, M. (2012). A new triterpene from Scorzonera latifolia (Fisch. and Mey.) DC. *Natural Product Research*, 26(20), 1892–1897. Retrieved from https://doi.org/10.1080/14786419.2011.625644

Batovska, D., Parushev, S., Stamboliyska, B., Tsvetkova, I., Ninova, M., & Najdenski, H. (2009). Examination of growth inhibitory properties of synthetic chalcones for which antibacterial activity was predicted. *European Journal of Medicinal Chemistry, 44*(5), 2211–2218. Retrieved from https://doi.org/10.1016/j.ejmech.2008.05.010

Ben Abdelkader, H., Salah, K. B. H., Liouane, K., Boussaada, O., Gafsi, K., Mahjoub, M. A., . . . Mighri, Z. (2010). Antimicrobial activity of Rhaponticum acaule and Scorzonera undulata growing wild in Tunisia. *African Journal of Microbiology Research*, 4(19), 1954–1958.

CLSI. (2000). *Reference method for Broth dilution antifungal susceptibility testing of yeasts: approved standard* M27-A NCCLS. Wayne (PA): CLSI.

CLSI. (2006). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard* M7-A5. Wayne (PA): CLSI.

Coşkunçelebi, K., Makbul, S., Gültepe, M., Okur, S., & Güzel, M. E. (2015). A conspectus of Scorzonera s.l. in Turkey. *Turkish Journal of Botany*, 39(1), 76–87. Retrieved from https://doi.org/10.3906/bot-1401-10

Erik, İ., Yalçın, C. Ö., Coşkunçelebi, K., Alpay Karaoğlu, Ş., & Yaylı, N. (2022). Phytochemical Analysis and Antimicrobial Activities of Scorzonera Aucheriana. *SSRN*.

Göç, F., & Mat, A. (2019). Türkiye'de Yanık Tedavisinde Geleneksel Olarak Kullanılan Bitkiler. *Sağlık Bilimlerinde İleri Araştırmalar Dergisi*, 2(1).

Granica, S., & Zidorn, C. (2015). Phenolic compounds from aerial parts as chemosystematic markers in the Scorzonerinae (Asteraceae). *Biochemical Systematics and Ecology*, 58, 102–113. Retrieved from https://doi.org/10.1016/j.bse.2014.11.005

Hamzaoğlu, E., Aksoy, A., Martin, E., Pınar, N. M., & Çölgeçen, H. (2010). A new record for the flora of Turkey: Scorzonera ketzkhovelii Grossh. (Asteraceae). *Turkish Journal of Botany* , 34(1), 57–61.

Makbul, S. (2012). Scorzonera L. In A. Güner, S. Aslan, T. Ekim, M. Vural, & M. T. Babaç (Eds.), *Türkiye Bitkileri Listesi (Damarlı Bitkiler)* (pp. 195–199). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği.

Miklasińska-Majdanik, M., Kępa, M., Wojtyczka, R., Idzik, D., Wąsik, T. (2018). Phenolic Compounds Diminish Antibiotic Resistance of Staphylococcus Aureus Clinical Strains. *International Journal of Environmental Research and Public Health, 15*(10), 2321. Retrieved from https://doi.org/10.3390/ijerph15102321

Nadiroğlu, M., Behçet, L., & Çakılcıoğlu, U. (2019). An ethnobotanical survey of medicinal plants in karlıova (Bingöl-turkey). *Indian Journal of Traditional Knowledge*, 18(1), 76–87.

Nielsen, S. F., Boesen, T., Larsen, M., Schønning, K., & Kromann, H. (2004). Antibacterial chalcones—bioisosteric replacement of the 4-hydroxy group. *Bioorganic & Medicinal Chemistry*, 12(11), 3047–3054. Retrieved from https://doi.org/10.1016/j.bmc.2004.03.071

Şahin, H., Boğa, M., & Sarı, A. (2022). Terpenoid and Fatty Acid Profiling along with Anti-Tyrosinase and Anti-Urease Potentials of Scorzonera pygmaea Sibth. & Sm. an Endemic Plant of Turkey. *Chemistry & Biodiversity*, 19(2), e202100758. Retrieved from https://doi.org/https://doi.org/10.1002/cbdv.202100758

Şahin, H., Sarı, A., Özsoy, N., Özbek Çelik, B., & Koyuncu, O. (2020a). Phenolic compounds and bioactivity of Scorzonera pygmaea Sibth. & Sm. aerial parts: In vitro antioxidant, anti-inflammatory and antimicrobial activities. Istanbul Journal of Pharmacy, 50(3), 294–299.

Şahin, H., Sarı, A., Özsoy, N., Özbek Çelik, B., & Koyuncu, O. (2020b). Two new phenolic compounds and some biolog-

ical activities of Scorzonera pygmaea Sibth. & Sm. subaerial parts. *Natural Product Research*, 34(5), 621–628. Retrieved from https://doi.org/10.1080/14786419.2018.1493585

Sarı, A., Şahin, H., Özsoy, N., & Özbek Çelik, B. (2019). Phenolic compounds and in vitro antioxidant, anti-inflammatory, antimicrobial activities of Scorzonera hieraciifolia Hayek roots. *South African Journal of Botany*, 125, 116–119. Retrieved 14 August 2022 from https://doi.org/10.1016/J.SAJB.2019.07.009

Sarı, Aynur. (2012). Phenolic compounds from Scorzonera latifolia Phenolic compounds from Scorzonera latifolia (Fisch. & Mey.) DC. *Natural Product Research*, 26(1), 50–55. Retrieved from https://doi.org/10.1080/14786419.2010.533666

Sarı, Aynur, Özbek, B., & Özgökçe, F. (2009). Antimicrobial Activities of Two Scorzonera Species Growing in Turkey. *Asian Journal of Chemistry*, 21(6), 4785–4788.

Suresh Babu, K., Hari Babu, T., Srinivas, P. V., Sastry, B. S., Hara Kishore, K., Murty, U. S. N., & Madhusudana Rao, J. (2005). Synthesis and in vitro study of novel 7-O-acyl derivatives of Oroxylin A as antibacterial agents. *Bioorganic & Medicinal Chemistry Letters*, 15(17), 3953–3956. Retrieved from https://doi.org/10.1016/j.bmcl.2005.05.045

Sweidan, A., El-Mestrah, M., Kanaan, H., Dandache, I., Merhi, F., & Chokr, A. (2020). Antibacterial and antibiofilm activities of Scorzonera mackmeliana. *Pakistan Journal of Pharmaceutical Sciences*, 33(1), 199–206. Retrieved from http://europepmc.org/abstract/MED/32122849

Takó, M., Kerekes, E. B., Zambrano, C., Kotogán, A., Papp, T., Krisch, J., & Vágvölgyi, C. (2020). Plant Phenolics and Phenolic-Enriched Extracts as Antimicrobial Agents against Food-Contaminating Microorganisms. *Antioxidants*, 9(2), 165. Retrieved from https://doi.org/10.3390/antiox9020165

Tuzlacı, E. (2016). *Türkiye Bitkileri Geleneksel İlaç Rehberi*. İstanbul: Istanbul Tıp Kitabevi.

Ugur, A., Sarac, N., Ceylan, O., Duru, M. E., & Beyatli, Y. (2010). Chemical Composition of Endemic Scorzonera sandrasica and Studies on the Antimicrobial Activity Against Multiresistant Bacteria. *Journal of Medicinal Food*, 13(3), 635–639. Retrieved from https://doi.org/10.1089/jmf.2008.0312

Zidorn, C., Ellmerer-Müller, E., & Stuppner, H. (2000). Sesquiterpenoids from Scorzonera hispanica L. . *Pharmazie*, 55(7), 550–551.

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İstanbul Journal of Pharmacy
Istanbul J Pharm, 54 (1): 69–79
DOI: 10.26650/IstanbulJPharm.2024.1404110

Original Article

Physiological, oxidative, and antioxidative responses of *Quercus vulcanica* Boiss. and *Quercus aucheri* Jaub. & Spach. under drought stress conditions

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ABSTRACT

Background and Aims: Drought stress is one of the most common global factors of abiotic stress affecting plant growth and productivity world-wide. The present study aims to assess the effects of 1, 2, and 4 weeks of drought stress on the two endemic tree species, *Quercus vulcanica* and *Q. aucheri*

Methods: After applying drought stress conditions, the study determines the physiological parameters, markers of oxidative damage, and levels of antioxidant enzymes in the leaves and stems of 6-month-old oak seedlings.

Results: The dry and fresh weights were observed to decrease by at least 11.44%, as well as the chlorophyll levels by at least 14%, in both the *Q. vulcanica* and *Q. aucheri* that were subjected to 1, 2, and 4 weeks of drought stress. The carotenoid, proline, and anthocyanin levels also increased in the leaves of both Quercus species; however, the amount of ascorbic acid, reduced glutathione (GSH), and total soluble protein decreased in the leaves and stems of both *Quercus* species. As oxidative stress markers, the levels of hydrogen peroxide (H_2O_2) and lipid peroxidation were seen to elevate by at least 1.21 fold under drought stress conditions. This revealed some of the alterations in the activities of antioxidant enzymes in the leaves and stems of both *Q. aucheri* and *Q. vulcanica*.

Conclusion: In conclusion, this study revealed increasing drought stress to have a substantial impact on the physiological parameters and oxidative and antioxidant responses in *Q. vulcanica* and *Q. aucheri*. This will contribute to understanding how oak species respond to drought stress and their adaptation strategies.

Keywords: *Q. vulcanica, Q. aucheri*, drought stress, oxidative stress, antioxidant enzyme system

INTRODUCTION

Due to increased industrial activity, the combustion of fossil fuels, the release of hazardous waste materials into the biosphere, and the release of greenhouse gases into the atmosphere as technology advances, global warming and droughts have become important environmental concerns. Plants cannot avoid stress in nature due to being in fixed locations and being exposed to various stress factors throughout their lifespan (Rao et al., 2006). Drought causes various changes in plants in terms of their ecological, morphological, physiological, biochemical, and molecular aspects (Lichtenthaler, 1996; Rao et al., 2006). These might result in different outcomes, such as inhibited growth, turgor loss, decreased pigment and protein content, and reductions in the quantity and quality of their yield. Additionally, drought increases the formation of free radicals and reactive oxygen species (ROS) that damage photosynthetic pigments, membrane lipids, proteins, and nucleic acids and also affects antioxidant enzyme systems (Egert & Tevini, 2002; Mittler, 2006; Noctor et al., 2018; Suzuki & Mittler, 2006; Yordanov et al., 2000). A balance exists between free radical formation and antioxidant defense systems in organisms. The excessive formation of free radicals and insufficient antioxidant defense due to various environmental factors or metabolic activities cause oxidative damage to the structure and functions of plant cells (Noctor et al., 2018; Suzuki & Mittler, 2006).

Drought causes negative effects on plants, and as a result, plants develop an adaptive response to stressful conditions. Therefore, studies on identifying plant species with high drought resistance or tolerance have become increasingly important.

Oaks are widely distributed trees in the Mediterranean region and can cope with a variety of environmental stressors due to

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the remarkable flexibility of their phenotypic and physiological features (Cotrozzi et al., 2016). The literature contains some studies on the negative effects of various drought stress conditions on some *Quercus* species, including *Q. ilex* (Echevarría-Zomeño et al., 2009; Cotrozzi, Pellegrini, et al., 2017; Simova-Stoilova et al., 2018); *Q. ilex, Q. cerris, Q. pubescens* (Cotrozzi et al., 2016), *Q. cerris* (Cotrozzi, Remorini, et al., 2017); *Q. infectoria* and *Q. libani* (Ghanbary et al., 2020); *Q. cerris* and *Q. pubescens* (Landi et al., 2019); *Q. ilex, Q. pubescens,* and *Q. robur* (Pellegrini et al., 2019); Q. lusitanica (Santamarina et al., 2022); and Q. serrata, *Q. serrata, Q. acutissima*, and *Q. variabilis* (Xiong et al., 2022). The aim of the study is to investigate drought stress on two endemic oak species (*Q. vulcanica* and *Q. aucheri*) in Türkiye by evaluating their physiological and biochemical responses. This approach will enable the investigation of the potential negative effects and adaptive responses in these species, ensuring the determination of resistant species. The research involves subjecting oak saplings to controlled drought conditions and comparing them with well-watered control groups. Thus, the study comparatively examines the effects of 1, 2, and 4 weeks of drought stress on the physiological and biochemical parameters (fresh weight, dry weight, chlorophyll, and carotenoid content, proline and anthocyanin content), oxidative damage (MDA, H_2O_2), and antioxidant systems (ascorbic acid, GSH, oxidized glutathione, superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase) in the leaves and stems of *Q. vulcanica* and *Q. aucheri*. This study will contribute to understanding the mechanisms underlying drought stress and determining the effects of drought stress on the biological diversity of endemic oaks.

MATERIALS AND METHODS

Collection, cultivation and preparation of plant materials for the experiment

The study was able to collect 100 seeds in autumn from different parts of 10 oak trees for each of the studied species in their natural distribution area. The seeds for the *Kasnak Meşesi* [Kotschy oak (Fagaceae)], or *Q. vulcanica* Boiss. & Heldr. ex, were collected from the Yukarıgökdere village of the Eğirdir municipality in Isparta province and for the Boz-Pırnal oak (Fagaceae), or *Q. aucheri* Jaub & Spach., from the city of Muğla in Muğla province. The seeds were then moved to a laboratory and stored at room temperature until the analysis. The seeds were identified and confirmed by Prof. Dr. Osman Erol and Asist. Prof. Dr. Erdal Üzen. The collected seeds were carefully cleaned of debris in the laboratory before being subjected to vernalization at +4°C for approximately one month. After vernalization, the seeds were soaked in a 10% sodium hypochlorite solution for 25 minutes and washed with distilled water three to four times for surface sterilization. The seeds were placed in petri dishes containing filter paper soaked with distilled water and left to germinate in a growth chamber at 25°C. To determine the germination rate of the seeds, they were placed in petri dishes with a diameter of 15 cm in an air-conditioned cabinet and placed in an oven at 25°C. Trials were carried out, with a germination rate of 90% being determined for both species.

The germinated seeds were transferred into pots and first grown individually in a mixture of sand (sieved and washed) and perlite (1:1 v/v) in a climate chamber under optimal conditions (Ozden & Baycu, 2004). The pot experiment was carried out in the phytotron (14 h light and 10 h dark period; light intensity = 7000 lux; temperature = 22°C ± 2°; humidity = 48% ± 2%), and the tree seedlings were watered every other day with a modified Ingestad nutrient solution (Ingestad, 1970; pH 5.8) for 4 months until they had developed 8–10 pinnate leaves. The tree seedlings were then carefully removed from their containers and transplanted into individual pots. Six plants were grown in black polypropylene vessels of 10 L volume under the same phytotron conditions for another 2 months, with the nutrient solutions being changed every other day. Later, the 6-month-old tree seedlings were divided into six experimental groups that were subjected to a drought stress for 1, 2 and 4 weeks alongside their corresponding control groups. Each treatment was comprised of six seedlings divided into three replicated culture vessels. The control groups maintained their regular development by receiving water and the Ingestad nutrient solution every other day. For the drought stress groups, the Ingestad nutrient solution was applied once a week. After the treatments, the oak seedlings were harvested, and some of leaves and stems were stored at -20°C until the day of the physiological and biochemical analyses; some of these leaves and stems were also used fresh for the physiological assessments.

Measurement of fresh and dry weights

The study measured the fresh and dry weights of the leaves and stem samples from the control and 1, 2, and 4 weeks of drought stress-treated oak seedlings (Horwitz, 1970). The relative water content (RWC) was then calculated as a percentage using Equation 1 (Dhanda & Sethi, 1998).

RWC (%)
= [(Fresh weight (fw) (g) - Dry weight (g)) / fw (g)] x 100 (1)

Measurement of chlorophyll and carotenoid contents

To measure the chlorophyll and carotenoid contents, fresh leaves weighing 2 g were taken, cut into small pieces, and homogenized in ice-cold acetone (100%, v/v) and $CaCO_3$ using an Ultra-Turrax (Janke & Kunkel, Germany). The leaf homogenates were then centrifuged at 3,000 g for 10 min. (Heraeus Labofuge 400 R, Germany) before collecting the supernatant. The absorbance of the supernatant was measured at 662 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for all carotenoids (Jenway 6105 UV-Vis Spectrophotometer, Great Britain). The results were calculated using Equations 2,

3, and 4 (Lichtenthaler & Wellburn, 1983) and expressed as mg/g FW for chlorophylls a and b and for all the carotenoids.

Chlorophyll-a = (11.75 x A662 - 2.35 x A645) x 10/mg fw (2)

Chlorophyll-b = (18.61 x A645 - 3.96 x A662) x 10/mg fw (3)

Total carotenoids
= [(1000 x A470 – 2.27 x Chl-a – 81.4 x Chl-b) /227] x 10/mg fw (4)

Measurement of the contents of proline and anthocyanins

The proline content in the leaves of the oak seedlings was determined according to the method described by Bates et al. (1973) using 200 mg of fresh weight (fw) samples and calculated based on the absorbance values at 520 nm and expressed as µmol/g fw. The anthocyanins content in the leaves of the oak seedlings was determined according to the method described by Mancinelli (1990). Briefly, 500 mg of fw samples were extracted in acidified methanol (1%, v/v) and kept at +4°C for 2 days before being centrifuged at 5,000 g. The supernatant was collected, and the absorbance was measured at 530 nm and 657 nm; the anthocyanins content is calculated using the Equation 5 and expressed as (A_{530}-0.33xA_{657})/g fw.

Anthocyanins content = A_{530} - 0.33 x A_{657} (5)

Measurement of hydrogen peroxide (H_2O_2) content and lipid peroxidation products

The H_2O_2 content in the leaves and stems of the oak seedlings was determined according to the method described by Velikova et al. (2000) using 500 mg of leaves and stem samples. The H_2O_2 content was calculated based on the standard curve prepared using H_2O_2 standard solutions and is expressed as µmol/g fw.

The malondialdehyde (MDA) content, which is an indicator of lipid peroxidation, was determined in the leaves and stems of the oak seedlings in accordance with the method described by Heath and Packer (1968). The principle of the method relies on the reaction of MDA with thiobarbituric acid (TBA) under acidic and high-temperature conditions. The MDA content was calculated using Equation 6 and is expressed as nmol/g fw.

MDA (nmol/g fw) = [(A_{532}-A_{600} / 155 mM^{-1} cm^{-1}) / fw (mg)] x 106 (6)

Measurement of glutathione and ascorbic acid contents

The contents of the reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG) in the leaves and stems of the oak seedlings was determined according to the method described by Gossett et al. (1994) using 500 mg of fw samples. The GSH + GSSG and GSSG contents were calculated using the standard curve prepared using GSH standard solutions. The GSH content was calculated by subtracting the GSSG content from the GSH + GSSG content. The results are expressed as nmol/g fw.

The ascorbic acid content in the leaves and stems of the oak seedlings was determined according to the method described by Gossett et al. (1994) using 500 mg of fw samples and calculated based on the standard curve prepared using standard solutions. The value is expressed as µmol/g fw.

Measurement of antioxidant enzymes

For the extraction of the antioxidant enzymes, 200 mg of leaves and stem samples were mixed with Triton X-100 (2%, v/v), ascorbate (5 mM), and 400 mg of polyvinylpolypyrrolidone in a KH2PO4/K2HPO4 buffer (10 mM, pH 7.8) for 1 minute and incubated on ice for 30 minutes. The homogenate was then centrifuged at 48,400 g at 4°C (Schwanz et al., 1996). The supernatant was collected and used to determine the total soluble protein and antioxidant enzyme activities.

The protein content in the leaves and stems of the oak seedlings was determined using the method described by Bradford (1976) and calculated using a standard curve prepared with bovine serum albumin (BSA) standard solutions and expressed as mg/g fw.

The superoxide dismutase (SOD) enzyme activity in the leaves and stems of oak seedlings was determined using the method described by Beyer and Fridovich (1987). The measurement of SOD activity is based on the reduction of nitro blue tetrazolium chloride (NBT) by O_2 radicals under light. The amount of enzyme that causes a 50% inhibition in the reduction of NBT was considered one unit, with SOD activity being expressed as U/g fw. The catalase (CAT) enzyme activity in the leaves and stems of oak seedlings was determined using the method described by Aebi (1984). The principle of the method is based on the enzymatic decomposition of the H_2O_2 substrate by CAT, which is monitored at 240 nm. The CAT activity was calculated using a standard curve prepared with H_2O_2 standard solutions and expressed as U/g fw.

The ascorbate peroxidase (APX) enzyme activity in the leaves and stems of oak seedlings was determined using the method described by Nakano and Asada (1987). The APX activity was calculated using the molar extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ at 290 nm and expressed as U/g fw. The guaiacol peroxidase (GuPX) enzyme activity in the leaves and stems of the oak seedlings was determined using the method described by Cakmak (1994) and expressed as U/g fw. The glutathione reductase (GR) enzyme activity in the leaves and stems of the oak seedlings was determined using the method described by Foyer and Halliwell (1976) and expressed as U/g fw.

Statistical analysis

All values in the obtained data were calculated as a mean ± standard deviation (*M* ± *SD*). The statistical evaluation of the

data was performed using the software package SPSS 14.0 for Windows and applying the one-way analysis of variance analysis (ANOVA) with post-hoc test and least significant difference (LSD) test. A p-value of less than 0.05 or 0.001 was considered statistically significant.

RESULTS

The effect of drought stress on the fresh and dry weight of oak seedlings

The fresh and dry weight of the *Q. vulcanica* and *Q. aucheri* leaves and stems significantly decreased in the groups that had been subjected to drought stress for 1, 2, and 4 weeks compared to the control group, indicating a significant decrease of at least 11.44% ($p < 0.001$), decreasing more with longer durations of drought stress (Figures 1a & 1b). The RWC in the leaves and stems of the *Q. vulcanica* seedlings was observed to significantly decrease by at least 3.77% ($p < 0.001$) after 1, 2, and 4 weeks of drought stress compared to the control group (Figure 1c). However, the decrease in RWC in the leaves of the *Q. aucheri* seedlings was not significant, while a significant decrease of at least 5.29% ($p < 0.001$) was observed in their stems after 2 and 4 weeks of drought stress (Figure 1c).

The effect of drought stress on the chlorophyll and total carotenoid contents

In the groups subjected to 1, 2, and 4 weeks of drought stress, a significant decrease of at least 14% ($p < 0.05$) was observed in the content of chlorophyll a in the leaves of the *Q. vulcanica* seedlings and of at least 49.17% ($p < 0.001$) in the leaves of the *Q. aucheri* seedlings compared to the control group (Figure 2a). The chlorophyll b content showed a non-significant decrease of 5.53% in the 1-week drought stress group, while significant decreases of at least 29.35% ($p < 0.05$) were observed in the 2- and 4- week groups for *Q. vulcanica* and of at least 46.68% ($p < 0.001$) for *Q. aucheri* in all drought stress groups (Figure 2b). The total chlorophyll content showed a significant decrease of 11.85% in the 1-week drought stress group and a significant decrease of at least 32.85% ($p < 0.05$) was observed in the 2- and 4-week groups for *Q. vulcanica*, while significant decreases of at least 34.63% ($p < 0.001$) were observed for *Q. aucheri* in all drought stress groups (Figure 2c). With regard to the total carotenoid content, increases of 3.7 fold ($p < 0.001$) and 1.52 fold ($p < 0.001$) were observed in all the drought stress groups for *Q. vulcanica* and *Q. aucheri*, respectively (Figure 2d).

