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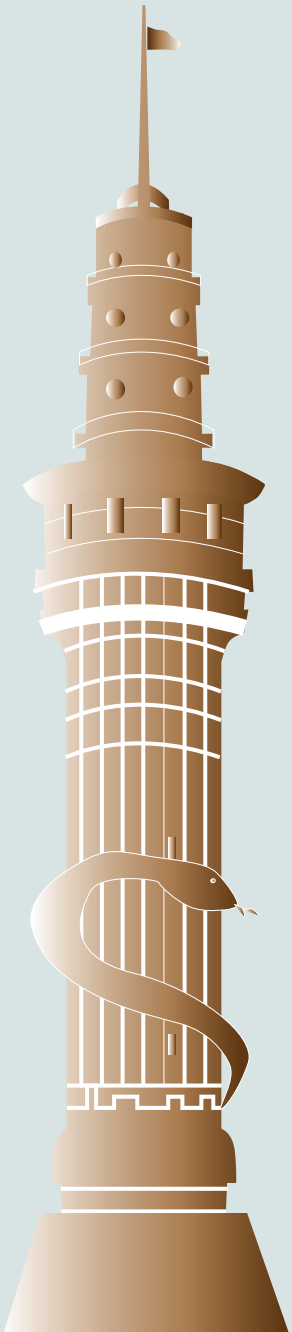

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VOLUME 53, ISSUE 3

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AIMS AND SCOPE

İstanbul Journal of Pharmacy (Istanbul J Pharm) is an international, scientific, open access periodical pub- lished 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, Au- gust, 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 disci- plines 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, TU- BITAK 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 publica- tion 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 avail- able 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.

PUBLICATION POLICIES

The journal is committed to upholding the highest standards of publication ethics and pays regard to Principles of Transparency and Best Practice in Scholarly Publishing published by the Committee on Publication Ethics (COPE), the Directory of Open Access Journals (DOAJ), the Open Access Scholarly Publishers Association (OASPA), and the World Association of Medical Editors (WAME) on https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing

The subjects covered in the manuscripts submitted to the Journal for publication must be in accordance with the aim and scope of the Journal.

Changing the name of an author (omission, addition or order) in papers submitted to the Journal requires written permission of all declared authors.

Plagiarism, duplication, fraud authorship/denied authorship, research/data fabrication, salami slicing/salami publication, breaching of copyrights, prevailing conflict of interest are unethical behaviors. All manuscripts not in accordance with the accepted ethical standards will be removed from the publication. This also contains any possible malpractice discovered after the publication.

Plagiarism

Submitted manuscripts that pass preliminary control are scanned for plagiarism using iThenticate software. If plagiarism/self-plagiarism will be found authors will be informed. Editors may resubmit manuscript for similarity check at any peer-review or production stage if required. High similarity scores may lead to rejection of a manuscript before and even after acceptance. Depending on the type of article and the percentage of similarity

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

Double Blind Peer-Review

After plagiarism check, the eligible ones are evaluated by the editors-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope. The editor provides a fair double-blind peer review of the submitted articles and hands over the papers matching the formal rules to at least two national/international referees for evaluation and gives green light for publication upon modification by the authors in accordance with the referees' claims.

Editorial Policy

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Council of Medical Journal Editors (ICMJE), the World As- sociation of Medical Editors (WAME), the Council of Science Editors (CSE), the Committee on Publication Ethics (COPE), the European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal conforms to the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice). Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be ac- cepted for publication. Manuscripts submitted for evalua- tion should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been sub- mitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization.

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The journal is an open access journal and all content is freely available without charge to the user or his/her institution. Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. This is in ac- cordance with the BOAI definition of open access.

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All expenses of the journal are covered by the İstanbul University. Processing and publication are free of charge with the journal. There is no article processing charges or submission fees for any submitted or accepted articles.

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Correction, Retraction, Expression of Concern

Editor should consider publishing correction if minor errors that do not effect the results, interpretations and conclusions of the published paper are detected. Editor should consider retraction if major errors and/or misconduction that invalidate results and conclusions are detected.

Editor should consider issuing an expression of concern if there is evidence of research or publication misconduct by the authors; there is evidence that the findings are not reliable and institutions of the authors do not investigate the case or the possible investigation seems to be unfair or nonconclusive.

The guidelines of COPE and ICJME are taken into consideration regarding correction, retractions or expression of concern.

Archiving Policy

To guarantee that all papers published in the journal are maintained and permanently accessible, articles are stored in Dergipark which serves as a national archival web site and at the same time permits LOCKSS to collect, preserve, and serve the content.