The effect of drought stress on the proline, anthocyanins, and total soluble protein contents

The groups subjected to 1, 2, and 4 weeks of drought stress showed a significant 1.17-fold increase in the proline content in the leaves for *Q. vulcanica* ($p < 0.05$) and 2.11-fold for *Q.*

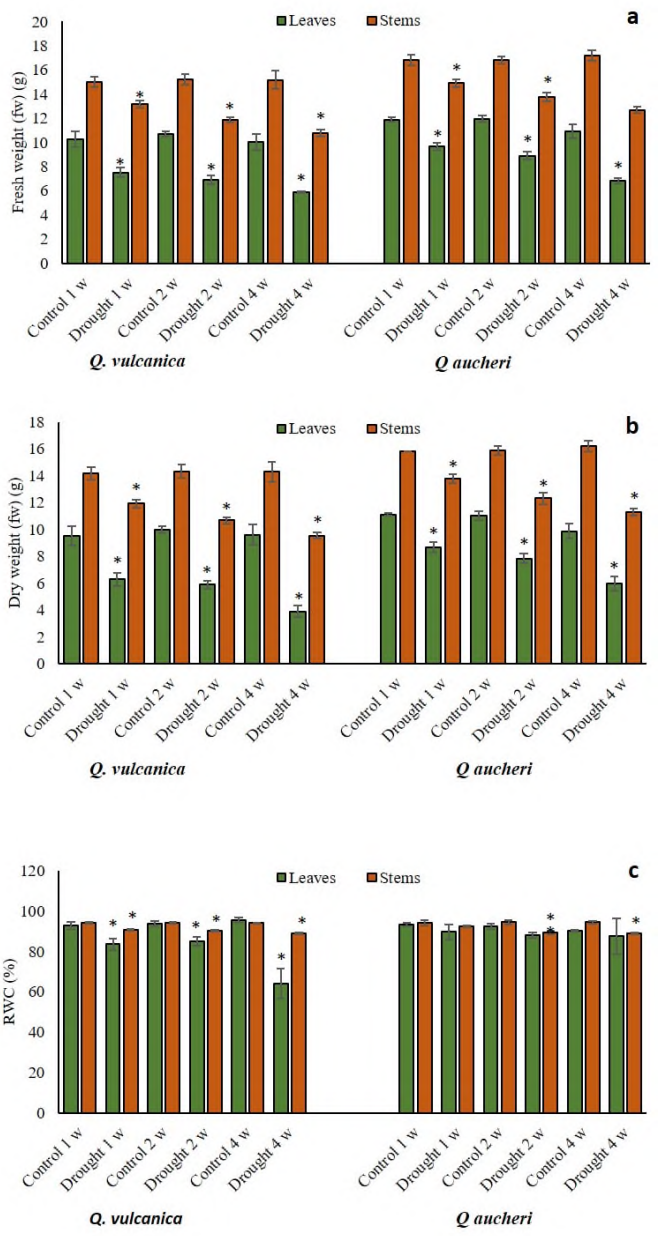

Figure 1. The effects of 1, 2, and 4 weeks of drought stress on (a) fresh weight, (b) dry weight, and (c) relative water content in the leaves and stems of *Q. vulcanica* and *Q.aucheri.* Data are presented as $M \pm SD$ (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by $*p < 0.05$ and $**p < 0.001$).

aucheri ($p < 0.001$) compared to the control group (Figure 3a). The groups subjected to 1, 2, and 4 weeks of drought stress saw the anthocyanins contents in the leaves of the *Q. vulcanica* and *Q. aucheri* seedlings to significantly increase compared to the control group at respective rates of at least 12.1 fold and 4.9 fold ($p < 0.001$) and to correlate with the applied duration of drought stress (Figure 3b). The total soluble protein content increased by 1.6 fold ($p < 0.001$) under one week of drought stress while decreasing by 17.42% under two weeks and 19.61% under four weeks of drought stress ($p < 0.05$) in the leaves

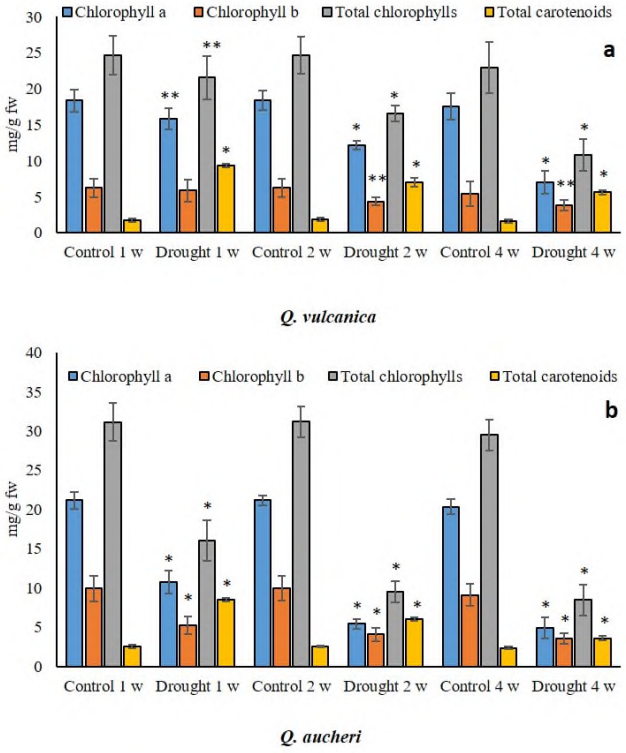

Figure 2. The effects of 1, 2, and 4 weeks of drought stress on the chlorophyll and total carotenoid levels in the leaves of (a) *Q. vulcanica* and (b) *Q.aucheri.* Data are presented as *M* ± *SD* (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by **p* < 0.05 and ***p* < 0.001).

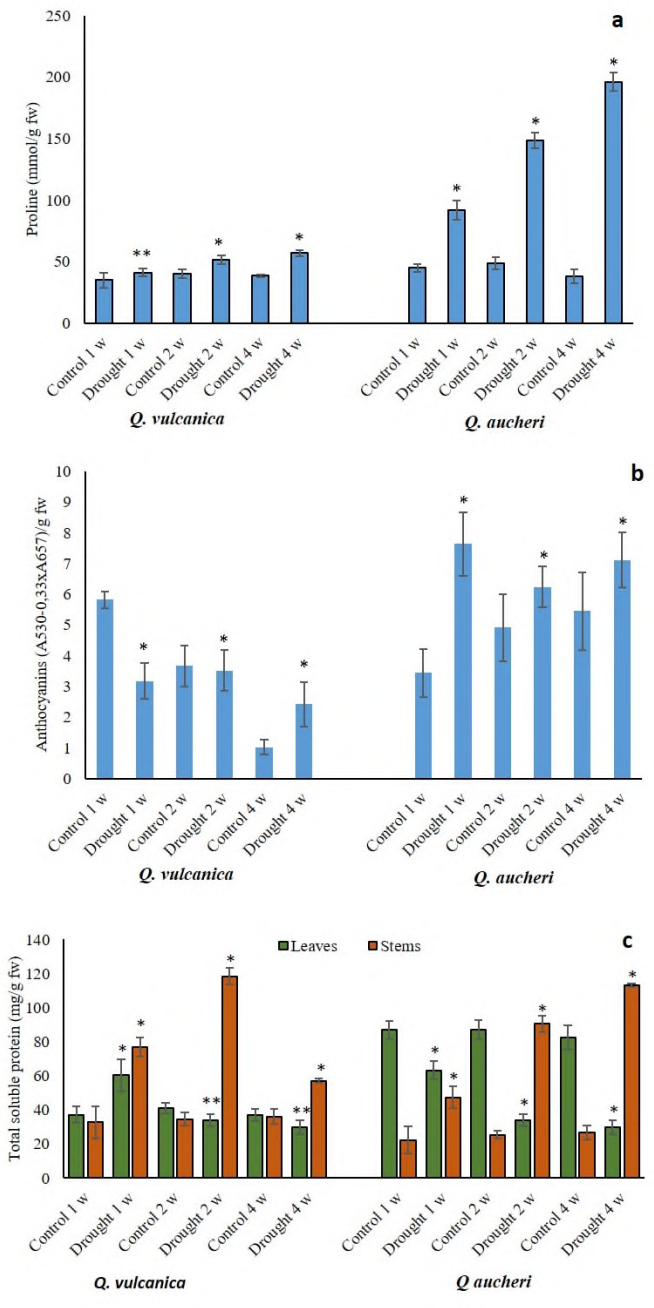

Figure 3. The effects of 1, 2, and 4 weeks of drought stress on the levels of (a) proline, (b) anthocyanins, and (c) total soluble protein in the leaves of *Q. vulcanica* and *Q.aucheri*. Data are presented as *M* ± *SD* (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by **p* < 0.05 and ***p* < 0.001).

of the *Q. vulcanica* seedling (Figure 3c). In the *Q. aucheri*'s leaves, a decrease of at least 27.23% ($p < 0.001$) in total protein content was observed for all drought stress groups. However, both Quercus species exhibited at least a 1.58-fold increase ($p < 0.001$) in total protein content in their stems for all drought stress groups (Figure 3c).

The effect of drought stress on the oxidative stress markers

As shown in Figure 4a, H_2O_2 content was observed to significantly increase in the 1-, 2-, and 4-week drought stress groups at least 1.4-fold ($p < 0.001$) for the *Q. vulcanica* leaves, at least 2.3-fold ($p < 0.001$) for the leaves, at least 1.41-fold ($p < 0.05$) for the *Q. vulcanica* stems and at least 2.2-fold ($p < 0.001$) for the *Q. aucheri* stems compared to the control group, increasing in correlation to the duration of the applied drought stress. The MDA content showed varying responses in the leaves of *Q. vulcanica* for the drought stress durations compared to the control group (Figure 4b), with the 1-week group resulting in a notable decrease (54.64%, $p < 0.001$), the 2-week group showing minimal change, and the 4-week group leading to a significant 2.38-fold increase ($p < 0.001$) in MDA content, indicating a significant increase in oxidative stress under prolonged (i.e., 4-week) drought conditions. Meanwhile, MDA levels had at least a 1.21-fold increase in the stems of *Q. vulcanica* under all drought stress groups ($p < 0.05$). As for *Q. aucheri*, the MDA content showed a 1.39-fold increase in the leaves ($p < 0.001$) and 1.21-fold increase in the stems ($p < 0.05$) in response to drought stress durations compared to the control group (Figure 4b).

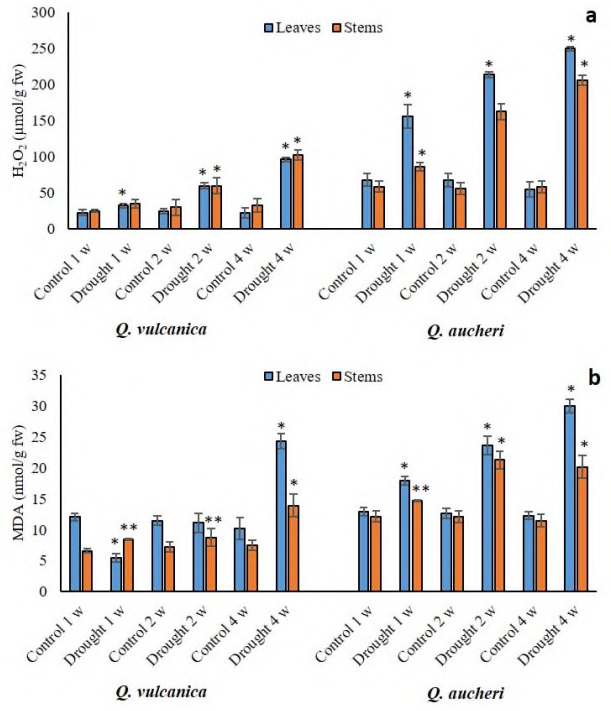

Figure 4. The effects of 1, 2, and 4 weeks of drought stress on (a) H_2O_2 and (b) MDA content in the leaves and stems of Q. vulcanica and Q.aucheri. Data are presented as M ± SD (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by $*p < 0.05$ and $**p < 0.001$).

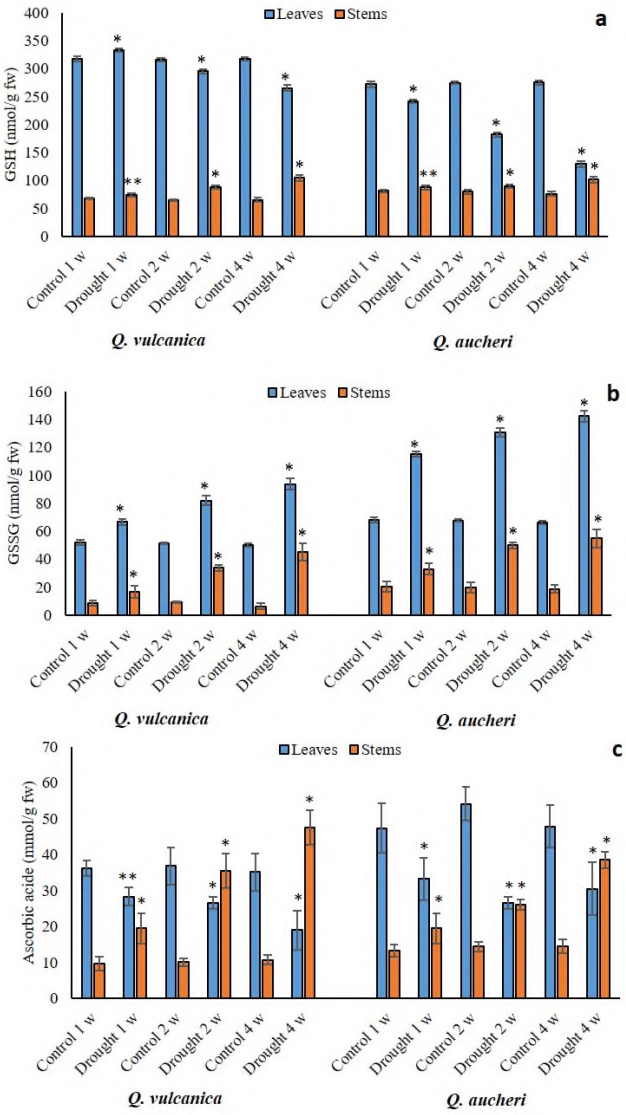

Figure 5. The effects of 1, 2, and 4 weeks of drought stress on the (a) GSH and (b) GSSG and (c) ascorbic acid content levels in the leaves and stems of *Q. vulcanica* and *Q.aucheri*. Data are presented as *M ± SD* (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by $*p < 0.05$ and $**p < 0.001$).

The effect of drought stress on the contents of GSH and GSSG and ascorbic acid

GSH content in the leaves was observed to significantly decrease by at least 6.75% in the 2-week and 4-week drought stress groups for *Q. vulcanica* ($p < 0.001$) and by at least 11.13% in all drought stress groups for *Q. aucheri* ($p < 0.001$), while GSH content increased at least 1.083 fold ($p < 0.05$) in the stems of both oak seedling types for all drought stress groups (Figure 5a).

The GSSG content was observed to significantly increase in the leaves of *Q. vulcanica* by at least 1.28 fold ($p < 0.001$), in the leaves of *Q. aucheri* by at least 1.69 fold ($p < 0.001$), in the stems of *Q. vulcanica* by at least 1.94 fold ($p < 0.001$), and in the stems of *Q. aucheri* by at least 1.67 fold ($p < 0.001$) for the 1-, 2-, and 4-week drought stress groups in line with the applied duration of drought stress (Figure 5b). In accordance with these results, alterations in the GSH+GSSG content were observed in both oak species.

The ascorbic acid content in the leaves of *Q. vulcanica* showed a significant decrease of at least 21.63% ($p < 0.05$), while the stems exhibited an increase of at least 2-fold ($p < 0.001$). Accordingly, the ascorbic acid content in the leaves of *Q. aucheri* showed a significant decrease of at least 29.91% ($p < 0.001$), while the stems exhibited an increase of at least1.47-fold ($p < 0.001$). These changes were observed to increase in line with the applied duration of drought stress (Figure 5c).

The effect of drought stress on antioxidant enzyme activity in oak seedlings' leaves and stems

The antioxidant enzyme activity is shown in Figures 6a-6e. The SOD activities were observed to significantly increase in the respective *Q. vulcanica* and *Q. aucheri* leaves by at least 8.6 fold ($p < 0.001$) or 1.6 fold ($p < 0.001$), and in the respective *Q. vulcanica* and *Q. aucheri* stems by at least 8.2 fold ($p < 0.001$) or 2.5 fold ($p < 0.001$). CAT activities were observed to significantly decrease in the respective *Q. vulcanica* and *Q.*

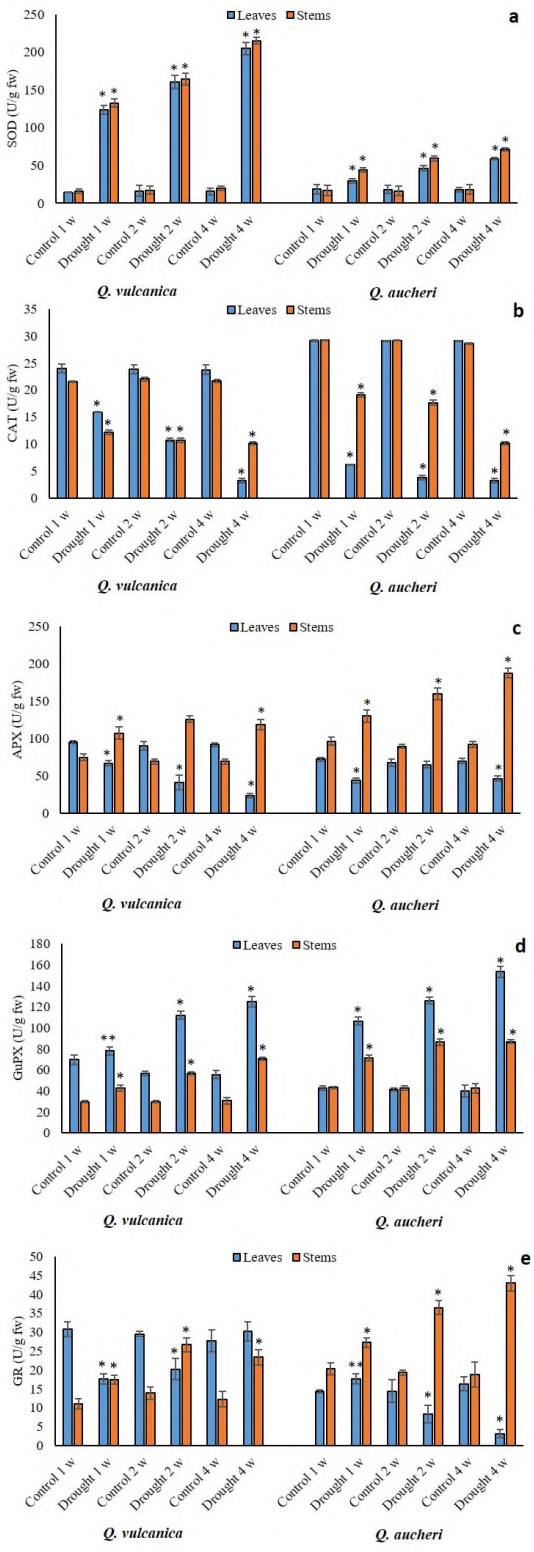

Figure 6. The effects of 1, 2, and 4 weeks of drought stress on the activities of (a) SOD, (b) CAT, (c) APX, (d) GuPx, and (e) GR content levels and (c) ascorbic acid levels in the leaves and stems of *Q. vulcanica* and *Q.aucheri*. Data are presented as mean ± SD. (Statistical analysis was performed using the ANOVA + LSD post hoc test. Statistically significant changes are indicated by $*p < 0.05$ and $**p < 0.001$).

aucheri leaves by at least 33.74% ($p < 0.001$) or 78.85% ($p < 0.001$), and in the respective *Q. vulcanica* and *Q. aucheri* stems by at least 43.58% ($p < 0.001$) or 34.7% ($p < 0.001$). APX activities were observed to significantly decrease by at least 29.7% ($p < 0.001$) in the leaves of *Q. vulcanica* and *Q. aucheri* (excluding the 2-week group), while increased at least 1.35-fold ($p < 0.001$) in the stems of both oak species. GuPx activity was observed to significantly increase in the leaves and stems of *Q. vulcanica* by at least 1.12 fold ($p < 0.05$) and of *Q. aucheri* by at least 1.65 fold ($p < 0.001$). Some alteration occurred regarding GR activity in the leaves of both oak species' seedlings, decreasing in particular in the 4-week drought stress group by at least 80.9% ($p < 0.001$) for *Q. aucheri*, while increasing significantly by at least 1.34-fold ($p < 0.001$) in the stems of both oak species' seedlings.

DISCUSSION

Drought stress is a significant environmental factor that can have profound effects on plant growth and survival. Among the various plant species, oaks (*Quercus* spp.) are particularly important due to their ecological and economic value. Understanding the impact of drought stress on oak species is crucial for effective conservation and management strategies. Therefore, this study has aimed to investigate the effects of drought stress on the physiological, oxidative and antioxidant parameters of two oak species.

Fresh and dry weights for the leaves and stems of *Q. vulcanica* and *Q. aucheri* seedlings is seen to decrease significantly as the applied duration of drought stress increases due to increased water loss and protein degradation (Perales-Vela et al., 2007). Accordingly, RWC also decreased in the leaves and stems of the two oak species under drought stress. Decreased water content has been detected in the leaves of *Q. pubescens* during drought (Gallé & Feller, 2007). Water content has also been seen to decrease after 14 days of drought stress in the leaves of *Q. ilex* (Echevarría-Zomeño et al., 2009) and in the leaves of *Q. ilex, Q. cerris,* and *Q. pubescens* (Cotrozzi et al., 2016), as well as in the shoots of *Q. lusitanica* after 22 weeks (Santamarina et al., 2022). In Cotrozzi, Pellegrini et al.'s (2017) study, a 15-day drought stress period showed no changes in RWC in *Q. ilex*. Landi et al. (2019) showed RWC to decrease in the leaves of *Q. cerris* and *Q. pubescens* (10% and 6%, respectively). The leaf water potential of *Q. infectoria* and *Q. libani* changed significantly in response to 1 month of drought stress (Ghanbary et al., 2020). The RWC and water potential of the leaves of *Q. fabri, Q. serrata, Q. acutissima,* and *Q. variabilis* decreased under continued drought stress (Xiong et al., 2022). As shown, water content in plant tissues decreases as a result of drought stress. This decrease in water quantity causes a decrease in turgor pressure, which is an indicator of water stress. The interaction between plants and drought stress results in an increase in solute concentrations within the cell. Additional im-

pacts of drought stress include reduced cell growth, decreased cell wall and protein synthesis, stomatal closure that reduces CO_2 absorption and respiration, and an increase in osmolytes, including proline and carbohydrates (Öpik & Rolfe, 2005).

Drought alters photosynthetic pigments and impairs photosynthesis due to disruptions in chloroplast structure, decreased chlorophyll production, and stomatal closure that prevents CO_2 from entering the plant. Determining chlorophyll content is one of the best methods used to determine the effects of environmental stress factors such as drought, low and high temperatures, soil and air pollution, and radiation on plants (Baycu et al., 2006). In the present study, the chlorophyll content in the leaves of both *Quercus* species considerably reduced as the length of drought stress increased. Consistent with this study, one study on young *Q. pubescens* trees demonstrated a decrease in chlorophyll content of leaves due to drought stress affecting photosynthetic performance (Gallé & Feller, 2007). Chlorophyll a (fluorescence of photosystem II) decreased in *Q. ilex* after a 15-day drought stress period (Cotrozzi, Pellegrini, et al., 2017). Landi et al (2019) showed chlorophyll a to decrease in the leaves of *Q. cerris* and *Q. pubescens* (by 3% and 37%, respectively). The decrease in chlorophyll content during drought can occur as a result of membrane damage due to oxidative stress (Alonso et al., 2001).

Carotenoids are pigments found in plants that protect the plant against oxidative damage. Carotenoids are weakly bound to proteins within the cell, protecting the pigment from reactions such as oxidation, degradation, and isomerization (Çinar, 2004). Carotenoids are highly efficient at scavenging singlet oxygen and can directly react with hydroxyl radicals, peroxyl radicals, and alkoxyl radicals, thus preventing lipid peroxidation chain reactions (Burton & Ingold, 1984). The current study has observed the carotenoid content in the leaves of *Q. vulcanica* and *Q. aucheri* seedlings to increase significantly compared to the control group, which indicates carotenoid content to protect the plant from damage. Furthermore, the lower carotenoid content in the 2- and 4-week drought stress groups compared to the 1-week stress group indicates that damage is more severe in the 2- and 4-week drought stress periods for both Quercus species. According to Pellegrini et al.'s (2019) results, total carotenoid content increased in severe drought conditions in *Q. ilex*, while slightly decreased in moderate drought for *Q. pubescens*; as for *Q. robur*, a significant decrease was shown under both moderate and severe drought conditions.

The accumulation of free proline, a nitrogenous osmoprotectant in plants, is a response to stress. This study observed the proline content in the leaves of both *Quercus* species to increase significantly under drought stress. Consistent with this, a study on the leaves of a 5-year-old *Q. robur*. clone showed an approximately 70% increase in proline content after 8 days of drought stress (Oufir et al., 2009). Proline content is seen to have increased under drought stress conditions in the leaves of *Q. ilex, Q. cerris,* and *Q. pubescens* (Cotrozzi et al., 2016); in *Q. ilex* (Cotrozzi, Pellegrini, et al., 2017); in *Q. cerris* (Cotrozzi, Remorini, et al., 2017); in *Q. cerris* and *Q. pubescens* (Landi et al., 2019); in *Q. infectoria* (Ghanbary et al., 2020); and in *Q. fabri, Q. serrata, Q. acutissima* and *Q. variabilis* (Xiong et al., 2022), with no change being observed in the proline content in *Q. libani* (Ghanbary et al., 2020). The proline content in the current study increased higher in *Q. aucheri* (2.05-5.15 fold) than in *Q. vulcanica* (1.17-1.48 fold). This shows that proline accumulation in plants can vary from species to species (Kocheva & Georgiev, 2008). Thomas et al. (2002) demonstrated the response of drought tolerance in oak species in Central Europe to be associated with an increase in endogenous nitrogenous osmoprotectants.

Anthocyanins are phenolic compounds found in all parts of higher plants and through their antioxidant properties protect plants against the harmful effects of ROS generated under various abiotic and biotic stresses. This study found the anthocyanin levels in the leaves of *Q. vulcanica* and *Q. aucheri* seedlings to increase significantly depending on the applied duration of drought stress. Thus, the plant increased its anthocyanin content to protect itself against drought stress. The content of free phenolics increased in the roots of *Q. ilex* under 9 days of limited water (Simova-Stoilova et al., 2018) and in the leaves of *Q. libani* (Ghanbary et al., 2020). Pellegrini et al. (2019) showed no change to occur in total phenols in the leaves of *Q. ilex, Q. pubescens,* and *Q. robur*, which they suggested was due to *Q. ilex* having a superior ability to counteract oxidative conditions. The total soluble protein content is considered an important indicator in plants for determining the physiological status of cells under stress conditions. The current study found the total protein content of *Q. vulcanica* and *Q. aucheri* under drought stress to decrease in the leaves while increasing in the stems. Xiong et al. (2022) reported that total soluble protein content increased under drought stress conditions in the leaves of *Q. serrata, Q. acutissima*, and *Q. variabilis*. Some variations have been observed in total soluble protein content in oak species, which suggests drought stress may have different effects on protein metabolism and synthesis in plants.

In biological systems, H_2O_2 forms as a result of enzymatic and non-enzymatic dismutation of superoxide radicals and plays a significant role in free radical biochemistry (Halliwell & Gutteridge, 2015; Karpinski et al., 1999). The present study observed the H_2O_2 content in the leaves and stems of *Q. vulcanica* and *Q. aucheri* seedlings to increase significantly based on the applied duration of drought stress. As a result, the onset of oxidative damage, which causes lipid peroxidation, becomes inevitable in plants under drought stress conditions. Lipid peroxidation is one of the most important mechanisms causing oxidative damage. Therefore, measuring the content of MDA is considered an indicator of cellular breakdown. This study observed high MDA contents in the leaves and stems of both Quercus species, especially in those under 4 weeks drought

stress. Accordingly, H2O2 and MDA contents were seen to increase in the leaves of *Q. ilex, Q. pubescens* and *Q. robur* (Pellegrini et al., 2019), while no change occurred regarding H_2O_2 content in the leaves of *Q. ilex* (Cotrozzi, Pellegrini, et al., 2017). MDA levels have been seen to increase in the leaves of *Q. ilex, Q. cerris, Q. pubescens* (Cotrozzi et al., 2016), and *Q. cerris* (Cotrozzi, Remorini, et al., 2017); in the cotyledons of *Q. ilex* (Simova-Stoilova et al., 2018); and in the leaves of *Q. serrata, Q. acutissima*, and *Q. variabilis* (Xiong et al., 2022) under drought stress. H_2O_2 levels have been seen to increase in the leaves of *Q. pubescens* (Landi et al., 2019) under drought stress. In the present study, the decrease in MDA content in the leaves of *Q. vulcanica* under 1- and 2-weeks of drought stress can be explained by antioxidant components inhibiting lipid peroxidation. In addition, the increase in MDA content in the 4-week drought stress group may be associated with an increase in lipid peroxidation, resulting in depletion of antioxidant capacity based on the duration of drought stress.

Ascorbic acid, which is found in all plant parts and helps protect plant cells from ROS and other electrophilic compounds, plays an important role in the Halliwell-Asada cycle as the electron donor and substrate of the APX enzyme. In addition, ascorbic acid also has important functions in various biochemical processes such as growth promotion, cell division, photosynthesis, and electron transport (Noctor & Foyer, 1998). The current study observed the ascorbic acid content in the leaves of both *Quercus* species to decrease based on the applied duration of drought stress but to increase in the stems, resulting in the leaves being more sensitive than stems in terms of ascorbic acid content under drought stress. Interestingly, Pellegrini et al. (2019) showed total ascorbic acid to increase in the leaves of *Q. robur* but to decrease in *Q. pubescens*. Water limitation caused a slight decrease in total ascorbic acid while increasing the percentage of oxidized ascorbate in root tips on day 3 in *Q. ilex* (Simova-Stoilova et al., 2018). This might suggest that changes in ascorbic acid content could be an important variable in the different parts of plant species. GSH is one of the mechanisms that protect cell DNA, lipoproteins in the cell membrane, and enzymes from ROS and other electrophilic compounds. GSH acts as a substrate for GPX (Glutathione Peroxidase) and dehydroascorbate reductase enzymes. In the Halliwell-Asada cycle, GSH is oxidized to ascorbate and reduced again by GR with the help of NADPH (nicotinamide adenine dinucleotide phosphate) during the regeneration of ascorbic acid (Creissen et al., 1994). The present study observed the GSH content to decrease in the leaves of both *Quercus* species but to increase in the stems. In the leaves of drought-tolerant plants acclimated to severe drought stress, the level of GSH and the GSH:GSSG ratio show a smaller decrease compared to non-acclimated drought-tolerant plants (Khanna-Chopra & Selote, 2007). The decrease in GSH content in the leaves of both *Quercus* species under drought stress in the current study might be attributable to increased oxidative damage, which also results in consistently high levels of GSSG. No change has been observed in the GSH levels in the leaves of *Q. ilex, Q. pubescens*, and *Q. robur* (Pellegrini et al., 2019). GSH levels were seen to increase in the roots of *Q. ilex* on day 6 after having decreased on day 3 without any changes to the GSH:GSSG ration (Simova-Stoilova et al., 2018).

Enzymatic antioxidants such as SOD, CAT, GR, and peroxidases play an important role in the elimination of free radicals and the prevention of oxidative damage at the cellular level (Halliwell & Gutteridge, 2015). According to the current study's results, SOD activity increased in the leaves and stems of both Quercus species, which supports a response to oxidative damage under drought stress. This response is also confirmed by the higher H_2O_2 level found in this study. The CAT enzyme activity in the leaves and stems of *Q. vulcanica* and *Q. aucheri* was observed to decrease significantly under drought stress. APX enzyme activity was observed to decrease in the leaves of *Q. vulcanica* and *Q. aucheri* but to increase in the stems. The increased amount of H_2O_2 can be concluded to deplete the CAT and APX enzyme activity in the leaves. In addition, GuPx enzyme activities in the leaves of *Q. vulcanica* and *Q. aucheri* increased significantly. Some alterations in GR activity were also shown to have occurred; this is thought to be relevant to maintaining the balance of the GSH:GSSG ratio in the cell. Xiong et al. (2022) showed the activities of peroxidase, SOD, and CAT to increase in the leaves of *Q. serrata, Q. serrata, Q. acutissima*, and *Q. variabilis* when under drought stress. According to Simova-Stoilova et al. (2018) the activities of CAT, SOD, and peroxidase in the roots and cotyledons had different profiles in *Q. ilex*, with the roots having high peroxidase and low CAT activity and cotyledones having the opposite effect. Ghanbary et al. (2020) showed drought stress to alter the antioxidant system in *Q. libani* and *Q. infectoria* seedlings. Similar to the current study. other studies have revealed different prevailing metabolic processes to appear in leaves and stems during drought stress and the need for distinct enzymes to deal with the generated ROS.

CONCLUSION

As a current significant environmental issue, drought has been exacerbated globally due to climate change and has varying impacts on countries including Türkiye. This is the first comparative study on the effects of drought stress and tolerance mechanisms on the endemic species of *Q. vulcanica* and *Q. aucheri* in Türkiye. This study has revealed that increasing drought stress significantly affects the physiological and biochemical parameters in *Q. vulcanica* and *Q. aucheri*, and this will contribute to understanding how oak species respond to drought stress and their adaptive strategies. By comparing *Q. vulcanica* and *Q. aucheri* based on the tolerance levels under drought stress, this study is able to suggest that *Q. aucheri* may be more drought tolerant than *Q. vulcanica*. This study is be-

lieved to be able to play a special role in the preservation and continuity of Türkiye's natural plant diversity.

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REFERENCES

Aebi, H. (1984). Catalase in vitro. In *Methods in Enzymology* (Vol. 105, pp. 121-126): Academic Press.

Alonso, R., Elvira, S., Castillo, F., & Gimeno, B. (2001). Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in *Pinus halepensis. Plant, Cell & Environment, 24*(9), 905-916.

Bates, L. S., Waldren, R. a., & Teare, I. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39*, 205-207.

Baycu, G., Tolunay, D., Özden, H., & Günebakan, S. (2006). Ecophysiological and seasonal variations in Cd, Pb, Zn, and Ni concentrations in the leaves of urban deciduous trees in Istanbul. *Environmental Pollution, 143*(3), 545-554.

Beyer Jr, W. F., & Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical Biochemistry, 161*(2), 559-566.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254.

Burton, G. W., & Ingold, K. (1984). β-Carotene: an unusual type of lipid antioxidant. *Science, 224*(4649), 569-573.

Cakmak, I. (1994). Activity of ascorbate-dependent H_2O_2-scavenging enzymes and leaf chlorosis are enhanced in magnesium-and potassium-deficient leaves, but not in phosphorus-deficient leaves. *Journal of Experimental Botany, 45*(9), 1259-1266.

Cotrozzi, L., Pellegrini, E., Guidi, L., Landi, M., Lorenzini, G., Massai, R., . . . Vernieri, P. (2017). Losing the warning signal: drought compromises the cross-talk of signaling molecules in *Quercus ilex* exposed to ozone. *Frontiers in Plant Science, 8*, 1020.

Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Lorenzini, G., Massai, R., . . . Landi, M. (2017). Cross-talk between physiological and metabolic adjustments adopted by *Quercus cerris* to mitigate the effects of severe drought and realistic future ozone concentrations. *Forests, 8*(5), 148.

Cotrozzi, L., Remorini, D., Pellegrini, E., Landi, M., Massai, R., Nali, C., . . . Lorenzini, G. (2016). Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress. *Physiologia Plantarum, 157*(1), 69-84.

Creissen, C. P., Boardbent, P., Kular, B., Reynolds, H., Welburn, A. R., & Mullineaux, P. M. (1994). Manupilation of glutathione reductase in transgenic plants: implications for plants response to environmental stress. *Proc. R. Soc. Edinburg, 102*, 167-175.

Çinar, I. (2004). Carotenoid pigment loss of freeze-dried plant samples under different storage conditions. *LWT-Food Science and Technology, 37*(3), 363-367.

Dhanda, S., & Sethi, G. (1998). Inheritance of excised-leaf water loss and relative water content in bread wheat *(Triticum aestivum). Euphytica, 104*, 39-47.

Echevarría-Zomeño, S., Ariza, D., Jorge, I., Lenz, C., Del Campo, A., Jorrín, J. V., & Navarro, R. M. (2009). Changes in the protein profile of Quercus ilex leaves in response to drought stress and recovery. *Journal of Plant Physiology, 166*(3), 233-245.

Egert, M., & Tevini, M. (2002). Influence of drought on some physiological parameters symptomatic for oxidative stress in leaves of chives *(Allium schoenoprasum). Environmental and Experimental Botany, 48*(1), 43-49.

Foyer, C. H., & Halliwell, B. (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta, 133*, 21-25.

Gallé, A., & Feller, U. (2007). Changes of photosynthetic traits in beech saplings *(Fagus sylvatica)* under severe drought stress and during recovery. *Physiologia Plantarum, 131*(3), 412-421.

Ghanbary, E., Tabari Kouchaksaraei, M., Zarafshar, M., Bader, K. F. M., Mirabolfathy, M., & Ziaei, M. (2020). Differential physiological and biochemical responses of *Quercus infectoria* and *Q. libani* to drought and charcoal disease. *Physiologia Plantarum, 168*(4), 876-892.

Gossett, D. R., Millhollon, E. P., & Lucas, M. C. (1994). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop science, 34*(3), 706-714.

Halliwell, B., & Gutteridge, J. M. (2015). *Free Radicals in Biology and Medicine*: Oxford university press.

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics, 125*(1), 189-198.

Horwitz, W. (1970). *Official methods of analysis of the Association of Official Analytical Chemists.* Washington, DC: The Association Washington, DC.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., & Mullineaux, P. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. *Science, 284*(5414), 654-657.

Khanna-Chopra, R., & Selote, D. S. (2007). Acclimation to drought stress generates oxidative stress tolerance in drought-resistant than-susceptible wheat cultivar under field conditions. *Environmental and Experimental Botany, 60*(2), 276-283.

Kocheva, K. V., & Georgiev, G. I. (2008). Changes in foliar proline concentration of osmotically stressed barley. *Zeitschrift für Naturforschung C, 63*(1-2), 101-104.

Landi, M., Cotrozzi, L., Pellegrini, E., Remorini, D., Tonelli, M., Trivellini, A., . . . Vernieri, P. (2019). When "thirsty" means "less

able to activate the signalling wave trigged by a pulse of ozone": A case of study in two Mediterranean deciduous oak species with different drought sensitivity. *Science of the Total Environment, 657*, 379-390.

Lichtenthaler, H. K. (1996). Vegetation stress: an introduction to the stress concept in plants. *Journal of Plant Physiology, 148*(1-2), 4-14.

Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. In: Portland Press Ltd.

Mancinelli, A. L. (1990). Interaction between light quality and light quantity in the photoregulation of anthocyanin production. *Plant Physiology, 92*(4), 1191-1195.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends in Plant Science, 11*(1), 15-19.

Nakano, Y., & Asada, K. (1987). Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant and Cell Physiology, 28*(1), 131-140.

Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology, 49*(1), 249-279.

Noctor, G., Reichheld, J. P., & Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Semin Cell Dev Biol, 80*, 3-12. doi:10.1016/j.semcdb.2017.07.013

Oufir, M., Schulz, N., Vallikhan, P. S. S., Wilhelm, E., Burg, K., Hausman, J.-F., . . . Guignard, C. (2009). Simultaneous measurement of proline and related compounds in oak leaves by high-performance ligand-exchange chromatography and electrospray ionization mass spectrometry for environmental stress studies. Journal of *Chromatography A, 1216*(7), 1094-1099.

Ozden, H., & Bayçu, G. (2004). Cadmium exposure and changes in some physiological parameters of *Quercus robur ssp. robur* L. (common oak) and Acer negundo L. (box elder) seedlings. *Fresenius Environmental Bulletin, 13*, 268-273.

Öpik, H., & Rolfe, S. A., (2005) *The physiology of flowering plants.* Cambridge, UK: Cambridge University Press.

Pellegrini, E., Hoshika, Y., Dusart, N., Cotrozzi, L., Gérard, J., Nali, C., Paoletti, E. (2019). Antioxidative responses of three oak species under ozone and water stress conditions. *Science of the Total Environment, 647*, 390-399.

Perales-Vela, H. V., González-Moreno, S., Montes-Horcasitas, C., & Cañizares-Villanueva, R. O. (2007). Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (Chlorophyceae). *Chemosphere, 67*(11), 2274-2281.

Rao, K. M., Raghavendra, A. S., & Reddy, K. J. (2006). *Physiology and molecular biology of stress tolerance in plants*: Springer Science & Business Media.

Santamarina, S., Montesinos, D., Alfaro-Saiz, E., & Acedo, C. (2022). Drought affects the performance of native oak seedlings more strongly than competition with invasive crested wattle seedlings. *Plant Biology, 24*(7), 1297-1305.

Schwanz, P., Picon, C., Vivin, P., Dreyer, E., Guehl, J.-M., & Polle, A. (1996). Responses of antioxidative systems to drought stress in pendunculate oak and maritime pine as modulated by elevated CO2. *Plant Physiology, 110*(2), 393-402.

Simova-Stoilova, L. P., López-Hidalgo, C., Sanchez-Lucas, R., Valero-Galvan, J., Romero-Rodríguez, C., & Jorrin-Novo, J. V. (2018). Holm oak proteomic response to water limitation at seedling establishment stage reveals specific changes in different plant parts as well as interaction between roots and cotyledons. *Plant Science, 276*, 1-13.

Suzuki, N., & Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum, 126*(1), 45-51.

Thomas, F. M., Blank, R., & Hartmann, G. (2002). Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. *Forest Pathology, 32*(4-5), 277-307.

Velikova, M., Bankova, V., Sorkun, K., Houcine, S., Tsvetkova, I., & Kujumgiev, A. (2000). Propolis from the Mediterranean region: chemical composition and antimicrobial activity. *Zeitschrift für Naturforschung C, 55*(9-10), 790-793.

Xiong, S., Wang, Y., Chen, Y., Gao, M., Zhao, Y., & Wu, L. (2022). Effects of drought stress and rehydration on physiological and biochemical properties of four oak species in China. *Plants, 11*(5), 679.

Yordanov, I., Velikova, V., & Tsonev, T. (2000). Plant responses to drought, acclimation, and stress tolerance. *Photosynthetica, 38*, 171-186.

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Original Article

Organ-specific antioxidant capacities and cytotoxic effects of *Thermopsis turcica* extracts in breast cancer

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ABSTRACT

Background and Aims: *Thermopsis turcica* is an endemic species present in Türkiye and it is seen as a source of functional compounds such as antioxidant phenolics. Even though some biological activities of the aerial parts of *T. turcica* have been determined, knowledge regarding the organ-specific chemical composition and effects on human breast cancer is still scarce. Therefore, the present study aims to evaluate the antioxidant capacities, phenolic acid profiles, and potential biological activities of methanol extracts obtained from the leaf, flower, and stem tissues of *T. turcica*.

Methods: The antioxidant capacities of methanol extracts of *T. turcica* was tested with complementary methods (TAC, CUPRAC, FRAP, and DPPH). While the total phenol (TPC) and flavonoid contents (TFC) of the extracts were determined spectrophotometrically, their phenolic acid profiles were determined by high-performance liquid chromatography (HPLC). The cytotoxic effects of extracts on the human normal breast cell line (MCF-10A cells) and the breast tumor cell lines (MCF7, MDA-MB-231, and SKBR3) were also analyzed after 24 h treatment.

Results: The leaf extracts were found to have higher antioxidant capacity, which was associated with the presence of higher amounts of TPC and TFC. The HPLC analysis revealed the presence of quercetin, hesperidin, and rosmarinic acid as the main compounds in the leaf extracts, while a high amount of benzoic acid was found in the flower extract. Leaf and flower extracts also showed stronger cytotoxic activity against MCF-7 cells (IC_{50} values were 0.65 mg/mL and 0.55 mg/mL, respectively) as compared to stem extract (IC_{50} value was 1.10 mg/mL). Leaf extracts were the most active extract against SKBR3 cells with IC_{50} of 0.75 mg/mL. All extracts exhibited weak cytotoxic effects against MDA-MB-231 cells and IC_{50} values (1.53-1.75 mg/mL) were similar to the MCF-10A cells (IC_{50} values: 1.59-1.69 mg/mL).

Conclusion: In conclusion, extracts derived from *T. turcica* have the potential to serve as a valuable source of bioactive metabolites with antioxidant and antiproliferative properties.

Keywords: Antioxidant capacity, breast cancer, cytotoxic activity, phenolic content, *Thermopsis turcica*

INTRODUCTION

Plants produce a wide variety of substances, including biologically active compounds formed during secondary metabolism (Salmeron-Manzano, Garrido-Cardenas, & Manzano-Agugliaro, 2020). In addition to their ecological importance, these phytochemicals have important applications in industries such as pharmacology (Leicach & Chludil, 2014). Among secondary metabolites, phenolic compounds are taken into consideration because of their significant effects on plant metabolism. Their response to biotic and abiotic factors and signaling mechanisms are excellent examples (Lone et al., 2023). Investigations can show the characteristics of various plants and can lead to new perspectives for several industrial materials due to their antifungal, antimicrobial, antibacterial, antiviral, antitumor, and antioxidant properties (Manzoor, Yousuf, Pandith, & Ahmad, 2023). Phenolic compounds have potential pharmacological properties especially in the daily diet due to their radical scavenging activity (Elgadir, Chigurupati, & Mariod, 2023). Therefore, they have considerable economic attention (Elshafie, Camele, & Mohamed, 2023).

Thermopsis is a genus of the Fabaceae family spread over

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the temperate areas of North America and East Asia (Wojciechowski, 2003). The *Thermopsis* genus includes an important plant species with high medicinal value. For instance, it is known that *Thermopsis lanceolate* has many pharmacological effects such as antimicrobial and anticancer (Zhang et al., 2022). Recently, it has been shown that ethanol extracts of *Thermopsis rhombifolia* aerial parts showed the *in vitro* cytotoxicity and antiproliferative effect against colorectal adenocarcinoma (HT-29), malignant glioblastoma (M059K) and normal lung fibroblast (WI-38) cell lines. Furthermore, flavone luteolin isolated from *T. rhombifolia* has shown to have the potential to arrest the cell cycle by inhibiting protein kinase activity (Tuescher et al., 2020). *Thermopsis turcica* is a poisonous plant and is an endemic species spreading in a narrow area in southwestern Turkey (Tan, Vural, & Küçüködük, 1983). Previous studies demonstrated that various extracts of *T. turcica* have antimicrobial, antioxidant, and anticancer activities (Liman, Eren, Akyil, & Konuk, 2012; Bali et al., 2014; Yıldız et al., 2020). In a previous study, Bali et al. (2014) showed that ethanol and ethyl acetate extracts (20-100 µg/mL) from the aerial parts of *T. turcica* had substantial antiproliferative effects on promyelocytic leukemia cells while being relatively nontoxic to human gingival fibroblast cells. However, methanol extracts (0.5-2.5 mg/mL) of the flower and leaf tissues of *T. turcica* have been shown to have cytotoxic activity against HeLa cells lines (Yıldız et al., 2020).