Additionally, authors are encouraged to self-archive the final PDF version of their articles in open electronic archives with that conform to standards of Open Archives Initiative (https://www.openarchives.org/). Authors should provide a link from the deposited version to the URL of IUPress journal website.

PEER REVIEW POLICIES

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 referees for evaluation and publication 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 sci- entific content without regard to ethnic origin, gender, sexual orientation, citizenship, religious belief or po- litical 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 be- tween 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 re- spect 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 ano- nymity of the referees must be ensured. In particular sit- uations, the editor may share the review of one reviewer with other reviewers to clarify a particular point.

PEER REVIEW PROCESS

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. Editor-in-Chief evaluates manu- scripts for their scientific content without regard to ethnic origin, gender, sexual orientation, citizenship, re- ligious belief or political philosophy of the authors and ensures a fair double-blind peer review of the selected manuscripts.

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

Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers and is re- sponsible for final decision for publication of the manu- scripts 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 ano- nymity 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 miscon- duct, e.g., plagiarism, citation manipulation, and data fal- sification/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.

Author's Responsibilities

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 co-decision 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 with the Editor to provide retractions or corrections of mistakes.

Human Subjects and Animal Use in Research, Ethics Committee Approval and Informed Consent

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 re- solve 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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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.

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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:

- The full title of the manuscript as well as a short title (running head) of no more than 50 characters,
- 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 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.

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

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

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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

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e) Journal Article wih DOI

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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

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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

Virtual drug screening for p65/rela subunit of nf-κb: Promising repurposable drugs in the treatment of stress-based diseases

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ABSTRACT

Background and Aims: Although NF-κB is composed of five subunits, RelA receives much more attention due to fact that its expression level is regulated under various stress conditions, such as exposure to radiation, reactive oxygen species (ROS), hypoxia, pathogens, and inflammatory cytokines, as well as regulating many inflammatory, proliferation, and apoptosis genes. To date, many pieces of evidence have demonstrated that RelA plays a significant role in in the prognosis of various proliferative and inflammatory diseases. Therefore, the design of novel inhibitors and the discovery of repurposable drugs are considered promising approaches in the treatment of RelA-based diseases.

Methods: A drug library including a total of 12,111 ligands has been screened for the RelA subunit of NF-κB. The sufficiency of the study's strategy has been revealed by analysis of commercially available inhibitors and re-docking applications.

Results: Findings demonstrate that ZINC000096928979 (Deleobuvir), ZINC000012503187 (Conivaptan), and ZINC000003974230 ligands have the highest binding affinity to RelA. Furthermore, many ligands with structural similarities to Valstar, Ergotamine drugs and Benzo[a]pyrene-7,8-Diol metabolite have been discovered.

Conclusion: While the ligands with the highest binding affinities could be repurposed in the treatment of RelA-based diseases, the structures of the ligands exhibiting similarity with Valstar, Ergotamine, and Benzo[a]pyrene-7, 8-D may be used as a scaffold in structure-based drug design studies. The stability of the interactions between the ligands and the receptor should be analyzed with further Molecular Dynamics Simulations (MD) studies and the possible ligands should be investigated by both in vitro and in vivo applications.

Keywords: RelA (p65), NF-κB, Virtual Drug Screening, Molecular Docking, Drug Repurposing

INTRODUCTION

Nuclear factor-κB (NF-κB) is one of the main transcription factors due to its regulatory activity on many significant cellular pathways such as apoptosis (Bernal-Mizrachi, Lovly, & Ratner, 2006), proliferation (Wan & Lenardo, 2010), differentiation (Kaltschmidt, Greiner, & Kaltschmidt, 2021), and inflammation (Liu, Zhang, Joo, & Sun, 2017). NF-κB is composed of five subunits: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (Perkins & Gilmore, 2006). Transcription of target genes requires nuclear translocation of NF-κB subunits through canonical and noncanonical signaling pathways (Sun, 2011; Zarnegar, Yamazaki, He, & Cheng, 2008). While these proteins may form several homodimers and heterodimers, these forms have distinct signaling mechanisms for the expression of various genes (Ghosh, Wang, Huang, & Fusco, 2012). The most abundant form of NF-κB is observed as heterodimers of NF-κB1 and RelA and the phosphorylation of RelA plays a significant role over the activity of this heterodimer since it provides chemical stability and causes conformational changes for protein-protein interactions (Chuang, Rehan, & Khorram, 2020; Darwish, Abo-Youssef, Messiha, Abo-Saif, & Abdel-Bakky, 2021).