Aksoy, Kolay, Ağılönü, Aslan, & Kargıoğlu (2013) reported that methanol and acetone extracts of the aerial parts of *T. turcica* have high phenolic content and accordingly high antioxidant capacity. Similarly, total *T. turcica* extracts prepared with different solvents were found to have antioxidant and cytotoxic effects (Bali et al., 2014). To our knowledge, no organ-specific antioxidant and biological activities have been reported in *T. turcica* extracts. In this study, therefore, it was aimed to determine total phenolic and flavonoid contents, total antioxidant activity, free radical scavenging activity, and phenolic acid profiles in methanol extracts of the leaf, flower, and stem tissues of *T. turcica*. Furthermore, the organ-specific cytotoxic effects of *T. turcica* extracts on human breast cancer cell lines were evaluated.

MATERIALS AND METHODS

Plant collection and preparation of extracts

The aerial parts of *Thermopsis turcica* were collected at undisturbed areas near Lake Eber, Afyonkarahisar, Türkiye. The plant specimen was identified by co-author Dr. Mustafa Yıldız. The aerial parts were separated into leaf, flower, and stem tissues and dried under laboratory conditions (in shade at room temperature). It has been suggested that methanol is the effective solvent for extracting phenolic compounds from plants (Cheynier, 2012). Therefore, dried tissues (3 g) were finely powdered and incubated overnight with 30 mL methanol at +4°C. After filtration with filter paper, extracts were vacuum-dried with a rotary evaporator at 50°C. For the determination of phenolic contents and antioxidant capacities, a portion of dry extracts (10 mg/mL) was dissolved in methanol. Another portion of extracts (10 mg/mL) was dissolved in 0.1% dimethyl sulfoxide (DMSO) to determine cytotoxic effects on breast cancer cell lines.

Determination of total phenolic and flavonoid contents

The total phenolic content (TPC) in the extracts (1 mg/mL) was determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). The TPC was determined by the gallic acid (GA) standard (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL) curve and presented as gallic acid equivalents (µg GAE/mg extract). The total flavonoid content (TFC) in the extracts (1 mg/mL) was evaluated by the aluminum chloride colorimetric method of Deng & van Verkel (1998). The TFC was determined by the quercetin (Q) standard (10, 20, 30, 40, and 50 µg/mL) curve and expressed as quercetin equivalents per mg of extracts (µg QE/mg extract).

Determination of antioxidant capacity

The antioxidant capacities of *T. turcica* extracts were determined via four *in vitro* methods (TAC, CUPRAC, FRAP, and DPPH assays). The total antioxidant capacities (TAC) of the extracts (1 mg/mL) were determined through the phosphomolybdenum assay (Prieto, Pineda, & Aguilar, 1999). The antioxidant capacities were expressed as µg ascorbic acid (AA) equivalents per mg of extract (µg AAE/mg extract). The cupric ion-reducing antioxidant capacities (CUPRAC) of the extracts (1 mg/mL) were determined according to the total antioxidant capacity measurement method based on the Cu^{2+} reducing capacity (Apak et al., 2007). The CUPRAC results were expressed as trolox (TR) equivalents per mg of extracts (mM TRE/mg extract). The ferric-reducing ability potential (FRAP) of the extracts (0.5 mg/mL) was determined according to the method based on the reduction of $[Fe\ (III)\ (TPTZ)_2]^{3+}$ to $[Fe\ (II)\ (TPTZ)_2]^{2+}$ (Tuberoso et al., 2010). The FRAP results were expressed as trolox equivalents per mg of extracts (mM TRE/mg extract). The free radical scavenging activities of the extracts (0.1-2 mg/mL) were determined according to the DPPH (2,2-diphenyl-1-picrylhydrazil) method (Espín, Soler-Rivas, & Wichers, 2000). Ascorbic acid was used as a positive control, and DPPH scavenging capacity was calculated using the equation:

$$\text{Inhibition of DPPH radical (\%)} = [(Abs_{Methanol} - Abs_{Extract})/Abs_{Methanol}] \times 100$$

Analysis of phenolic compounds via HPLC

Quantitative analysis of phenolic components was carried out using a chromatographic system (Agilent 1200) coupled with an UV-diode array detector (DAD) and a reversed-phase column Supelco LC18 (250 × 4.6 mm^2, 5 µm). The leaf, flower, and stem extracts (10 mg/mL) of *T. turcica* were prepared in HPLC-grade methanol. After centrifugation at 10,000 × g for 10 min, the resulting supernatants were filtered using 0.45 µm filters. The injection volume was 20 µL and the flow rate was 0.8 mL min^{-1}. UV region at 278 nm was used for peak detection. The mobile phase consisted of acetic acid (2%) and methanol. The quantifications were calculated by comparing the peak surface areas with phenolic compounds standards of 3-hydroxy benzoic acid, benzoic acid, caffeic acid, catechin hydrate, chlorogenic acid, epicatechin, gallic acid, hesperidin, p-coumaric acid, quercetin, rosmarinic acid, sinapic acid, syringic acid, t-cinnamic acid, and t-ferulic acid (Caponio, Alloggio, & Gomes, 1999). The method was evaluated according to Koc et al. (2020). The correlations of standard curves of each phenolic substance are given in Table 2. The phenolic compounds were identified by comparing their retention time and UV spectra with those obtained from standard solutions. Quantification of phenolic components was performed by normalization method based upon the area percent reports obtained by HPLC-DAD.

Cell culture and viability assay

The human normal breast cell line (MCF-10A cells) and the breast tumor cell lines (MCF7, MDA-MB-231, and SKBR3) were obtained from Medicinal Genetics Department, Afyonkarahisar Health Sciences University. The human normal breast cell line (MCF-10A cells) was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, and 1% penicillin-streptomycin. The breast tumor cell lines (MCF7, MDA-MB-231, and SKBR3) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% penicillin-streptomycin. Cells were grown in a humidified incubator set at 37°C with 5% CO_2. The viability of cells was assessed using the WST-1 assay (Roche Diagnostics, Switzerland). Briefly, cells were seeded in a 96-well plate at a density of $1x10^4$ cells per well. After 24 hours of incubation at 37°C, the cells were treated with varying concentrations (0 – 4 mg/mL) of leaf, flower, and stem extracts of *T. turcica* or 0.1% DMSO for 24 hours. Following this treatment period, 10 µL of WST-1 reagent was added to each well and further incubated for 4 hours. Optical absorbance was measured using a Multiscan GO microplate reader (Thermo Scientific, USA) at a wavelength of 450 nm. The IC_{50} values were calculated from the linear regression of the dose-log response curves.

Statistical analysis

All statistical analyses were performed using SPSS software (version 22.0, SPSS, USA). For the comparisons of means, one-way ANOVA followed by post hoc test (Tukey's test) was employed. Values are expressed as the mean ± standard error.

RESULTS

Alterations in total phenolic and flavonoid contents

TPC and TFC of the different tissue extracts of *T. turcica* are presented in Table 1. The highest concentrations for TPC and TFC were found for leaf extract (145.8 ± 5.9 µg GAE/mg extract, and 76.6 ± 1.3 µg QE/mg extract, respectively), followed by flower extract (87.2 ± 3.6 µg GAE/mg extract, and 53.7 ± 4.2 µg QE/mg extract, respectively). The lowest values were determined in stem extract (TPC; 70.8 ± 4.9 µg GAE/mg extract, TFC; 32.2 ± 2.4 µg QE/mg extract).

Table 1. Total phenolic content (TPC), total flavonoid content (TFC), and *in vitro* antioxidant capacities (TAC, CUPRAC, and FRAP) of the different tissue extracts of *T. turcica*.

Parameters	Plant tissues		
	Leaf	Flower	Stem
TPC (µg GAE/mg extract)	145.8 ± 5.90	87.2 ± 3.63 [a]	70.8 ± 4.88 [a]
TFC (µg QE/mg extract)	76.6 ± 1.26	53.7 ± 4.24 [a]	32.2 ± 2.36 [a,c]
TAC (µg AAE/mg extract)	110.3 ± 2.10	87.2 ± 3.53 [a]	94.7 ± 2.82 [b]
CUPRAC (mM TRE/mg extract)	1.13 ± 0.06	0.62 ± 0.03 [a]	0.48 ± 0.06 [a]
FRAP (mM TRE/mg extract)	1.26 ± 0.04	0.58 ± 0.03 [a]	0.39 ± 0.04 [a,d]

a P<0.001 vs Leaf group, b P<0.01 vs Leaf group, c P<0.001 vs Flower group, d P<0.01 vs Flower group.

Alterations in antioxidant capacity

Antioxidant capacities of the leaf, flower, and stem extracts from *T. turcica* evaluated using four complementary assays are given in Table 1. All tissue extracts exerted a total antioxidant capacity, the most active being leaf extract (110.3 ± 2.1 µg AAE/mg extract), followed by stem extract (94.7 ± 2.8 µg AAE/mg extract) and flower extract (87.2 ± 3.5 µg AAE/mg extract). The highest antioxidant capacity was detected for leaf extract, both in CUPRAC and FRAP assays (1.13 ± 0.06 mM TRE/mg extract and 1.26 ± 0.04 mM TRE/mg extract, respectively), followed by flower extract (0.62 ± 0.03 mM TRE/mg extract and 0.58 ± 0.03 mM TRE/mg extract, respectively). The stem extract displayed the lowest antioxidant capacity in CUPRAC and FRAP assays (Table 1). All tested *T. turcica* extracts showed the potential to reduce DPPH (Figure 1). Results

showed that tissue extract differentially affected the antioxidant capacity. The best reducer of DPPH was leaf extract after the positive control ascorbic acid.

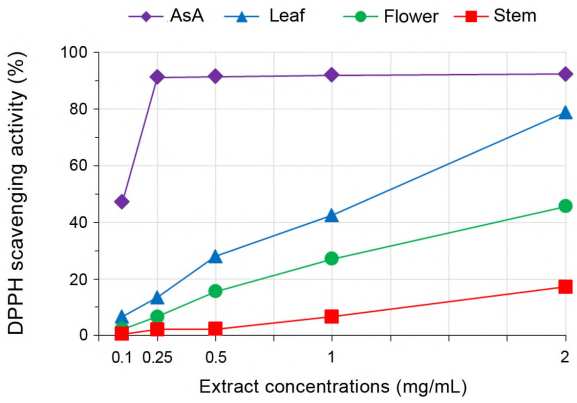

Figure 1. DPPH radical-scavenging activity of the leaf, flower, and stem extracts of *T. turcica.*

Phenolic acid composition of *T. turcica* extracts

Sixteen phenolic acids were analyzed by reverse-phase HPLC. The HPLC chromatograms obtained from the leaf, flower, and stem extracts showed similar phenolic profiles (Figure 2). In order of retention time, the phenolic compounds are given in Table 2. Among them, 3-hydroxybenzoic acid was detected in the leaf and stem samples, while it was not detected in the flower samples. Catechin hydrate and caffeic acid were determined only in the leaves, whereas sinnapic acid was determined only in the flowers. Moreover, syringic acid was not detected in all tissues. Among the sixteen phenolic compounds, the most abundant phenolic acids were quercetin (58.11 ± 0.48 µg/g DW), hesperidin (29.12 ± 1.29 µg/g DW), and rosmarinic acid (11.77 ± 2.34 µg/g DW) in the leaf tissues. Additionally, hesperidin, quercetin, rosmarinic acid, t-cinnamic acid, and gallic acid were found in the leaves more than in stem and flower samples. Moreover, benzoic acid (46.24 ± 3.86 µg/g DW) was found as the main compound in the flower extract of *T. turcica* (Table 2).

The cytotoxic effects of *T. turcica* extracts on breast cancer cell lines

The cytotoxic effects of the different tissue extracts of *T. turcica* on the cell lines are shown in Figure 3. We observed that *T. turcica* extracts induced a significant decrease in the viability of MCF7, MDA-MB-231, and SKBR3 cells with increasing extract concentration. Determination of IC_{50} values for different tissue extracts of *T. turcica* on the cell lines exhibited various inhibitory patterns (Table 3). The leaf extracts of *T. turcica* manifested IC_{50} values of 1.63 ± 0.01 mg/mL, 0.65 ± 0.19 mg/mL, 1.62 ± 0.03 mg/mL, and 0.75 ± 0.18 mg/mL for MCF-10A, MCF7, MDA-MB-231, and SKBR3 cells, respectively. Of note, MCF7 ($P < 0.05$) and SKBR3 cells ($P < 0.05$) showed significantly lower IC_{50} values compared to those of MCF-10A cells. For the flower extracts, the IC_{50} values were found to be 1.59 ± 0.01 mg/mL for MCF-10A, 0.55 ± 0.02 mg/mL for MCF7, 1.53 ± 0.02 mg/mL for MDA-MB-231, and 1.11 ± 0.08 mg/mL for SKBR3 cells. Notably, the IC_{50} values were significantly lower in both MCF7 ($P < 0.001$) and SKBR3 ($P < 0.05$) cells when compared to MCF-10A cells. Similarly, *T. turcica* stem extracts showed IC_{50} values of 1.69 ± 0.04 mg/mL, 1.10 ± 0.58 mg/mL, 1.75 ± 0.06 mg/mL, and 1.30 ± 0.04 mg/mL for MCF-10A, MCF7, MDA-MB-231, and SKBR3 cells, respectively. The IC_{50} value of SKBR3 cells ($P < 0.05$) was significantly lower compared to that of MCF-10A cells (Table 3).

DISCUSSION

TPC is a crucial factor in determining the overall antioxidant capacity and is commonly employed to assess the antioxidant attributes of plant-based materials (Lamuela-Raventós, 2018). Given the diverse array of phenolic compounds and antioxidant constituents present in plants, each varying in structure, size, and polarity, the choice of extraction solvents can significantly impact the outcomes of such analyses (Xu et al., 2017). Our results showed significant differences in TPC and TFC of the different tissue extracts from *T. turcica*. The highest TPC and TFC of the extracts were obtained from the leaf extracts. In a previous study, Bali et al. (2014) evaluated the TPC of ethyl acetate, ethanol, and methanol extracts of the total aerial parts of *T. turcica* plants. Authors determined the highest TPC value in ethyl acetate followed by methanol extracts and the results ranged from 162.5 ± 1.2 to 44.9 ± 0.90 µg gallic acid/mg of dry extract. However, the highest TPC values were obtained when acetone was used as a solvent (Aksoy et al., 2013). Methanol extracts in plants have been found to contain high TPC (Molole, Gure & Abdissa, 2022), indicating better solubility of these compounds in polar solvents. Overall, the higher phenolic substance content in leaves is a well-documented phenomenon supported by scientific evidence. Understanding the role of phenolic compounds in leaves can provide valuable insights into plant defense mechanisms and potential health benefits. Further research in this area is warranted to explore the full potential of phenolic compounds in leaves.

It is known that there is a significant correlation between antioxidant capacity and phenolic substance content of medicinal plants (Cai, Luo, Sun, & Corke, 2004). *T. turcica* has been suggested as a natural source of antioxidants due to the phytochemicals of the aerial parts of the plant (Aksoy et al., 2013). Previous studies have shown that ethanol and water extracts of *T. turcica* had antioxidant effects (Çelik & Küçükkurt, 2016). Ethyl acetate, methanol, and ethanol extracts were also mentioned to be effective antioxidants due to the quantity of their

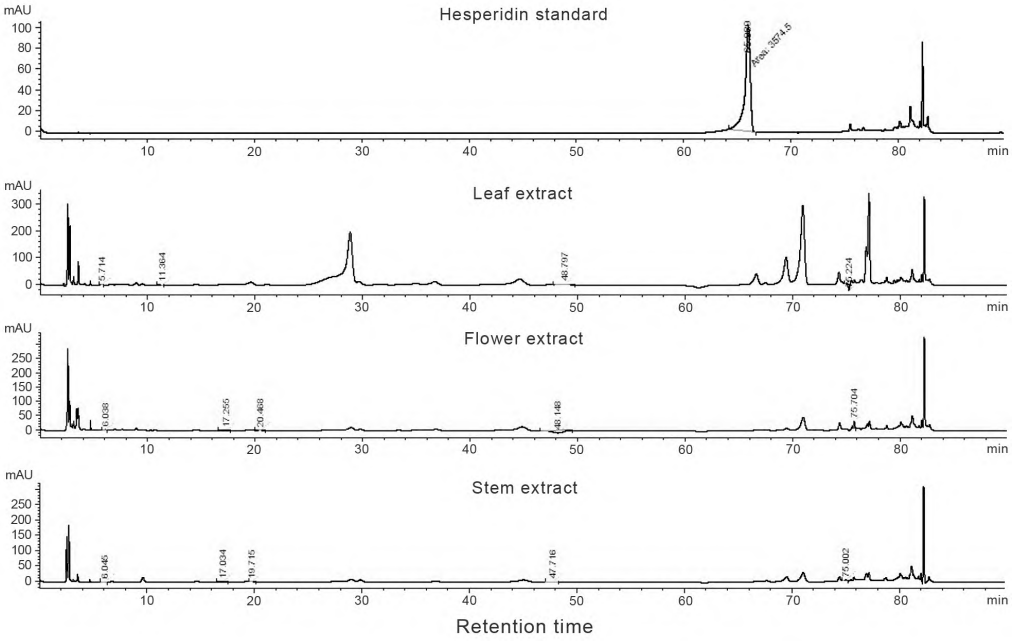

Figure 2. Representative HPLC chromatograms of the hesperidin standard and phenolic acids in the methanolic extracts of *T. turcica* tissues.

Table 2. Quantitative changes in phenolic compounds in different tissue extracts of *T. turcica.*

Phenolic compounds	Correlation (r^2)	RT (min)	Leaf (µg/g DW)	Flower (µg/g DW)	Stem (µg/g DW)
Gallic acid	0.99966	5.912	1.21 ± 0.17	0.92 ± 0.16	0.59 ± 0.12 [c]
Catechin hydrate	0.99906	11.499	3.00 ± 0.41	*ND*	*ND*
Chlorogenic acid	0.99970	16.239	*ND*	1.03 ± 0.02	*UC*
4-Hydroxy benzoic acid	0.99994	17.647	*UC*	0.89 ± 0.08	*ND*
Epicatechin	0.99879	20.169	*ND*	5.26 ± 0.30 [c]	7.64 ± 1.23 [b]
Caffeic acid	0.99892	21.476	1.09 ± 0.01	*ND*	*ND*
3-Hydroxy benzoic acid	0.99928	22.545	1.71 ± 0.45	*ND*	1.16 ± 0.04
Syringic acid	0.99839	22.628	*ND*	*ND*	*ND*
p-Coumaric acid	0.99982	33.597	0.57 ± 0.02	1.69 ± 0.12 [a]	0.62 ± 0.13 [d]
t-Ferrulic acid	0.99993	37.202	0.36 ± 0.09	0.28 ± 0.11	0.06 ± 0.02
Sinnapic acid	0.99925	38.264	*ND*	0.38 ± 0.05	*ND*
Benzoic acid	0.99986	47.629	21.07 ± 2.84	46.25 ± 3.86 [b]	*UC*
Hesperidin	0.99705	65.989	29.12 ± 1.29	4.79 ± 0.25 [a]	3.59 ± 0.13 [a]
Rosmarinic acid	0.99907	70.655	11.78 ± 2.34	3.21 ± 0.16 [b]	2.70 ± 0.07 [b]
t-Cinnamic acid	0.99998	75.207	2.21 ± 0.45	1.07 ± 0.01 [c]	0.46 ± 0.04 [b]
Quercetin	0.99962	76.313	58.11 ± 0.48	8.66 ± 0.66 [a]	14.10 ± 0.38 [a,e]

[a] P<0.001 vs Leaf group, [b] P<0.01 vs Leaf group, [c] P<0.05 vs Leaf group, [d] P<0.001 vs Flower group, [e] P<0.01 vs Flower group. *ND* = Not determined. *UC* = Uncalculated.

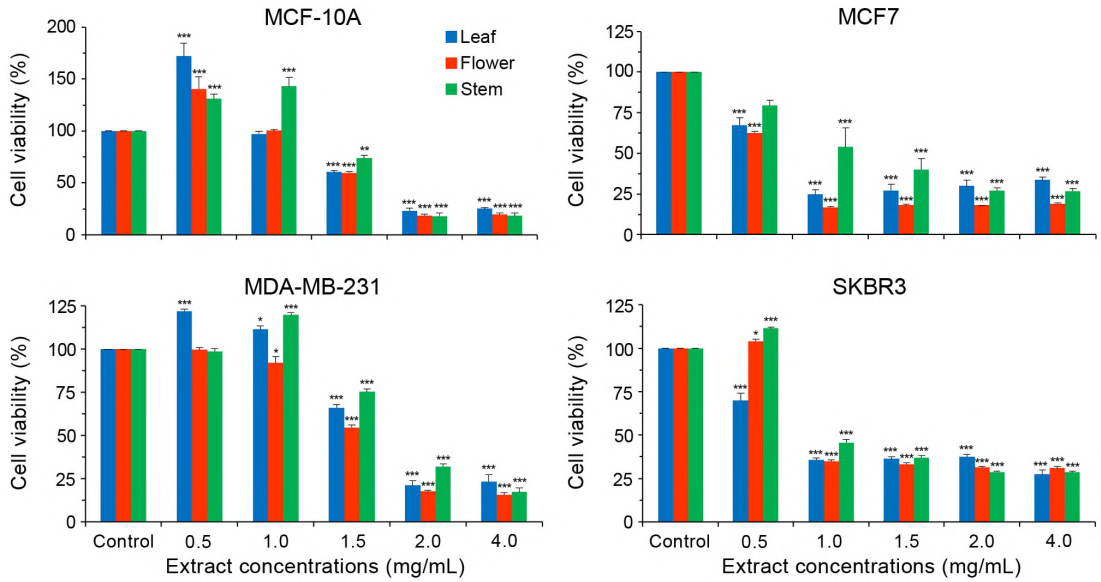

Figure 3. Cytotoxic effects of the leaf, flower, and stem extracts of *T. turcica* on the normal and breast tumor cell lines. Data is presented as mean ± SE. ***P < 0.001, **P < 0.01, and *P < 0.05 compared with the control group.

Table 3. IC_{50} values of the different tissue extracts of *T. turcica* for the normal and breast tumor cell lines.

Cell lines	Leaf	Flower	Stem
	IC_{50} values (mg/mL)		
MCF-10A	1.63 ± 0.01	1.59 ± 0.01	1.69 ± 0.04
MCF7	0.65 ± 0.19 [a]	0.55 ± 0.02 [b]	1.10 ± 0.58
MDA-MB-231	1.62 ± 0.03	1.53 ± 0.02	1.75 ± 0.06
SKBR3	0.75 ± 0.18 [a]	1.11 ± 0.08 [a]	1.30 ± 0.04 [a]

[a] P<0.05 vs MCF-10A group, [b] P<0.001 vs MCF-10A group.

total phenolic compounds (Bali et al., 2014). In our study, the results showed that leaf extracts exhibited antioxidant capacity more than flower and stem extracts. Indeed, TPC and TFC were highly correlated with the antioxidant capacity measured by TAC, CUPRAC, FRAP, and DPPH assays. This result suggested that there is a relationship between antioxidant capacity and the content of phenolic acids or flavonoid compounds for all extracts. Sinan et al. (2023) suggested the high antiradical and antioxidant activity of methanol extracts could be attributed to their high total phenolic and flavonoid contents. Kumar and Goel (2019) reported that substituents on the aromatic ring in phenolic acids impact the stabilization of the structure, thus influencing the radical-quenching ability. In fact, the antioxidant activity of the extracts may also be associated with other compounds with a specific antioxidant potential (Huang, Ou, & Prior, 2005).

Plant phenolics such as simple phenols, phenolic acids, and flavonoids are a special class of secondary metabolites. In addition to their important functions in plant metabolism, phenolic acids are the precursors of many bioactive compounds beneficial for human health (Kumar & Goel, 2019). There are no studies in the literature on the phenolic acid profiles of *T. turcica* extracts. In the present study, therefore, phenolic acid profiles of the leaf, flower, and stem extracts of *T. turcica* were analyzed qualitatively and quantitatively. Our findings revealed that there are organ-specific differences in the phenolic acid profiles of extracts. Among the analyzed sixteen phenolic compounds, hesperidin, quercetin, and rosmarinic acid were found as the main compounds in leaf extracts, while benzoic acid content was remarkable in the flower extracts of *T. turcica*. The health benefits of phenol compounds are linked to their function in preventing various ailments associated with the destructive impact of free radicals and ROS (Valko et al., 2007). Hesperidin, a flavonoid that falls under the flavanone group, has been demonstrated to have significant antioxidant, anti-inflammatory, and neuroprotective effects in various models of central nervous system disorders (Muhammad et al., 2019). Furthermore, hesperidin's anticancer potential has been described through different mechanisms of action (Pandey & Khan, 2021). Quercetin, another flavonoid, possesses potent antioxidant properties that allow it to scavenge free radicals, decrease oxidative stress, and safeguard against cellular damage. Quercetin's anti-inflammatory properties involve the inhibition of inflammatory cytokines and enzymes, making it a potential therapeutic agent for various inflammatory conditions (Aghababaei & Hadidi, 2023). Rosmarinic acid, which possesses antioxidant and anti-inflammatory properties, has been observed to have positive effects on cancer disease (Ijaz et al., 2023).

Breast cancer is one of the most marked common malignant tumors among women (Wang et al., 2022). The use of plant-

derived products in cancer treatment has gained great importance in recent years. Plant phenolics exert a great potency for the prevention and treatment of oxidative stress-related disorders such as cancer (Abotaleb, Liskova, Kubatka, & Büsselberg, 2020). Among the flavonoid components, quercetin is suggested to overcome tumor cells via modulation of proliferation and apoptosis. Previous research has demonstrated that quercetin modulates several signal pathways to inhibit the progression of breast cancer (Ranganathan, Halagowder, & Sivasithambaram, 2015; Liu, Lee, & Ahn, 2019). Hesperidin is a flavonoid that possesses various biological activities, suggesting therapeutic potential in the treatment of cancer (Madureira et al., 2023). Recently, Önder et al. (2023) reported that hesperidin exerts cytotoxic effects by inhibiting cellular proliferation and inducing apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines. Benzoic acid and its derivatives, which are included in a class of simple phenolic acids, have been reported to have biological activities such as inhibiting the growth of breast cancer cells (Lin, Chen, Chou, & Wang, 2011). In the present study, exposure of the human breast cancer cell lines (MCF7, MDA-MB-231, and SKBR3) to the *T. turcica* extracts caused a decrease in cell proliferation depending on the concentration and the type of each extract. The IC_{50} value (0.65 mg/mL and 0.55 mg/mL, respectively) of leaf and flower extracts in MCF-7 cells was found to be lower than the value of normal MCF-10A cells. Similar results were also determined for SKBR3 cell lines. However, IC_{50} values for MDA-MB-231 cells were similar to control cells for all extracts. There are very few studies providing data on the anticancer potential of *Thermopsis* species. For instance, ethanol extracts (50 and 500 µg/mL) of *T. rhombifolia* leaves were found to exert cytotoxic activity on human colon cancer (HT-29) and brain tumor cell lines (SHSY5Y). Twenty-four hours exposure of HT-29 and SHSY5Y cells to the extracts resulted in a decrease in cell viability with IC_{50} values of 220 and 183 µg/mL, respectively (Kernéis et al., 2015). Furthermore, ethanol extracts (0.1 - 1.000 µg/mL) of *T. rhombifolia* aerial parts also demonstrated anticancer activity on HT-29 (IC_{50}: 130 µg/mL), M059K malignant glioblastoma (IC_{50}: 90 µg/mL), and WI-38 normal lung fibroblast (IC_{50}: 240 µg/mL) cell lines after 96 hours exposure (Tuescher et al., 2020). However, luteolin extracted from *T. rhombifolia* has been shown to inhibit cyclin dependent kinase and arrested cells in the G1 phase of the cell cycle (Tuescher et al., 2020). The predominant compounds of *T. turcica* extracts such as quercetin, hesperidin, and benzoic acid may be recognized as inhibitors of breast cancer cell proliferation.

CONCLUSION

In summary, the current study presented a comparative analysis of the antioxidant capacity, phenolic acid profile, and biological activities of the different tissue extracts of *T. turcica*. High levels of TPC and TFC were highly correlated with the antioxidant capacity measured by TAC, CUPRAC, FRAP, and DPPH assays. The leaf extracts exerted the highest antioxidant activity for all assays. HPLC analyses showed high amounts of quercetin and hesperidin in leaf extract, while benzoic acid was found as the predominant compound in flower extract. These phytochemicals may be responsible for the cytotoxic effects of *T. turcica* on human breast cancer. However, there is a need to test the individual and synergistic effects of these phytochemicals.

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REFERENCES

Abotaleb, M., Liskova, A., Kubatka, P., & Büsselberg, D., (2020). Therapeutic potential of plant phenolic acids in the treatment of cancer. *Biomolecules, 10*(2), 221. https://doi.org/10.3390/biom10020221

Aghababaei, F., & Hadidi, M. (2023). Recent advances in potential health benefits of quercetin. *Pharmaceuticals, 16*(7), 1020. https://doi.org/10.3390/ph16071020

Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z., & Kargıoğlu, M. (2013). Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica. Saudi Journal of Biological Sciences, 20*(3), 235–239. https://doi.org/10.1016/j.sjbs.2013.02.003

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., ... Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules, 12*, 1496–1547. https://doi.org/10.3390/12071496

Bali, E. B., Açik, L., Akca, G., Sarper, M., Elçi, M. P., Avcu, F., & Vural, M. (2014). Antimicrobial activity against periodontopathogenic bacteria, antioxidant and cytotoxic ef-

fects of various extracts from endemic *Thermopsis turcica*. *Asian Pacific Journal of Tropical Biomedicine, 4*(7), 505–514. https://doi.org/10.12980/APJTB.4.2014APJTB-2013-0010

Cai, Y., Luo, Q., Sun, M., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences, 74*, 2157–2184. https://doi.org/10.1016/j.lfs.2003.09.047

Caponio, F., Alloggio, V., & Gomes, T. (1999). Phenolic compounds of virgin olive oil: influence of paste preparation techniques. *Food Chemistry, 64*, 203–209. https://doi.org/10.1016/S0308-8146(98)00146-0

Cheynier, V. (2012). Phenolic compounds: from plants to foods. *Phytochemistry Reviews, 11*, 153–177. https://doi.org/10.1007/s11101-012-9242-8

Çelik, Y., & Küçükkurt, İ. (2016). Investigation of the antioxidant effects of extract obtained from *Thermopsis turcica plant in rats.* Kocatepe Veterinary Journal, 9(4), 259–265. https://dergipark.org.tr/en/pub/kvj/issue/32995/370465

Deng, H., & van Verkel, G.J. (1998). Electrospray mass spectrometry and UV/visible spectrophotometry studies of aluminum (III)-flavonoid complex. *Journal Mass Spectrometry, 33*, 1080–1087. https://doi.org/10.1002/(SICI)1096-9888(1998110)33:11<1080::AID-JMS720>3.0.CO;2-2

Elgadir, M. A., Chigurupati, S., & Mariod, A. A. (2023). Selected potential pharmaceutical and medical benefits of phenolic compounds: Recent advances. *Functional Food Science, 3*(7), 108. https://doi.org/10.31989/ffs.v3i7.1118

Elshafie, H.S., Camele, I., & Mohamed, A.A. (2023). A comprehensive review on the biological, agricultural and pharmaceutical properties of secondary metabolites based-plant origin. *International Journal of Molecular Sciences, 24*(4), 3266. https://doi.org/10.3390/ijms24043266

Espín, J. C., Soler-Rivas, C., & Wichers, H. J. (2000). Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-Diphenyl-1-picrylhydrazyl radical. *Journal of Agricultural and Food Chemistry, 48*(3), 648–656. https://doi.org/10.1021/jf9908188

Huang, D., Ou, B., & Prior, R. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856. https://doi.org/10.1021/jf030723c

Ijaz, S., Iqbal, J., Abbasi, B.A., Ullah, Z., Yaseen, T., Kanwal, S., . . . Cho, W.C. (2023). Rosmarinic acid and its derivatives: Current insights on anticancer potential and other biomedical applications. *Biomedicine & Pharmacotherapy, 162*, 114687. https://doi.org/10.1016/j.biopha.2023.114687

Kernéis, S., Swift, L.H., Lewis, C.W., Bruyère, C., Oumata, N., Colas, P., . . . Golsteyn, R.M. (2015). Natural product extracts of the Canadian prairie plant, *Thermopsis rhombifolia*, have anti-cancer activity in phenotypic cell-based assays, *Natural Product Research, 29*(11), 1026–1034, https://doi.org/10.1080/14786419.2014.979423

Koc, B., Akyuz, L., Cakmak, Y.S., Sargin, I., Salaberria, A.M., Labidi, J., . . . Kaya, M. (2020). Production and characterization of chitosan-fungal extract films. *Food Bioscience, 35*, 100545, https://doi.org/10.1016/j.fbio.2020.100545

Kumar, N., & Goel, N. (2019). Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnology Reports, 24*, e00370. https://doi.org/10.1016/j.btre.2019.e00370

Leicach, S.R., & Chludil, H.D. (2014). Plant secondary metabolites: structure–activity relationships in human health prevention and treatment of common diseases. In Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry (pp. 267–270). Amsterdam, Elsevier. https://doi.org/10.1016/B978-0-444-63281-4.00009-4

Lamuela-Raventós, R.M. (2018). Folin–Ciocalteu method for the measurement of total phenolic content and antioxidant capacity. In R. Apak, E. Capanoglu & F. Shahidi (Eds.), Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications (pp. 107–15.). New York, Wiley. https://doi.org/10.1002/9781119135388.ch6

Liman, R., Eren, Y., Akyil, D., & Konuk, M. (2012). Determination of mutagenic potencies of aqueous extracts of *Thermopsis turcica* by Ames test. *Turkish Journal of Biology, 36*, 85–92. https://doi.org/10.3906/biy-1011-158

Lin, H. H., Chen, J. H., Chou, F. P., & Wang, C. J. (2011). Protocatechuic acid inhibits cancer cell metastasis involving the down-regulation of Ras/Akt/NFkappaB pathway and MMP-2 production by targeting RhoB activation. *Brazilian Journal of Pharmacology, 62*(1), 237–254. https://doi.org/10.1111/j.1476-5381.2010.01022.x

Liu, H., Lee, J.I., & Ahn, T.G. (2019). Effect of quercetin on the anti-tumor activity of cisplatin in EMT6 breast tumor-bearing mice. *Obstetrics & Gynecology Science, 62*(4), 242–248. https://doi.org/10.5468/ogs.2019.62.4.242

Lone, R., Baba, S.H., Khan, S., Al-Sadi, A.M., & Kamili, A.N. (2023). Phenolics: Key players in interaction between plants and their environment. In R Lone, S Khan & A Mohammed Al-Sadi (Eds.), Plant phenolics in abiotic stress management. Singapore, Springer. https://doi.org/10.1007/978-981-19-6426-8_2

Madureira, M. B., Concato, V. M., Cruz, E. M. S., Bitencourt de Morais, . JM., Inoue, F. S. R., Concimo Santos, N., . . . Pavanelli, W.R. (2023). Naringenin and hesperidin as promising alternatives for prevention and co-adjuvant therapy for breast cancer. *Antioxidants*, 12(3), 586. https://doi.org/10.3390/antiox12030586

Manzoor, A., Yousuf, B., Pandith, J. A., & Ahmad, S. (2023). Plant-derived active substances incorporated as antioxidant, antibacterial or antifungal components in coatings/films for food packaging applications. *Food Bioscience, 53*, 102717. https://doi.org/10.1016/j.fbio.2023.102717

Molole, G.J., Gure, A., & Abdissa, N. (2022). Determination of total phenolic content and antioxidant activity of *Commiphora mollis (Oliv.)* Engl. Resin. BMC Chemistry, 16, 48. https://doi.org/10.1186/s13065-022-00841-x

Muhammad, T., Ikram, M., Ullah, R., Rehman, S.U., & Kim, M.O. (2019). Hesperetin, a Citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-κB signaling. *Nutrients, 11*(3), 648. https://doi.org/10.3390/nu11030648

Önder, G. Ö., Göktepe, Ö., Baran, M., Bitgen, N., Aydın, F., & Yay, A. (2023). Therapeutic potential of hesperidin: Apoptosis induction in breast cancer cell lines. *Food and Chemical Toxicology, 176*, 113791. https://doi.org/10.1016/j.fct.2023.113791

Pandey, P., & Khan, F. (2021). A mechanistic review of the anticancer potential of hesperidin, a natural flavonoid from citrus fruits. *Nutrition Research, 92*, 21–31. https://doi.org/10.1016/j.nutres.2021.05.011

Prieto, P., Pineda, M., & Aguilar, M. (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Analytical Biochemistry, 269, 337–341. http://dx.doi.org/10.1006/abio.1999.4019

Ranganathan, S., Halagowder, D., & Sivasithambaram, N.D. (2015). Quercetin suppresses twist to induce apoptosis in MCF-7 breast cancer cells. *PLoS One, 10*(10), e0141370. https://doi.org/10.1371/journal.pone.0141370

Salmeron-Manzano, E., Garrido-Cardenas, J. A., & Manzano-Agugliaro, F. (2020). Worldwide research trends on medicinal plants. *International Journal of Environmental Research and Public Health, 17*, 3376. https://doi.org/10.3390/ijerph17103376

Sinan, K.I., Yagi, S., Llorent-Martínez, E.J., Ruiz-Medina, A., Gordo-Moreno, A.I., Stefanucci, A., . . . Zengin, G. (2023). Understanding the chemical composition and biological activities of different extracts of *Secamone afzelii* leaves: A potential source of bioactive compounds for the food industry. *Molecules, 28*, 3678. https://doi.org/10.3390/molecules28093678

Singleton, V. L. & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158. https://doi.org/10.5344/ajev.1965.16.3.144

Tan, K., Vural, M., & Küçüködük, M. (1983). An unusual new *Thermopsis* from Turkey. *Notes from the Royal Botanic Garden of Edinburgh, 40*, 515–518.

Tuberoso, C.I.G., Rosa, A., Bifulco, E., Melis, M.P., Atzeri, A., Pirisi, F.M., & Dessi, M.A. (2010) Chemical composition and antioxidant activities of *Myrtus communis L.* berries extracts. *Food Chemistry, 123*, 1242–1251. https://doi.org/10.1016/j.foodchem.2010.05.094

Tuescher, J. M., Tailfeathers, D., Kernéis, S. M., Baratte, B., Ruchaud, S., Bach, S., . . . Golsteyn, R.M. (2020). The Canadian prairie plant *Thermopsis rhombifolia* contains luteolin, a flavone that inhibits cyclin dependent kinase 9 and arrest cells in the G1-phase of the cell cycle. *Journal of Natural Health Product Research, 2*(2), 1–14. https://doi.org/10.33211/jnhpr.12

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*, 4484. https://doi.org/10.1016/j.biocel.2006.07.001

Wang, Y. J., Wang, F., Yu, L. X., Xiang, Y. J., Zhou, F., Huang, S.Y., . . . Liu, L.Y. (2022). Worldwide review with meta-analysis of women's awareness about breast cancer. *Patient Education and Counseling, 105*(7), 1818–1827. https://doi.org/10.1016/j.pec.2021.12.012

Wojciechowski, M. F. (2003). Reconstructing the phylogeny of legumes (Leguminosae): an early 21st century perspective. In BB Klitgaard & A Bruneau (Eds.), Advances in Legume Systematics, Part 10, Higher Level Systematics (pp. 5–35). Kew, UK: Royal Botanic Gardens.

Xu, D.-P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., . . . Li, H.-B. (2017). Natural antioxidants in foods and medicinal plants: extraction, assessment and resources. *International Journal of Molecular Sciences, 18*(1), E96. https://doi.org/10.3390/ijms18010096

Yıldız, M., Terzi, H., Yıldız, S. H., Varol, N., Özdemir Erdoğan, M., Kasap, M., . . . Solak, M. (2020). Proteomic analysis of the anticancer effect of various extracts of endemic *Thermopsis turcica* in human cervical cancer cells. *Turkish Journal of Medical Sciences, 50*(8), 1993–2004. https://doi.org/10.3906/sag-2005-321

Zhang, P., Zou, J. B., An, Q., Yi, P., Yuan, C. M., Huang, L. J., . . . Hao, X. J. (2022). Two new cytisine-type alkaloids from the seeds of *Thermopsis lanceolata. Journal of Asian Natural Products Research, 24*(12), 1141–1149. https://doi.org/10.1080/10286020.2021.2020759

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İstanbul Journal of Pharmacy
Istanbul J Pharm, 54 (1): 89–95
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Original Article

Antibiofilm activities of denture cleaning tablets against *Streptococcus anginosus* - Cytotoxic effects on human gingival fibroblast cells

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ABSTRACT

Background and Aims: *Streptococcus anginosus* and *Candida albicans* exert negative effects on oral health and can cause biofilm formation on tooth surfaces. Partial or total denture surfaces used by people who have lost their teeth due to various reasons are sensitive to microorganism invasion and biofilm formation, similar to tooth surfaces. Therefore, this study was conducted to investigate the antibiofilm activities of various denture cleaning tablets (DCTs) used to disinfect dentures against *S. anginosus* clinical isolates and *C. albicans* standard strain and their cytotoxic effects against oral epithelial cells.

Methods: The biofilm-forming abilities of strains were determined using the crystal violet assay. The modified time-killing curve (TKC) method was used to evaluate the dynamic bactericidal or fungicidal activities of DCTs against biofilms of *S. anginosus* isolates and *C. albicans* ATCC 10231. In vitro cytotoxicity experiments of DCTs on human gingival fibroblast cell lines (HGF-1) were also conducted using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining method.

Results: Overall, 18.18% and 36.36% of *S. anginosus* isolates were strong and moderate biofilm formers, respectively. *C. albicans* ATCC 10231 also formed a strong biofilm. The TKC analysis revealed that all the examined DCTs inhibited almost all living cells in mature biofilms at every time point. The cytotoxic activities of DCTs against HGF-1 cell lines were in the range of 93%–95% at their direct usage concentrations.

Conclusion: DCTs exhibit rapid and strong activity against biofilms, which is extremely important for biofilm-related infections. Nevertheless, it is necessary to consider the cytotoxic effects of DCTs on HGF-1 cells for consumers' oral health.

Keywords: Biofilm, denture cleaning tablet, cytotoxic effects, *Streptococcus anginosus*

INTRODUCTION

Streptococci are generally the most prevalent and dominant bacteria among oral or dental pathogens, and the majority of studies focus on *Streptococcus mutans* or *Streptococcus mitis* species. Conversely, several studies have also demonstrated that *S. anginosus* is a major colonizer in the early stages of natural oral biofilm formation. *S. anginosus* not only contributes to the early colonization of teeth but also forms the biofilm structure that potentially exerts adverse effects on oral health (Heller et al., 2016).

S. anginosus, similar to all streptococci, possess strong and wide-ranging adherence properties, including binding to human tissue components, epithelial cells, and other bacteria (Jenkinson, 1994). In the oral cavity, streptococci produce specific adhesion proteins such as α-amylase-binding protein A, antigen I/II, SspA/SspB, and surface lectins (Nobbs, Lamont, & Jenkinson, 2009). These proteins bind to the pellicles on tooth surfaces, allowing the bacterial adhesion on teeth and maturing the plaque formation that increases the number of other bacteria involved in biofilm formation (Ritz, 1967). Consequently, *Streptococcus* species can form aggregates both within the same or among different species, and these characteristics play a crucial role in biofilm formation on teeth (Li et al., 2004; Ruhl et al., 2014; Heller et al., 2016).

When mechanical or chemical dental cleaning is not performed adequately, biofilm formation, a well-understood process, commonly occurs on tooth surfaces with the participation of especially *Streptococcus* spp, yeasts such as *Candida albicans*, and other microorganisms. Partial or total denture surfaces, which are used by people who have lost their teeth due to various reasons, to continue their normal chewing, speaking, and other functions, are also susceptible to microorganism in-

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vasion and biofilm formation, similar to tooth surfaces. Some studies indicate that inadequate or inappropriate care of prostheses results in inflammation (stomatitis) in the prosthetic area (Gendreau and Zg, 2011; Ramage et al., 2018), with the occurrence of bacterial, fungal, and polymicrobial infections (Srinivasan and Gulabani, 2010). In addition to insufficient prosthesis cleaning, factors such as salivary pH, smoking, and consumption of sugary foods are crucial for predisposition to oral and dental infections (Martori et al., 2014; Ramage et al., 2005). Because of the bacteria that adhere to the surfaces of dentures, providing an appropriate environment for microbial plaque formation and biofilms, studies have emphasized the importance of cleansers that are effective against microorganisms and possess noncorrosive properties for teeth or prostheses (Emami et al., 2014).

Although various cleaning agents are used for oral and prosthesis health, most of the users perform prosthesis cleaning through brushing with toothpaste, but this method can cause surface abrasions and make it easier for microbial adhesion (Verran et al., 2014; Orgini et al., 2012). It has been suggested that regional use of prosthesis cleaners can facilitate the accumulation of mature prosthesis biofilms (Apratim et al., 2013). Conversely, although some cleaning agents used in combating biofilms are effective against planktonic forms of oral microorganisms, they exert limited effects on mature biofilms (Jose et al., 2010). These previous studies have highlighted the difficulty in oral hygiene and the challenge in combating biofilms.

The present study was conducted to investigate the antibiofilm activities of various denture cleaning tablets (DCTs) used for disinfecting prostheses against *S. anginosus* isolates and a *C. albicans* standard strain. In addition, their cytotoxic properties against oral epithelial cells were evaluated.

MATERIALS AND METHODS

DCTs

Five DCTs, used for the cleaning of removable dental prostheses in Turkiye, were selected and purchased commercially for this study. Table 1 shows the detailed information regarding the ingredients of these effervescing tablet products. Before each test, all DCTs were disintegrated in 100 ml of tap water at 25°C–30°C for 5 min, according to the instructions for their use.

Bacterial strains

A total of 11 *S. anginosus* isolates obtained from specimens submitted to the routine Clinical Microbiology Laboratories of Marmara University Pendik Training and Research Hospital, Cerrahpaşa Medical Faculty Hospital, and Istanbul Başakşehir Çam and Sakura City Hospital were used in this study. All clinical isolates were typed at the species level by MALDI-TOF MS. Furthermore, *S. pneumoniae* ATCC 49619 and *C. albicans* ATCC 10231 standard strains were used in this study.

Media

Brain heart infusion (BHI) broth medium containing 2.5%–5% lysed horse blood (Difco Laboratories) and Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) buffered to pH 7.0 with morpholine propane sulfonic acid (Sigma) were used to determine the antibiofilm activities against bacteria and fungi, respectively.

Biofilm formation

S. anginosus isolates were cultured in BHI broth for 24 h at 5% CO_2 and 37°C, adjusted for 0.5 McFarland turbidity, and diluted in fresh media, resulting in a final concentration of 1×10^6 CFU/mL. Next, a 200-µL sample of this suspension was added to the wells of 96-well tissue culture microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubated for 24 h at 37°C in the presence of 5% CO_2. After incubation, the residual media was aspirated gently, and wells were washed three times with 200 µL physiological buffered saline (PBS) solution. Then 200 µL of 99% methanol was added to the wells for chemical fixation for 15 min and aspirated, after which the plates were allowed to air-dry. For biofilm staining, 200 µL of 0.1% crystal violet was added to each well for 5 min, and after removing the dye, the plates were washed with tap water, and the bound crystal violet was solubilized by adding 95% ethanol for 30 min. The optical density (OD) was measured at 600 nm. Tests were repeated three times. Standard *S. pneumoniae* ATCC 49619 and *C. albicans* ATCC 10231 strains were used as the positive control, and BHI broth or RPMI-1640 medium was used as the negative control. Biofilm formation was interpreted as follows:

OD (isolate) ≤ OD (negative control) = negative biofilm formation; OD (negative control) ≤ OD (isolate) ≤ 2× OD (negative control) = weak biofilm formation; 2× OD (negative control) ≤ OD (isolate) ≤ 4× OD (negative control) = moderate biofilm formation; 4× OD (negative control) ≤ OD (isolate) = strong biofilm formation (Nirwati et al., 2019)

Time-killing curve analyses

The modified time-killing curve (TKC) method was used to determine the dynamic bactericidal or fungicidal activities of DCTs against the biofilms of six moderate or strong biofilm former *S. anginosus* isolates and *C. albicans* ATCC 10231 (Dosler & Karaaslan, 2014). For this purpose, 24–48 h biofilms of *S. anginosus* and *C. albicans* were prepared using BHI broth medium in 24-well tissue culture microtiter plates. DCT solutions were added to each corresponding well, and the plates were incubated for 0, 2, 4, 6, and 24 h at 37°C in the presence of

Table 1. The active ingredients of examined DCTs

DCTs	Ingredients
1	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Citric Acid, Sorbitol, VP/VA Copolymer, Sodium Lauryl Sulfate, Sodium Lauryl Sulfoacetate, Aroma, CI 73015.
2	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Citric Acid, Sorbitol, VP/VA Copolymer, Sodium Lauryl Sulfate, Sodium Lauryl Sulfoacetate, Aroma, CI 73015
3	Sodium Bicarbonate, Citric Acid, Potassium Caroate (potassium monopersulfate), Sodium Carbonate, Sodium Carbonate Peroxide, TAED, Sodium Benzoate, PEG-180, Sodium Lauryl Sulfate, VP/VA Copolymer, Aroma, Subtilisin, Cellulose Gum, CI 42090, CI 73015.
4	Sodium Bicarbonate, Citric Acid, Potassium Caroate (potassium monopersulfate), Sodium Carbonate, Peroxide Sodium Carbonate, TAED, sodium benzoate, PEG-180, Sodium Lauryl Sulfate, Aroma, VP/VA Copolymer, Cellulose Gum, CI 42090, CI 73015.
5	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Sodium Carbonate Peroxide, Sodium Sulfate, Malic Acid, PEG-150, Citric Acid, Sodium C10-13 PEG 90, Aroma, TAED, Potassium Persulfate, Alkyl Benzenesulfonate, Aqua, Sodium Chloride, Cl 42090, Cl 28440

5% CO_2. After each incubation period, wells were washed two times with sterile PBS, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole, assay was performed to evaluate the viability of live microorganisms in biofilms. The MTT solution purchased as a powder from Sigma-Aldrich Chemical A.Ş. (St. Louis, MO, USA) was prepared using the PBS solution. Biofilms were incubated with 0.5 mg/mL of MTT solution for 10 min at 37°C. After washing, the purple formazan crystals that formed inside the living cells were dissolved with dimethyl sulfoxide (DMSO), and then the OD values were measured at 570 nm.

Cytotoxicity assay

In vitro cytotoxicity experiments of DCTs on human gingival fibroblast cell lines (HGF-1) (ATCC CRL-2014) were conducted using the MTT staining method. Cells were grown in Eagle's minimum essential medium (Gibco) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin G under a humidified, 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 1×10^4 cells per well in 96-well tissue culture microplates and kept for 24 h to ensure cell attachment. This was followed by incubation in the absence or presence of two-fold serial dilutions of DCTs between direct usage concentration and five dilutions less (1–1/32-fold) for 24 h at 37°C under 5% CO_2 atmosphere. Cell viability was determined using the MTT assay according to the manufacturer's protocol. The microplates were covered with foil to protect from light and incubated for 3 h at 37°C in a 5% CO_2 environment. After incubation, the MTT solution was removed from the wells, DMSO solution (Sigma, 100 µL) was added to dissolve the formazan crystals, and the plates were placed on a shaker for 15 min to completely dissolve the dye. The OD values were measured at 570 nm using a microplate reader (EON-BioTek Instruments, Winooski, VT, USA) (Andrighetti et al., 2003). In each assay, three replicates were used for each concentration, and the process was repeated three times. The cytotoxic effects of each compound were obtained as % cytotoxicity compared with the control.

RESULTS

Biofilm formation assay

Of the 11 *S. anginosus* isolates obtained from various clinical specimens, 18.18% (2 isolates) formed strong biofilms, 36.36% (4 isolates) formed moderate biofilms, and 45.46% (5 isolates) formed weak biofilms. *C. albicans* ATCC 10231 strain also formed strong biofilms.

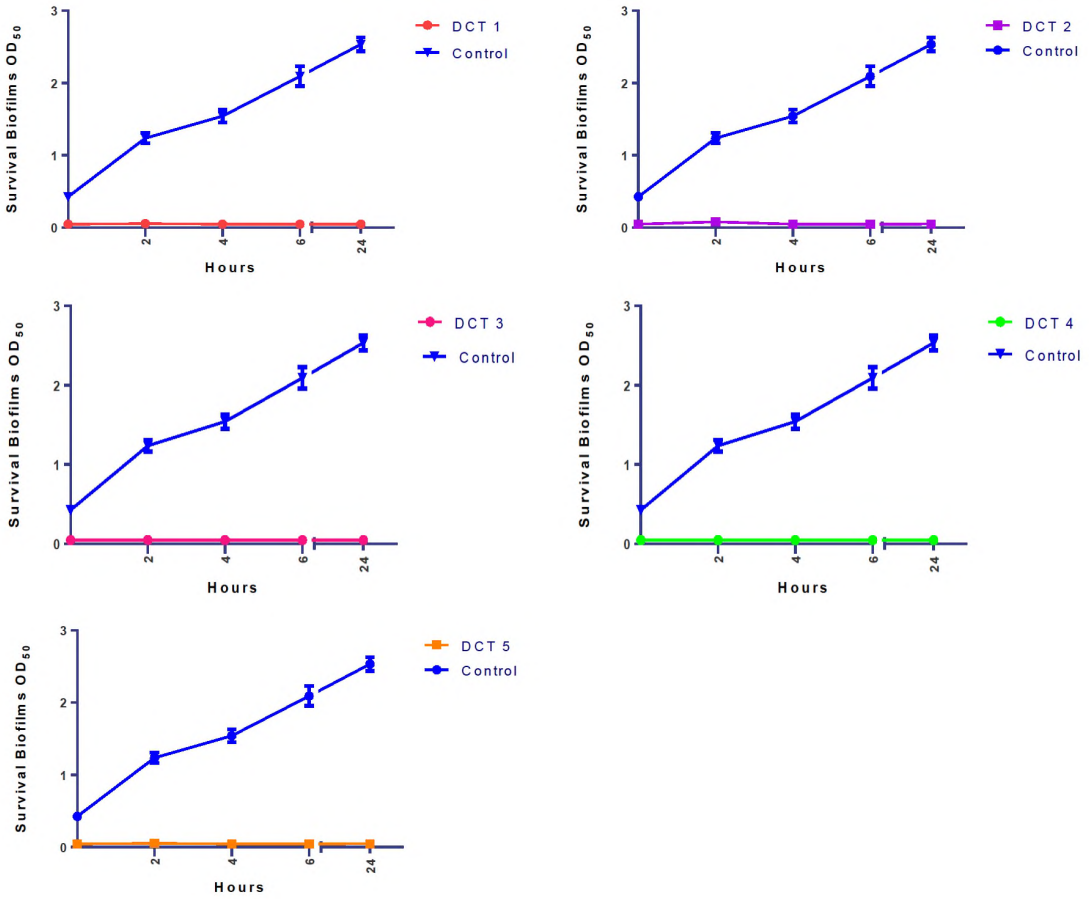

Figure 1. Time killing curves of the studied 5 DCTs against *S. anginosus* isoiates. X-axis represents time, and Y-axis represents the ODs' of biofilm mass. The control shows the result for bacteria have not treated with any product. The results are shown as the averages of two experiments against 6 different isolates.

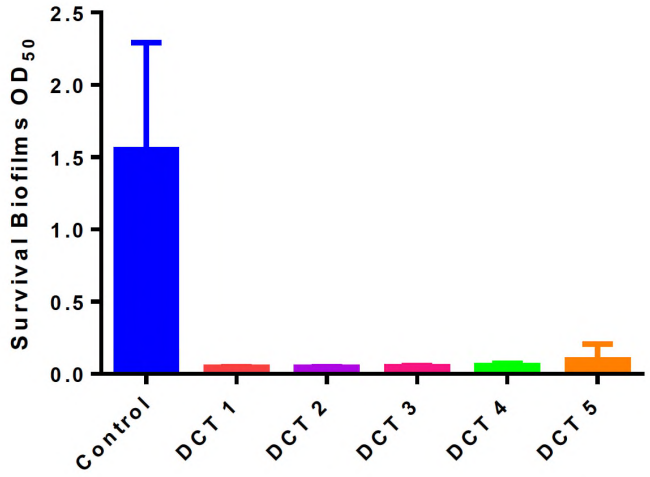

Figure 2. Antibiofilm activities of 5 DCTs against *C. albicans* ATCC 10231. These results were obtained by TKC analysis at the 1st hour and remained the same until the 24th hour. X-axis represents different DCTs, and Y-axis represents the ODs' of biofilm mass. The control shows the result for *C. albicans* not treated with any product. The results are shown as the averages of two experiments.

TKC analyses

The modified TKC analyses revealed that all the examined DCTs exhibited >3-log10 killing ability against six moderate and strong biofilms of *S. anginosus* isolates and *C. albicans* ATCC 10231 within 1 h. All DCTs also inhibited almost all living cells in mature biofilms at every time point (Figures 1 and 2, respectively).

Cytotoxicity assay

The cytotoxicity assay revealed that DCTs exhibited 93%–95% cytotoxicity for HGF-1 cell lines at their direct usage concentrations. These cytotoxic effects continued to be observed at 2.93%–10.26% even at their 1/32 dilutions. These findings demonstrated that the DCTs possess no cell proliferative activity but they exert severe cytotoxic side effects at the usage concentrations and below (Figure 3).

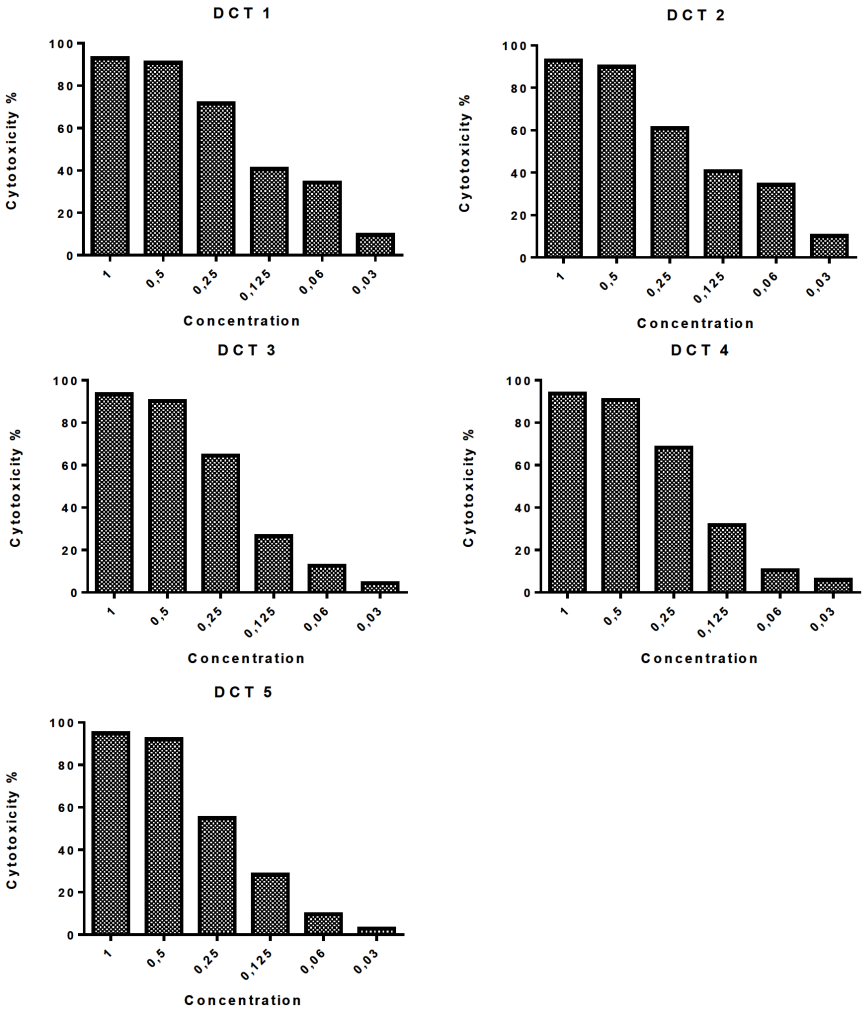

Figure 3. Cytotoxic effects of the 5 studied DCTs against HGF-1 cell line. X-axis represents the diluted product concentrations (direct-1/32). Y-axis show the % cytotoxicity values calculated by comparing with the untreated control cells.

DISCUSSION

Teeth are the major organs of chewing function and are also extremely important for phonetics and aesthetics. Although oral and dental health is an indicator for individual and social health, sometimes tooth losses may occur due to various reasons. In such cases, removable total or partial dentures are widely used for the treatment of missing teeth, especially in elderly people, and the oral health of these people is extremely important for their general systemic health and the quality of their life. Because dental prostheses often function as structures that facilitate biofilm formation for various microorganisms (Takamiya et al., 2011, Shankar et al, 2017), removing and cleaning the dentures after meals is extremely essential for health, and this process should disrupt the biofilm structure. Although several mechanical and chemical methods are used for denture cleaning, chemical cleaners are preferred to facilitate this process, especially for elderly people who have weakened motor coordination. For this purpose, several denture cleaners are available on the market, which contain alkaline peroxides, disinfectants, enzymes, and diluted acids as active ingredients (Budtz-Jorgensen et al., 1972). In practice, the simplest denture cleaning can be achieved by placing one of the DCTs in a glass of warm water and waiting for 3 min, although it is necessary to wait for at least 15 min for deep cleaning. Moreover, the prosthesis can be left overnight to achieve the best cleaning and disinfection, after which it must be rinsed with clean water before use.

In this study, we investigated the antibiofilm activities of five commercially available denture cleaning products against the mature biofilms of strong biofilm former *S. anginosus* isolates and *C. albicans* standard strain. The TKC analyses revealed that all the tested DCTs were highly effective against the biofilms, starting at the 15th min and continuing for 24 h. As all the examined DCT formulations, antibiofilm activities, and contact times were extremely similar to each other, no significant differences were observed between their effectiveness. In the

continuation of our study, we intend to determine the effectiveness of DCTs by generating biofilms on acrylic surfaces to imitate their usage areas.

Because DCTs are used externally and rinsed products, there is no legal specific limitation concerning their toxic effects on body cells. Although these products might exhibit extremely rapid and strong activity against planktonic cells or biofilms of microorganisms, it also implies that they contain high levels of antimicrobial substances and must be rinsed extremely well before use. For this purpose, although the product instructions mention that they should be rinsed thoroughly, it is always necessary to be aware of their cytotoxic effects in cases where everyone cannot read and follow those instructions. In this study, the cytotoxic properties of the products that were effective against the biofilms of *S. anginosus* isolates were investigated on HGF-1 cell lines. Results showed that the cytotoxic effects of the substances were extremely high (>90%) at their usage concentrations, with a cytotoxicity of ≤10% achieved by a dilution of only 1/32, which can be considered relatively safe. These data suggested that rinsing and washing are extremely critical steps to eliminate the toxic effects during the use of the product after the cleaning process, and they must be performed correctly.

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REFERENCES

Andrighetti, Fröhner., C.R., Antonio, R.V., Creczynski-Pasa, T.B., Barardi, C. R.M., Simões, C.M.O. (2003). Cytotoxicity and potential antiviral evaluation of violacein produced by Chromobacterium violaceum. *Memórias do Instituto Oswaldo Cruz*, 98, 843-848.

Apratim, A., Shah, S. S., Sinha, M., Agraval M, Chhaparia, N., Abubakkar, A. (2013). Denture hygiene habits among elderly patients wearing complete dentures. *Journal of Contemporary Dental Practice*, 14, 1161–1164.

Budtz-Jorgensen E., Loe, H. (1972). Chlorhexidine as a denture disinfectant in the treatment of denture stomatitis. *Scandinavian Journal of Dental Research*, 80, 457-464.

Dosler, S., Karaaslan, E. (2014). Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. *Peptides*, 62, 32-37.

Emami, E., Kabawat, M., Rompre, P.H., Feine, J.S. (2014). Linking evidence to treatment for denture stomatitis: A meta-analysis of randomized controlled trials. *Journal of Dentistry*, 42, 99–106.

Gendreau, L., & Zg, L. (2011). Epidemiology and etiology of denture stomatitis. *Journal of Prosthodontics*, 20, 251–260.

Heller, D., Helmerhorst, E. J., Gower, A. C., Siqueira, W. L., Paster, B. J., & Oppenheim, F. G. (2016). Microbial Diversity in the Early In Vivo-Formed Dental Biofilm. *Applied and Environmental Microbiology*, 82, 1881-1888.

Jenkinson, H.F. (1994). Cell surface protein receptors in oral streptococci. *FEMS Microbiology Letters,121*(2),133-140.

Jose, A., Coco, B. J., Milligan, S., Young, B., Lappin, D.F., Bagg, J. ... Ramage, G. (2010). Reducing the incidence of denture stomatitis: Are denture cleansers sufficient? *Journal of Prosthodontics*, 19, 252–257. 10.1111/j.1532-849X.2009.00561.x

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., & Oppenheim, F. G. (2004). Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology*, 97, 1311-1318. https://doi.org/10.1111/j.1365-2672.2004.02420.x

Martori, E., Ayuso-Montero, R., Martinez-Gomis, J., Vinas, Miquel., Peraire, M. (2014). Risk factors for denture-related oral mucosal lesions in a geriatric population. *The Journal of Prosthetic Dentistry*, 111, 273–279. doi: 10.1016/j.prosdent.2013.07.015.

Nirwati, H., Sinanjung, K., Fahrunissa, F., Fernando, W., Napitupulu, S., Hati, V.P. . . . Nuryastuti, T. (2019). Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. BMC, 13, 1-8 .https://doi.org/10.1186/s12919-019-0176-7

Nobbs, A. H., Lamont, R. J., & Jenkinson, H. F. (2009). Streptococcus adherence and colonization. *Microbiology and Molecular Biology Reviews, 73*, 407-450. https://doi.org/10.1128/MMBR.00014-09.

Orgini, D. B., Silva-Lovato, C. H., de Souza, R. F., & et al. (2012). Abrasiveness of conventional and specific denture-cleansing dentifrices. *Brazilian Dental Journal*, 23, 154–159.

Ramage, G., Saville, S.P., Thomas, D. P., et al. (2005). Candida biofilms: An update. *Eukaryotic Cell*, 4, 633-688.

Ramage, G., O'Donnell, L., Sherry, L., Culshaw, S., Bagg, J., Czesnikiewicz-Guzik, M., Brown, C. ... Robertson, D. (2018). Impact of frequency of denture cleaning on microbial and clinical parameters – a bench to chairside approach. *Journal of Oral Microbiology*, 11, 1-15. https://doi.org/10.1080/20002297.2018.1538437

Ritz, H. L. (1967). Microbial population shifts in developing human dental plaque. *Archives of Oral Biology, 12*, 1568-1570.

Ruhl, S., Eidt, A., Melzl, H., Reischl, U., & Cisar, J. O. (2014). Probing of microbial biofilm communities for coadhesion partners. *Applied and Environmental Microbiology*, 80, 6583–6590. https://doi.org/10.1128/AEM.01826-14.

Shankar, T., Gowd, S., Suresan, V., Mantri, S., Saxena, S., Mishra, P,. Panday, P. (2017) Denture Hygiene Knowledge and Practices among Complete Denture Wearers attending a Postgraduate Dental Institute. *Journal of Contemporary Dental Practice*, 18, 714-721.

Srinivasan, M., & Gulabani, M. (2010). A microbiological evaluation of the use of denture cleansers in combination with an oral rinse in complete denture patients. *Indian Journal of Dental Research*, 21, 353-356.

Takamiya, A.S.; Monteiro, D.R.; Barão, V.A.R.; Barbosa, D.B.; Pero, A.C, Compagnoni, M.A. (2011). Complete denture hygiene and nocturnal wearing habits among patients attending the Prosthodontic Department in a Dental University in Brazil. *Gerodontology*, 28, 91-96.

Verran, J., Jackson, S., Coulthwaite, L., & et al. (2014). The effect of dentifrice abrasion on denture topography and the subsequent retention of microorganisms on abraded surfaces. *Journal of Prosthetic Dentistry*, 112, 1513–1522.

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Original Article

A new and rapid HPLC method for the determination of phenoxyethanol in topical formulation

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ABSTRACT

Background and Aims: This study develops and validates a new liquid chromatographic method for determining the phenoxyethanol used as an antimicrobial preservative in pharmaceutic and cosmetic products. The study applies the developed high-performance liquid chromatography (HPLC) method to pomade formulation using a diode array detector to determine the phenoxyethanol.

Methods: The phenoxyethanol in the sample was analyzed in a C18 column (150 x 4.6 mm, 5 µm ID) under chromatographic conditions where the flow rate was determined as 1.0 mL/min. The column oven was 30.0°C, and phenoxyethanol was detected at 270 nm. Isocratic application of acetonitrile-water (50:50, v/v) was used as the mobile phase system. The validation of the developed method was performed according to the guidelines from the International Conference on Harmonisation (ICH, 2005) *Guidelines Q2 (R1).*

Results: The linearity range of the phenoxyethanol was 0.125-0.375 mg/mL, and the limits of detection and quantification were calculated as 31.25 ng/mL and 125.0 ng/mL, respectively. The assay recovery and precision of the phenoxyethanol from the pomade formulation were evaluated at 0.125 mg/mL, 0.250 mg/mL, and 0.375 mg/mL concentrations. The mean recoveries for phenoxyethanol in the pomade formulation were calculated at 99.99%-102.86%.

Conclusion: The validated method was successfully applied for determining phenoxyethanol in a topical formulation. The proposed method is cheap, fast, and simple and can be used safely for routine analysis.

Keywords: Phenoxyethanol, HPLC, DAD, cream formulation, validation, antimicrobial preservative

INTRODUCTION

Phenoxyethanol is one of the most popular preservatives used in many pharmaceutical and cosmetic products to protect against microbial growth (Figure 1). A large number of pharmaceutical and cosmetic preparations are known today to contain this preservative. Moreover, the products of some cosmetic brands are stated to be preserved with very high amounts of phenoxyethanol (Dreno et al., 2019).

Meanwhile, determining the amounts of active ingredients or excipients used for any reason, especially in pharmaceutical products, is an indispensable element of quality control. Although some studies have previously shown the wide use of phenoxyethanol to be safe, a lot of information is also found about its negative effects on human health (Dreno et al., 2019; Jakubczykz & Michalkiewicz, 2019). The amount of phenoxyethanol permitted in pharmaceutical preparations must be in the range of 0.5%-1% (Regulation of the European Community [EC], 2009). European Union Directive No. 1223/2009 states within its scope that the use of this substance in cosmetics should not exceed 1% (w/w; Agence nationale de sécurité du médicament et des produits de santé [ANSM], 2012). In fact, its use in baby products has been further restricted (ANSM, 2019).

When considering all these limitations, the ability to determine the amount of this substance in pharmaceutical or cosmetic products quickly, precisely, and accurately using validated methods becomes even more important. To date, researchers have reported many methods for the determination of phenoxyethanol alone or in combination with other preservatives in pharmaceutical formulations (Akhtar et al., 1996; Sharma et al., 2008; Shabir, 2010; Roy & Chakrabarty, 2013; Jakubczykz & Michalkiewicz, 2019; Algethami et al., 2023)

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and cosmetic products (Abad-Gil et al., 2021; Abad-Gil et al., 2022; Borremans et al., 2004; Jakubczykz & Michalkiewicz, 2019). Although most of these are based on the HPLC technique, studies on other methods are also found (Jakubczykz & Michalkiewicz, 2019; Algethami et al.,2023).

This study plans to develop an extremely rapid, easy, accurate, and precise method for analyzing phenoxyethanol when found alone in pharmaceutical preparations. After validating the developed method, the study applies it to the analysis of a topical cream containing phenoxyethanol. The proposed method is superior in terms of time and cost, as its analysis time is shorter than other methods. In addition, the rapid and ease of the sample preparation procedure in the topical formulation are one advantage the method has compared to other methods.

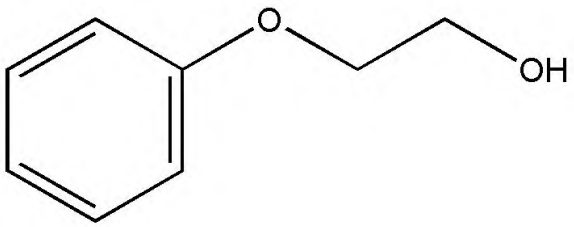

Figure 1. Chemical structure of phenoxyethanol.

MATERIAL AND METHODS

Chemicals

Phenoxyethanol (99%-100.5%) was provided by Brenntag Kimya. Ultrapure water from the Elga brand water system was used located in the research laboratory. Liquid chormatography (LC) grade acetonitrile was obtained from Merck (Germany). The pharmaceutical formulation was supplied by a pharmacy and Kurtsan İlaçları A.Ş.

Solutions

The phenoxyethanol stock solution (2.5 mg/mL) was dissolved in water. The linearity range for phenoxyethanol was prepared in the concentration ranges of 0.125, 0.187, 0.250, 0.312 and 0.375 mg/mL. The solutions were diluted using the mobile phase. Isocratic application of the acetonitrile-water mix (50:50, v/v) was used as the mobile phase system. This was then sonicated for 20 min.

HPLC System

The study uses a liquid chromatographic system equipped with an autosampler, a column oven compartment, and a diode-array detector (DAD). The device used in the analysis is the Shimadzu LC 20A system (Kyoto, Japan). Separation was performed with a C18 column (150 x 4,6 mm, 5 µm ID) under chromatographic conditions where the flow rate was determined as 1.0 mL/min. The column oven was 30.0°C, and phenoxyethanol was detected at 270 nm. The chromatographic data, analysis, and reporting were performed via the LC-Solution system software.

Preparing the sample solutions

The sample solution was prepared in mobile phase. The cream formulation is dissolved in the acetonitrile-water mix (50:50, v/v) to obtain a concentration of 0.25 mg/mL. Next, the sample solution is stirred in a vortex mixer for 60.0 s and then kept in an ultrasonic bath for 10 min. The mixture is then filtered with a 0.45 µm nylon filter and injected into the HPLC system. The amount of substance in the cream formulation is calculated by substituting the resulting area into the calibration equation.

Validation

The developed method has been validated using the guidelines from the International Conference on Harmonisation (ICH, 2005) *Text and Methodology Q2 (R1)*. The linearity of the phenoxyethanol is studied in the range of 0.125-0.375 mg/mL by taking into account the phenoxyethanol concentration in the preparation containing the solution for injection. The limits of detection (LOD) and limits of quantitation (LOQ) are calculated using the signal-to-noise ratio formula. Absolute recoveries are evaluated using the placebo addition by selecting the initial, middle, and final concentrations of the calibration curve. A placebo solution is prepared similar to the sample solution. Three different concentrations of standard phenoxyethanol solutions (0.125, 0.250, and 0.375 mg/mL) were filled to the same volume using the placebo solution, and these mixtures have also been analyzed using the recommended method.

Precision studies of the method were examined with inter-day and intra-day precision. Therefore, separate standard solutions were prepared at concentrations of 0.125, 0.250 and 0.375 mg/mL in the mobile phase solution. Standards from the same day and different days were analyzed and assessed by calculating the relative standard deviation percentages (%RSDs) of the field values.

The stability of phenoxyethanol in the cream formulation solution was studied at the end of the 12^{th} and 24^{th} hours of the samples being kept under autosampler conditions. The stability was assessed by comparing the initial results with those obtained upon the conclusion of the analyses. The robustness parameter was assessed by changing the mobile phase flow rate and column temperature. The standard solution was initially injected using a method flow rate of 1.0 mL/min. Subsequently, injections were conducted after adjusting the system to flow rates of 0.9 mL/min and 1.1 mL/min. A similar approach was taken for column temperature, with initial injections made at the method-specified temperature of 30.0°C. The standard solution was injected by adjusting the column temperature first to 28°C

and then to 32°C. The obtained chromatograms were analyzed for the theoretical plate numbers and tailing factors, which are the essential parameters for evaluating system suitability.

RESULTS AND DISCUSSION

The developed and validated HPLC method involves a simple sample preparation and is a selective, reproducible, and reliable method enabling the analysis of phenoxyethanol from a cream formulation based on HPLC using DAD.

Selectivity

To assess the method selectivity, the system received injections of the mobile phase, phenoxyethanol standard solution, cream formulation, and placebo solutions. During the retention time of phenoxyethanol, no peaks were observed due to the solvent or placebo, as depicted in Figure 2.

Linearity and sensitivity

Linearity was studied in the proposed method in the concentration range of 0.125-0.375 mg/mL by taking the analyzed phenoxyethanol formulation into consideration. The average regression formula can be expressed as:

$$A = 10472711C + 91526 (r = 0.9997) \quad (1)$$

where C represents the concentration of phenoxyethanol (mg/mL) and A represents the peak area. The results for linearity from the proposed method are displayed in Table 1. In accordance with the study parameters, LOD and LOQ results were determined as 31.25 ng/mL and 125.0 ng/mL, respectively.

Table 1. Linearity results obtained from the developed method

Parameter	Phenoxyethanol
Linearity range (mg mL^{-1})	0.125-0.375
Regression equation	A = 10472711C + 91526
Slope ± SD	10472711± 2755
Intercept ± SD	91526± 762
Mean correlation coefficient, r	0.9997
LOD[a] (ng mL^{-1})	31.25
LOQ[b] (ng mL^{-1})	125.0

[a] Limits of Detection; [b] Limits of Quantitation

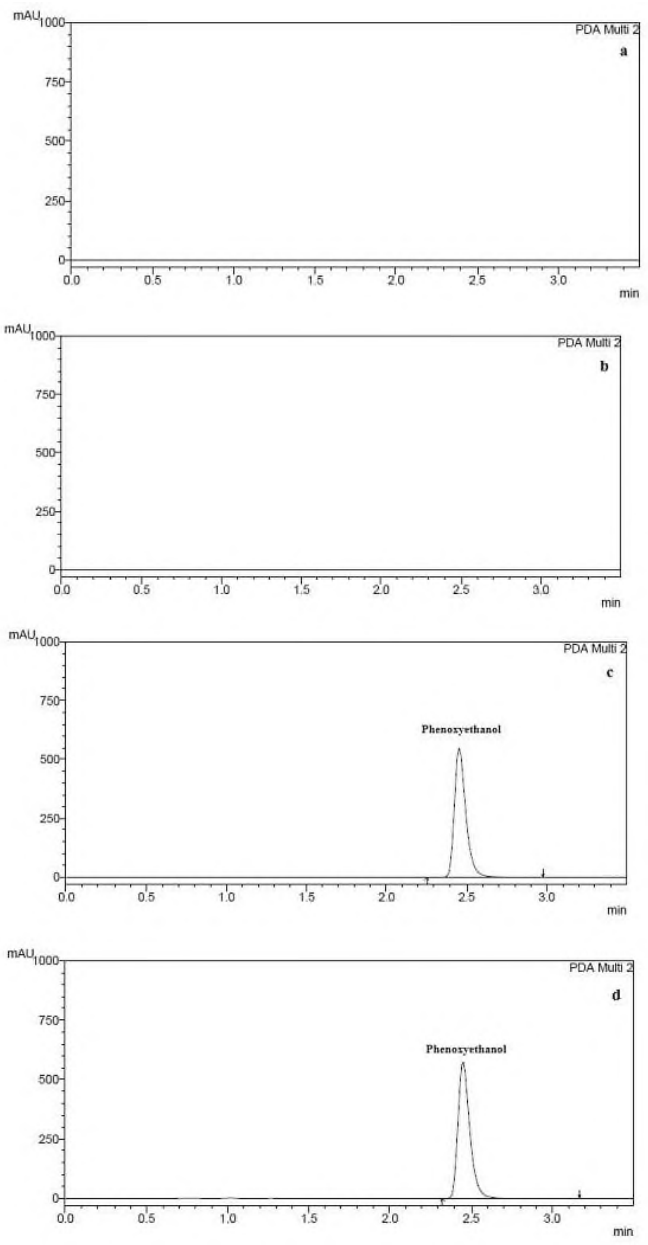

Figure 2. The chromatograms obtained from (a) the mobile phase, (b) the placebo solution, (c) the standard phenoxyethanol solution (0.250 mg/mL), and (d) the cream formulation (1.0 g).

Recovery

As shown in Table 2, the absolute recovery values of phenoxyethanol in the cream formulation were found between 99.99-102.86%. The average phenoxyethanol recovery was calculated as 101.07%.

Table 2. Recovery results for the phenoxyethanol assay.

Concentration (mg mL^{-1})		Recovery (%)	RSD[b] (%)
Added	Found (mean ± SD[a])		
0.125	0.129±0.000$_1$	102.86	0.039
0.250	0.251±0.000$_1$	100.36	0.026
0.375	0.375±0.000$_1$	99.99	0.007

[a] Standard deviation; [b] Relative standart deviation

Precision

Precision assessments were obtained with both intra-day and inter-day repeatability, as outlined earlier. The %RSD ranged from 0.008%-0.442% for intra-day repeatability and from 0.010%-0.448% for inter-day repeatability. Table 3 displays the precision values for the method. These results are in accordance with the statement that the %RSD value should be less than 2.0%.

Table 3. Intra-day & inter-day precision and accuracy of phenoxyethanol (n = 6)

Concentration (mg/mL)		RSD[b] (%)	RME[c] (%)
Added	Found (mean ± SD[a])		
Intra-day			
0.125	0.123 ± 0.000$_0$	0.008	-1.441
0.250	0.250 ± 0.001	0.442	0.123
0.375	0.375 ± 0.000$_0$	0.008	-0.012
Inter-day			
0.125	0.123 ± 0.000$_1$	0.069	-1.489
0.250	0.251 ± 0.001	0.448	0.407
0.375	0.375 ± 0.000$_0$	0.010	-0.004

[a] Standard deviation; [b] Relative standart deviation; [c] Relative mean error

Stability

Assessing the stability of the proposed method was computed under the conditions determined for the phenoxyethanol solution by comparing the initial results with those obtained at the conclusion of the analyses after 12 and 24 hours had passed. Upon analyzing the obtained values, the variations were seen to range between 0.50‰-0.39‰ (Table 4). These results show no noticeable alteration to have occurred in the peak areas.

Table 4. Stability results for the phenoxyethanol obtained using the proposed method

Time (hour)	Concentration (mg/mL) (mean±SD)	RSD (%)	Variation (%)
0	0.273±0.000$_1$	0.029	0.00
12	0.274±0.000$_1$	0.023	0.50
24	0.274±0.000$_1$	0.022	0.39

Robustness

The robustness of the method was tested by evaluating the results obtained from changing the flow rate and column oven temperature. The average tailing factor using the proposed method was determined as 1.530 ± 0.007 at a flow rate of 1.0 mL/min and a column temperature of 30.0°C. While assessing the robustness of the method, adjustments were made to the column temperature and mobile phase, as previously outlined. The resulting values for the tailing factor were within the range of 1.531-1.540 and 1.492-1.537, respectively. Additionally, the theoretical plate number, which was initially determined to be 29,816 ± 896 using the proposed method, varied between 27,433-31,337 for the mobile phase changes and between 28,438-29,252 for the column temperature changes. The method was determined to have remained unaffected by minor changes.

Determination of phenoxyethanol from the topical formulation

The percentage of phenoxyethanol in the cream formulation as determined by the proposed method was calculated in the range of 98.65%-98.90% (Table 5). These outcomes align with the specified range of 95.0% to 105.0% as outlined in ICH's (2005) *Text and Methodology Q2* (R1). This proves the applied method to have been successful at analyzing the phenoxyethanol within the cream preparation.

CONCLUSION

This study has developed an extremely rapid, easy, and accurate method for analyzing phenoxyethanol in pharmaceutical prepa-

Table 5. Determination of the phenoxyethanol in a topical formulation (n = 6)

n	g / 100 g	%
1	0.9868	98.68
2	0.9869	98.69
3	0.9865	98.65
4	0.9890	98.90
5	0.9885	98.85
6	0.9887	98.87
Mean	0.9877	
SD[a]	0.001	
RSD[b]	0.115	

[a] Standard deviation; [b] Relative standart deviation

rations. After validating the developed method in accordance with ICH rules, the method was then successfully applied to the analysis of a topical cream containing phenoxyethanol.

Compared to other HPLC techniques, the developed method is shorter in terms of analysis time (Akhtar et al., 1996; Sharma et al., 2008; Sabir, 2010; Roy & Chakrabarty, 2013; Algethami et al., 2023; Abad-Gil et al., 2021; Abad-Gil et al., 2022; Borremans et al., 2004) using a standard HPLC instrument, with the analysis time being completed in 3.5 minutes. This is very useful in terms of time and costs for pharmaceutical companies' routine analyses. In addition, the sample preparation of the developed method includes the injection into the device after a 10-minute degassing of the mobile phase. This is an extremely simple procedure for topical formulations that might otherwise require quite a challenging sample preparation.

As a result, the developed method is extremely rapid and simple, in addition to being highly accurate and precise, and will bring advantages to the routine analysis of phenoxyethanol in topical formulations, especially in terms of time and cost.

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REFERENCES

Abad-Gil, L., Lucas-Sanchez, S., Gismera, M.J., Sevilla, M.T., & Procopio, J.R. (2021). Determination of paraben-, isothiazolinone- and alcohol-type preservatives in personal care products by HPLC with dual (diode-array and fluorescence) detection. *Microchemical Journal, 160*, 105613. doi:/10.1016/j.microc.2020.105613

Abad-Gil, L., Lucas-Sanchez, S., Gismera, M.J., Sevilla, M.T., & Procopio, J.R. (2022). HPLC method with electrochemical detection on gold electrode for simultaneous determination of different antimicrobial agents in cosmetics. *Microchemical Journal, 182*, 107881. doi:10.1016/j.microc.2022.107881

Akhtar, M. J., Khan, S., Roy, I.M., & Jafri, I.A. (1996). High performance liquid chromatographic determination of phenoxetol, methyl paraben, ethyl paraben, n-propyl paraben, *iso-butyl* paraben, n-butyl paraben and croconazole" HC1. *Journal of Pharmaceutical and Biomedical Analysis, 14*, 1609-1613.

Algethami, F.K., AlSalem H. S., Gamal, M., Nabil, N., Zaazaa, H. E., Ibrahim, M. A., & Mandour, A. A. (2023). Simultaneous Analysis of Flumethasone Pivalate and Clioquinol in the Presence of Phenoxyethanol Preservative in Their Pharmaceuticals Using TLC and UHPLC Methods. *Processes* 11, 1888-1901. doi :10.3390/pr11071888

ANSM/ FEBEA, Risk assessment on the use of phenoxyethanol in cosmetic products (2012), ansm.sante.fr/var/ansm_site/storage/original/application/0b46fedc079e8bb174a40b7b6f16d04c.pdf

ANSM, A mandatory warning on leave-on cosmetics containing phenoxyethanol (2019),ansm.sante.fr/content/download/158253/2075101/version/1/file/DPS_Phenoxyethanol-200319.pdf

Borremans, M., Loco, V.J., Roos, P., & Goeyens, L. (2004). Validation of HPLC Analysis of 2-Phenoxyethanol, 1-Phenoxypropan-2-ol, Methyl, Ethyl, Propyl, Butyl and Benzyl 4-Hydroxybenzoate (Parabens) in Cosmetic Products, with Emphasis on Decision Limit and Detection Capability. *Chromatographia*, 59, 47-53.

doi:10.1365/s10337-003-0127-2

Dreno, B., Zuberbier, T., Gelmetti, C., Gontijo, G., & Marinovich, M. (2019). Safety review of phenoxyethanol when used as a preservative in cosmetics. *Journal of the European Academy of Dermatology & Venereology, 33* (7), 15-24. doi: 10.1111/jdv.15944.

ICH Q2 (R1). (2005). Validation of Analytical Procedures: Text and Methodology, https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.Accessed October 8, 2023.

Jakubczykz, M., & Michalkiewicz, S. (2019). First Voltammetric Method of Phenoxyethanol Determination in Pharmaceutical and Cosmetic Preparations. *Journal of The Electrochemical Society*, 166 (8) 291-296. doi:10.1149/2.05219008jes

Regulation (EC) No 1223/2009 of The European Parliament and of the Council of 30 November 2009 on Cosmetic Products (Dz. U. L 342/59 of 22.12.2009 r.).

Roy, C., & Chakrabarty, J. (2013). Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Phenoxyethanol, Methylparaben, Propylparaben, Mometasone Furoate, and Tazarotene in Topical Pharmaceutical Dosage Formulation, *Scientia Pharmaceutica*. 81, 951–967. doi:10.3797/scipharm.1303-22.

Shabir, G.A. (2010). A New Validated HPLC Method for the Simultaneous Determination of 2-phenoxyethanol, Methylparaben, Ethylparaben and Propylparaben in a Pharmaceutical Gel. *Indian Journal of Pharmaceutical Sciences,* 72 (4): 421-425.

Sharma, B., Joseph, A., & Sood, A. (2008). A simple and rapid method for quantifying 2-phenoxyethanol (2-PE) in Diphtheria, Tetanus and w-Pertussis (DTwP) vaccine. *Biologicals* 36: 61-63.

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Original Article

An anatomical study of *Lepidium graminifolium* L. (Brassicaceae)

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ABSTRACT

Background and Aims: Many taxonomic studies on *Lepidium* species grown in Türkiye have been conducted to illuminate its morphological characteristics. Although many morphological studies occur, only a few anatomical studies are found on *Lepidium* species worldwide. The aim of this study is to reveal the anatomical features of *Lepidium graminifolium.*

Methods: In order to determine the anatomical features of *L. graminifolium*, plant samples were collected and preserved in 70% ethanol, and then all sections were cut separated by hand with a razor blade and stained with Sartur reagent. Microscopic slides were prepared and examined using the light microscope, Olympus BH-2. Photographs were taken with the light microscope and measurements were made using the ImageJ© program. Stomatal indices were calculated using the formula SI (%) = (S/S+E) × 100, where S is the number of stomata per unit area, and E is the number of epidermal cells per unit area.

Results: The results elucidate on the anatomical features of the root, stem, leaf and petiole. A table was prepared by taking measurements of the anatomical structures to present the anatomical characteristics of *L. graminifolium*. This study is the first to elucidate upon the anatomical features of *L. graminifolium*.

Conclusion: Two important structures are mentioned among *Lepidium* species that can be used as distinguishing characteristics: the morphology of the trichomes and the type of stomata. Compared with other studies, the stoma type of *L. graminifolium* was found to differ from other *Lepidium* species. The anatomical features that may differ among other *Lepidium* species are the sclerenchymatous fibers observed in the root, the bifacial type of the leaf mesophyll, and the sparse distribution of unicellular glandular hairs on both the adaxial and abaxial surfaces of the leaves.

Keywords: Anatomy, Brassicaceae, *Lepidium*, Türkiye

INTRODUCTION

Brassicaceae (or Cruciferae), commonly known as the mustard family, is a large plant family comprised of 372 plant genera and 4,060 accepted plant species (International Plant Names Index [IPNI], 2023). Cruciferae are annual, biennial, or perennial grasses and shrubs containing glucosinolates of great scientific and economic importance (Koch & Mummenhoff, 2006). Most of these are distinctly woody (Simpson, 2019). The flowers are actinomorphic and usually consist of four free sepals, four free petals, and six free tetradynamous stamens (Appel & Al-Shehbaz, 2003; Hedge, 1976). The major distribution centers of the family occur in the Irano-Turanian, Mediterranean, and Saharo-Sindian regions (Bona, 2014). Among the distribution regions of the family, the largest number of endemic taxa is found in the Irano-Turanian phytogeographical region and represented by 150 genera and 530 endemic species, followed by the Mediterranean phytogeographical region with 110 genera and 290 endemic species (Al-Shehbaz, Mutlu, & Dönmez, 2007).

The genus *Lepidium* L. is one of the largest genera of the Brassicaceae, consisting of 262 species (IPNI, 2023). It is distributed worldwide, primarily in temperate and subtropical regions. The genus is poorly represented in Arctic climates and grows in mountains in tropical areas. In Türkiye, the genus *Lepidium* is represented by 15 taxa, one of which is considered a naturalized invasive alien plant (Bona, 2013).

Anatomical data have been applied to better understand the interrelationships of plants, and combined analyses have provided confirming evidence in the molecular age of the natural relationships plant families have. Anatomical characteristics are most useful when determining the relationship between different genera, families, orders, and/or other taxonomic categories. Anatomical data have also solved several phylogenetic

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problems. Metcalfe identified several herbarium specimens using vegetative anatomy (Chaffey, 2008). Many studies are also found to have addressed the importance of leaf anatomy and trichome morphology as a distinctive feature for identifying *Lepidium* species (Abdel, 2005; Al-Shehbaz, Beilstein, & Kellogg, 2006).; Beilstein, Al-Shehbaz, & Kellogg, 2006).

Lepidium species have medicinal importance for their sulfur heteroside content. *Lepidium meyenii*, known as Peruvian maca, is used to regulate sexual dysfunction and for a memory enhancement and antidepressant; it also has neuroprotective, antioxidant, anti-cancer, and anti-inflammatory effects (Peres et al., 2020). According to another study, maca is used to manage anemia, infertility, and female hormone balance (Lee, Shin, Yang, Lim, & Ernst, 2011). Due to the *Lepidium* species potential for medical use, determining its morphological and anatomical characteristics and elucidating upon its distinguishing characteristics are important.

L. graminifolium is a perennial species with a wide distribution from Europe to Northwest Africa and Southwest Asia. It grows on dry slopes up to 2000 m above mean sea level. The species is multi-stemmed and highly branched above. The stem is glabrous, and rarely has sparse hairs (Figure 1A-1B). Flowering occurs from April to September (Bona, 2014).

A number of taxonomic studies on *Lepidium* species have been carried out in Eastern Europe (Smirnov, 1948; Dorofeev; 2012; De Carvalho & Vasconcellos, 1964). Hewson (1981) also studied the Australian *Lepidium* species. German (2014) mentioned unresolved taxonomic complexity regarding *Lepidium* species in Central and Southwest Asia, and Bona (2014) made a taxonomic revision of the genus in Türkiye.

Although many morphological studies have occurred, only a few anatomical studies are found on *Lepidium* species worldwide (Grigore & Toma, 2008; Sangekar, Devarkar, Shaikh, Shahane, & Kshirsagar, 2018) The anatomical study of *Aethionema lepidioides* conducted by Tekin (2022) shows the importance of the anatomical characteristics in plant taxonomy for Brassicaceae family members. This is the first detailed study to elucidate on the root, stem, and leaf anatomical features of *Lepidium graminifolium.*

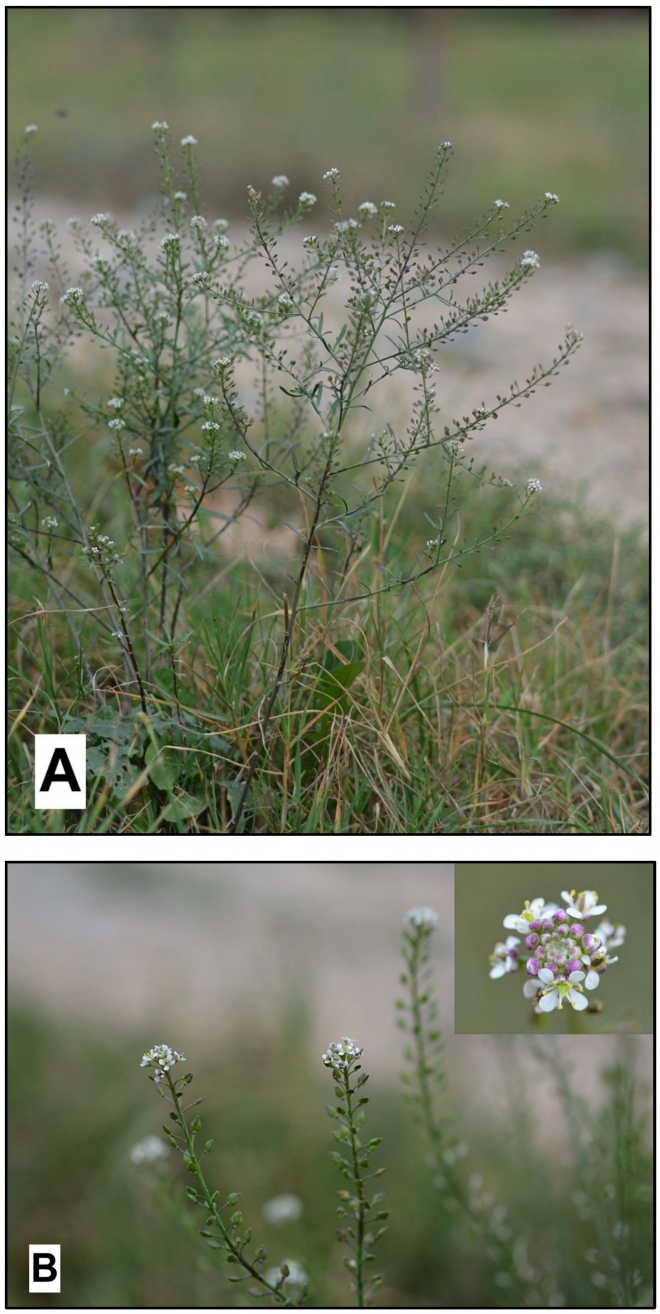

Figure 1. General view of *Lepidium graminifolium.* (A) General view of *L. graminifolium* in its own habitat; (B) Stem and flowers of *L. graminifolium.*

MATERIALS AND METHODS

Plant samples were collected by the first author on November 19, 2022 from Validebağ Grove in Istanbul's Üsküdar municipality. Photos of the plant were taken during its flowering period with a Nikon D7100 camera and 60 mm Nikkor macro lens. The collected plant samples were identified using *Flora of Turkey and the East Aegean Islands* (Vol. I; Hedge, 1965). The prepared herbarium specimens were deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE) and recorded with number ISTE: 118370. For anatomical investigations, plant materials were preserved in 70% ethanol, and then all sections were cut separated by hand with a razor blade. All sections were stained using Sartur reagent (Çelebioğlu & Baytop, 1949; Özkan, 2017) and examined with the Olympus BH-2 light microscope. Photographs were taken with the light microscope and measurements were made using the ImageJ© program. Stomatal indices were calculated using the formula: SI (%) = (S/S+E) × 100, where S is the number of stomata per unit area, and E is the number of epidermal cells per unit area. For anatomical descriptions, the study follows the terminology proposed by Metcalfe & Chalk (1957).

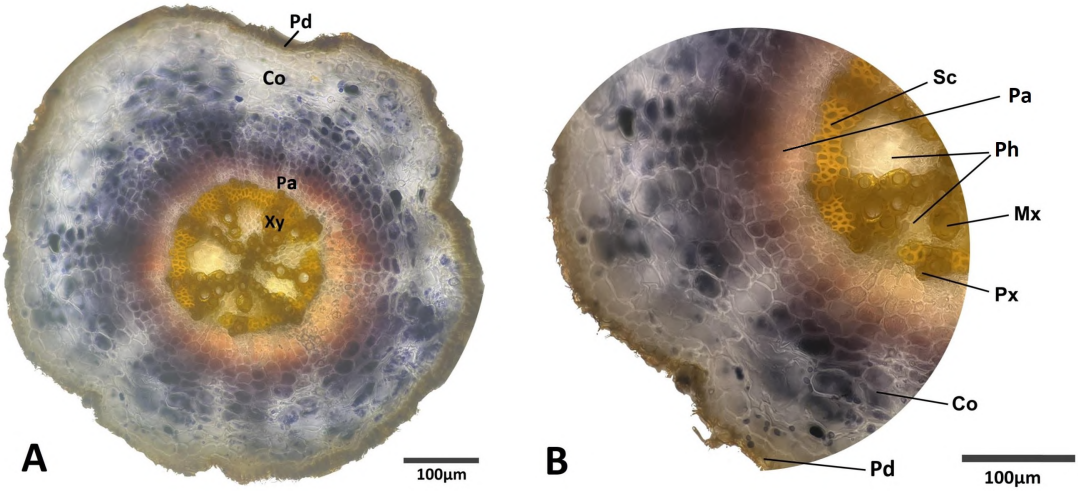

Figure 2. Root cross section of *Lepidium graminifolium*: (A) General view (B) Cortex and vascular bundles (Co = cortex, Mx = metaxylem, Pa = parenchyma Pd = periderm, Ph = phloem; Sc = sclerenchymatous fibers, Px = protoxylem, Xy = xylem).

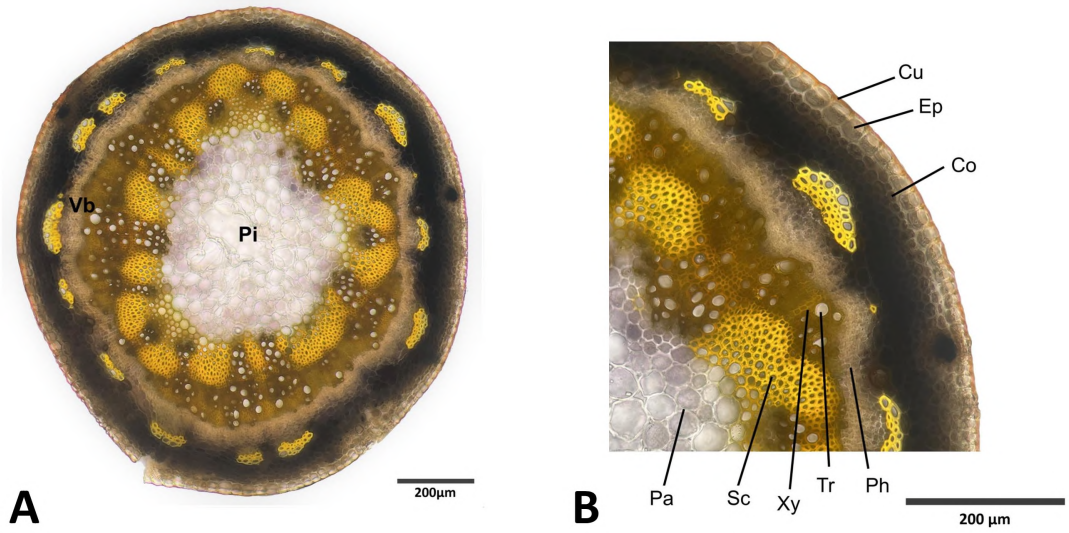

Figure 3. Stem cross section of *Lepidium graminifolium*: (A) General view; (B) Pith and vascular bundles (Cu = cuticle, Ep = epidermis, Co = cortex, Xy = xylem, Ph = phloem, Sc = sclerenchyma, Tr = trachea, Pa = parenchyma, Pi = pith, Vb = vascular bundle).

RESULTS AND DISCUSSION

Anatomical properties of the root

The periderm, which replaces the epidermis as the outer protective tissue, consists of 2 to 3 flattened, unformed cell layers with an average thickness of 17.31 µm (± 7.78 µm SD). The inner layer after the periderm is a cortex consisting of 10-15 layers of isodiametric or irregularly shaped cells with intercellular spaces. The cortex includes sclerenchymatous fiber cells. The inside of the cortex has a well-developed central cylinder, with phloem elements arranged radially between the polyarch xylem arms. The central cylinder is surrounded by 4-6 rows of sclerenchymatous fiber cells. The tracheary elements have an average diameter of 11.09 µm (± 5.36 µm SD; Figure 2). The measurements are listed in Table 1.

Anatomical properties of the stem

The outer protective tissue consists of a single layer of epidermis covered with a thin cuticle (2.15 ± 1.02 µm SD). The average width of the epidermis cells is 19.70 µm (± 6.46 µm SD), and the average length is 20.88 (± 3.44 µm SD). Four to seven layers of collenchyma cells are observed below the epidermis. The cortex, which is an outer layer of the stem, lies below the epidermis and is composed of 5-7 rows of large thin-walled parenchyma cells, followed by sclerenchymatous fibers. These sclerenchymatous fiber groups are located opposite the vascular bundles and above the phloem, which consists of 4-6 rows of isodiametric cells. The xylem and phloem are arranged side by side on the same radius. The xylem is located under the phloem, and no cambium was observed between the xylem and phloem. The xylem has tracheary elements with an average diameter of 11.79 µm (± 3.17 µm SD). The vascular bundles are collateral in type. Between these vascular bundles, sclerenchymatous fibers are seen to spread throughout the stem in the form of a ring. The pith consists of isodiametric parenchyma cells (Figure 3). The measurements are listed in Table 1.

Anatomical properties of leaf

The leaf is bifacial. The mesophyll has two distinct parts. Below the adaxial surface of the leaf is a single line of isodiametric epidermis cells under the outermost thin cuticle layer. The epidermis is followed by palisade parenchyma cells consisting of 3-4 layers of elongated cells. Beneath the palisade parenchyma cells is a sponge parenchyma cell consisting of 3-4 layers of oval or oval-rectangular cells, followed by isodiametric epidermis cells arranged in a single row on the abaxial surface. Stomata are present on the both surfaces of the leaf, so the leaf is amphistomatic. The stoma is anisocytic in type. The stomatal index was calculated as 32.2 for the adaxial surface and as 28.1 for the abaxial surface. In the mesophyll, elongated palisade parenchyma cells are arranged in 2 rows. This is followed by oval-shaped sponge parenchyma cells arranged in 4-5 rows. The mean mesophyll thickness was calculated as 185.70 µm (± 9.72 µm SD). Collateral-type vascular bundles are present within the mesophyll tissue. These represent the midrib and veins of the leaf. Unicellular eglandular trichomes are sparsely distributed on both the adaxial and abaxial surfaces of the leaves. The petiole has a single line of epidermis cells under the outermost thin cuticle layer. From the epidermis towards the center parenchyma, cells are observed to be lined up in 5-6 rows. The center and edges of the petiole have 3-5 vascular bundles, one big central vascular bundle followed by 2-4 little lateral vascular bundles (Figure 4). The measurements are listed in Table 1.

German's (2014) study mentions two important characters that can be used as distinguishing characteristics: the morphology of the trichomes and the type of stomata. When comparing the anatomical data obtained in this study with the anatomical data on different *Lepidium* species found in other studies, anatomical differences are observed to be present. For example, Sangekar's (2018) study on *L. sativum* stated the stoma to be of the tetracytic type, while the current study observed anisocytic-type stoma in *L. graminifolium*. Although most of the anatomical structures of *L. sativum* species are similar to *L. graminifolium*, the difference between stoma types for these two species shows that more anatomical studies can play a role in resolving the taxonomic issues surrounding *Lepidium* species around the world.

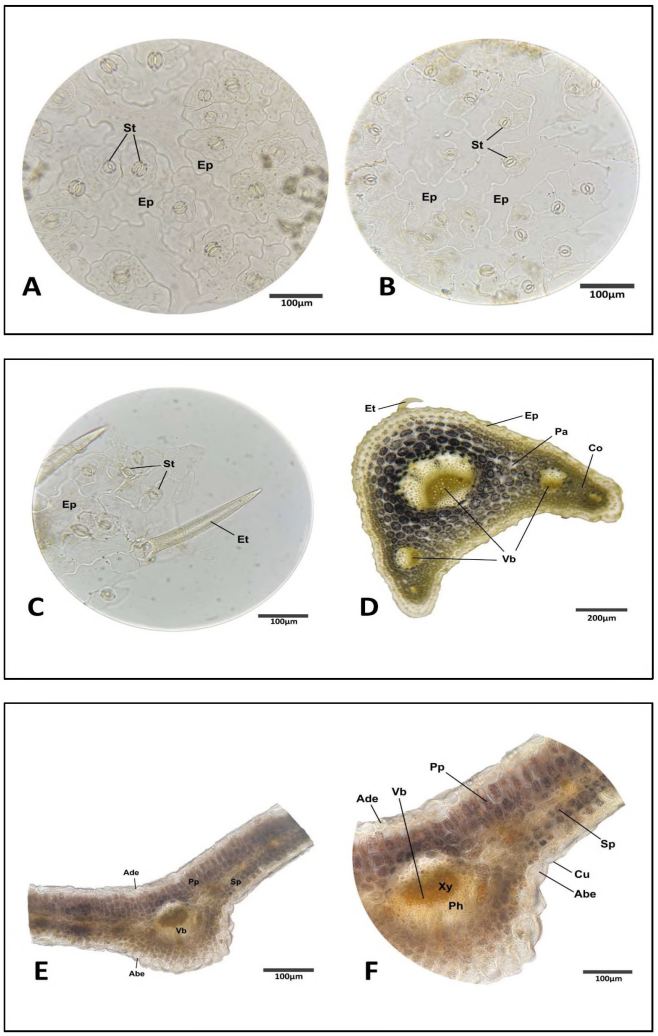

Figure 4. Leaf superficial and cross sections of *Lepidium graminifolium*: (A) Superficial section of abaxial epidermis, (B) Superficial section of adaxial epidermis, (C) eglandular trichome on the adaxial surface, (D) Cross section of the petiole, (E) and (F) Cross sections of the leaf lamina (Ade = adaxial epidermis, Abe = abaxial epidermis, Pp = palisade parenchyma, Sp = sponge parenchyma, Cu = cuticle, Xy = xylem, Ph = phloem, St = stoma, Ep = epidermis, Co = collenchyma, Pa = parenchyma, Vb = vascular bundle, Et = eglandular trichome, St = stoma, Ep = epidermis).

Table 1. Anatomical measurements of *Lepidium graminifolium*

		Width (µm)		Length (µm)	
		Min-Max	**Mean ± *SD***	**Min-Max**	**Mean ± *SD***
Root	Periderm thickness	10.24-28.46	17.31 ± 7.78	–	–
	Cortex cells	6.59-26.32	14.61 ± 7.58	12.98-47.94	31.07 ± 11.28
	Sclerenchymatous fiber cells	2.93-7.79	4.90 ± 1.76	4.73-8.64	7.07 ± 1.67
	Trachea (diameter)	3.44-20.18	11.09 ± 5.36	–	–
Stem	Cuticle thickness	2.66-5.33	3.77 ± 1.09	–	–
	Epidermis cells	9.43-27.55	19.70 ± 6.46	15.61-24.34	20.88 ± 3.44
	Endodermis cells	11.40-13.20	12.36 ± 0.82	14.02-24.05	18.15 ± 3.86
	Trachea (diameter)	6.86-16.23	11.79 ± 3.17	–	–
	Parenchyma cells(diameter)	13.73-49.71	29.54 ± 11.75	–	–
Leaf	Adaxial epidermis cells	20.87-37.67	27.16 ± 6.26	18.42-27.42	22.45 ± 3.90
	Abaxial epidermis cells	24.53-53.47	36.51 ± 12.12	18.91-46.15	32.41 ± 10.87
	Palisade parenchyma cells	11.04-18.43	15.15 ± 3.01	22.11-40.53	34.26 ± 6.88
	Spongy parenchyma cells	12.71-24.53	19.44 ± 3.98	11.71-20.16	15.93 ± 3.26
	Mesophyll thickness	175.47-200.34	185.70 ± 9.72	–	–
	Eglandular trichome	24.40-35.63	30.74 ± 4.38	244.67-297.56	267.01 ± 21.27
	* *SD* = Standard Deviation				

CONCLUSION

This is the first study to elucidate upon the anatomical features of *L. graminifolium.* The sclerenchymatous fibers observed in the root, the bifacial type of leaf mesophyll, and the sparse distribution of unicellular glandular hairs on both the adaxial and abaxial surfaces of the leaves are the anatomical features that differ from other *Lepidium* species. This study believes that elucidating upon the anatomical features of other *Lepidium* species may reveal results that are able to support morphological data regarding taxonomy.

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REFERENCES

Abdel, K. K. (2005). Morphological studies on trichomes of Brassicaceae in Egypt and taxonomic significance. *Acta Botanica Croatica, 64*(1), 57-73.

Al-Shehbaz, I. A., Beilstein, M. A., & Kellogg, E. A. (2006). Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. *Plant Systematics and Evolution, 259*, 89-120. http://dx.doi.org/10.1007/s00606-006-0415-z

Al-Shehbaz, I. A., Mutlu, B., & Dönmez, A. A. (2007). The Brassicaceae (Cruciferae) of Turkey, updated. *Turkish Journal of Botany, 31*(4), 327-336.

Appel, O., & Al-Shehbaz, I. A. (2003). Cruciferae. In K. Kubitzki & C. Bayer (Eds.), *Flowering Plants· Dicotyledons: Malvales, Capparales and Non-betalain Caryophyllales* (pp. 75-174). Berlin, Heidelberg: Springer.

Beilstein, M. A., Al-Shehbaz, I. A., & Kellogg, E. A. (2006). Brassicaceae phylogeny and trichome evolution. *American Journal of Botany, 93*(4), 607-619.

Bona, M. (2013). Seed-coat microsculpturing of Turkish Lepidium (Brassicaceae) and its systematic application. *Turkish Journal of Botany, 37*(4), 662-668. http://dx.doi.org/10.3906/bot-1207-2

Bona, M. (2014). Taxonomic revision of *Lepidium L.*(Brassicaceae) from Turkey. *Istanbul Ecz. Fak. Derg. J. Fac. Pharm, 44*, 31-62.

Çelebioğlu, S., & Baytop, T. (1949). Bitkisel tozların tetkiki için yeni bir reaktif. *Farmakolog, 19*, 301.

Chaffey, N. (2008). *Plant anatomy: an applied approach.* Oxford, United Kingdom: Blackwell Publishing,

De Carvalho, J., & Vasconcellos, J. D. (1964). Lepidium L. In: T. G. Tutin, V. H. Heywood, N. A. Burges, D.H. Valentine, S. M. Waters & D. A. Webb (Eds.). *Flora Europaea*, vol. 1: 330-333. Cambridge: Cambridge University Press.

Dorofeev V. I. (2012). Brassicaceae Burnett. In: D. V. Geltman (ed.). *Conspectus florae Europae Orientalis*, vol. 1 (pp. 364-437). Consociatio editionum scientificarum KMK: Petropoli-Mosqua

German, D. A. (2014). Taxonomic remarks on some Asian Lepidium sl (Brassicaceae). *Phytotaxa, 186*(2), 97. http://dx.doi.org/10.11646/phytotaxa.186.2.4

Grigore, M. N., ¨& Toma, C. (2008). A Histo-Anatomical Study On Some Halophylous Species Of The Lepidium Genus. *Studia Universitatis Vasile Goldis Seria Stiintele Vietii (Life Sciences Series), 18*, 27-32.

Hedge I. A. (1976). Systematic and Geographical Survey of the Old World Cruciferae. In: MacLeod AJ, Jones BMG (Eds). *The Biology and Chemistry of the Cruciferae*, (pp 1-45). London: Academic Press.

Hedge I. A. (1965). Lepidium L. In: P. H. Davis (Ed). *Flora of Turkey and the East Aegean Islands*, Vol. 1. Edinburg: Edinburg University Press.

Hewson, H. J. (1981). The genus *Lepidium L.*(Brassicaceae) in Australia. *Brunonia, 4*(2), 217-308.

IPNI. (2023, July 19). *Lepidium L.*: International Plant Name Index. Retrieved from https://www.ipni.org/n/60437330-2

Koch, M. A., & Mummenhoff, K. (2006). Evolution and phylogeny of the Brassicaceae. *Plant systematics and evolution, 259*(2/4), 81-83. http://dx.doi.org/10.1007/s00606-006-0433-x

Lee, M. S., Shin, B. C., Yang, E. J., Lim, H. J., & Ernst, E. (2011). Maca (Lepidium meyenii) for treatment of menopausal symptoms: a systematic review. *Maturitas, 70*(3), 227-233. http://dx.doi.org/10.1016/j.maturitas.2011.07.017

Metcalfe, C. R. & Chalk, L. (1957). *Anatomy of the Dicotyledones*. Vol. 2. London: Oxford University Press.

Özkan, Y. (2017). *Türk Farmakopesi*. Genel monograflar I, 1st ed., Ankara: T.C. Sağlık Bakanlığı Yayını.

Peres, N. D. S. L., Bortoluzzi, L. C. P., Marques, L. L. M., Formigoni, M., Fuchs, R. H. B., Droval, A. A., & Cardoso, F. A. R. (2020). Medicinal effects of Peruvian maca (Lepidium meyenii): A review. *Food & function, 11*(1), 83-92. http://dx.doi.org/10.1039/c9fo02732g

Sangekar, S. N., Devarkar, V. D., Shaikh, T. J., Shahane, M. K., & Kshirsagar, U. M. (2018). Phytochemical and taxonomical studies of Lepidium sativum L.(Brassicaceae). *International Research Journal of Science and Engineering*, 166-172.

Simpson, M. G. (2019). Diversity and classification of flowering plants: Eudicots. J Plant System, *3*: 285-466. http://dx.doi.org/10.1016/B978-0-12-812628-8.50008-0

Smirnov, P. A. (1948). About Lepidium meyeri Claus. *Byulleten Moskovskogo obschestva ispytatelej prirody. 53*(4): 72-78.

Tekin, M. (2022). A morphological, anatomical and palynological study of Aethionema lepidioides (Brassicaceae)-an endangered species endemic to Turkey. *Acta Botanica Croatica, 81*(1), 0-0. http://dx.doi.org/10.37427/botcro-2022-003

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