While the activation of RelA is observed in endothelial cells (Bijli, Fazal, & Rahman, 2012), macrophages (Dorrington & Fraser, 2019), and smooth muscle cells (Zhang et al., 2010), various stresses such as radiation (Kim et al., 2004), reactive oxygen species (ROS) (Morgan & Liu, 2010), hypoxia (Choi et al., 2019), the existence of pathogens within the host body (Rahman & McFadden, 2011), and recognition of inflammatory cytokines (Ronin et al., 2019) enhance the expression level of RelA. Considering the activation of RelA by stresses, its expression regions and regulatory effect on proliferation, apoptosis

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and inflammatory genes, the connection between RelA and several proliferative diseases such as various cancer types (Zhang, Ma, Zhang, Zhang, & Hu, 2021), inflammatory diseases such as rheumatoid arthritis (Makarov, 2001) and inflammatory bowel diseases (Balta, 1998), and muscle tissue diseases such as multiple sclerosis (Zhou, 2020) have been reported in the literature. As such, inhibition of RelA shows promising potential in the treatments of related diseases (Giridharan & Srinivasan, 2018).

In this study, a molecular docking-based virtual drug screening targeting the RelA subunit of NF-κB was performed. Primarily, a drug library including 12,111 ligands composed of four distinct datasets; FDA-Approved Drugs (1,615 ligands), World-not-FDA Approved Drugs (4,288 ligands), Drugs in Clinical Trials (3,897 ligands), and Non-human Metabolites (2,311 ligands) was created and screened during the study. In addition, 16 commercially available inhibitors as well as the ligand found in the chemical structure of RelA (S-Adenosylmethionine (SAM)) were analyzed through the same experimental procedure for validation. Findings point that ZINC000096928979 (Deleobuvir), ZINC000012503187 (Conivaptan), and ZINC000003974230 ligands have the highest affinities in order to interact with RelA. In addition, many structurally similar ligands with Valstar, Ergotamine, and Benzo[a]pyrene-7,8-Diol ligands have binding affinity to RelA. Thus, results demonstrate that while the three best scored ligands might be considered as promising to be tried in the treatment of RelA based diseases, the structures of Valstar, Ergotamine, and Benzo[a]pyrene-7,8-Diol might be considered as scaffolding for further structural based drug design studies.

Materials and Methods

Receptor Preparation

The crystal structure of RelA subunit of NF-κB was retrieved from the Protein Data Bank (PDB) in .pdb format (PDB ID: 3QXY). The resolution, R-value (free), R-value (observed) parameters of the selected RelA subunit's were 2.09 Â, 0.229, and 0.173, respectively. Preparation of the receptor was carried out through the Dock Prep module of UCSF Chimera software version 1.16 by adding hydrogen atoms, partial charges and replacing the side chains with the Dunbrack 2010 rotamer library to remove the ligands, heteroatoms, and water. The prepared receptor was exported in .pdb format for further molecular docking studies (Pettersen et al., 2004).

Ligand Library Preparation

A drug library including 12,111 ligands was created by retrieving FDA-Approved Drugs (1,615 ligands), World-not-FDA Approved Drugs (4,288 ligands), Drugs in Clinical Trials (3,897 ligands), and Non-human Metabolites (2,311 ligands) datasets from the ZINC15 database. The ligands of the library were prepared through the energy minimization module of PyRx Virtual Screening Tool after importing the data separately (Dallakyan & Olson, 2015).

Molecular Based Drug Screening

Molecular docking based virtual drug screening of the prepared library was carried out with the AutoDock Vina package of PyRx Virtual Screening Tool by targetting the region interacting with S-Adenosylmethionine (SAM) inhibitor (Trott & Olson, 2011). For this purpose, the ligands were converted to .pdbqt format, and grid box parameters were defined as 20 x 20 x 20 as size, and x= 61.728, y= 7.720, z= 61.982 as coordinates. The data showing binding affinity, rmsd/ub, and rmsd/lb values of the ligands were exported in .csv format. The modes of the best scored ligands with 0 rmsd/ub, and 0 rmsd/lb values were selected, and the interactions between selected ligands with the receptor were analyzed in Biovia Discovery Studio Visualiser software.

Validation

A validation study was carried out by exporting the S-Adenosylmethionine (SAM) inhibitor found in chemical structure of RelA, following the same ligand preparation, and molecular docking procedures. The RMSD difference between SAM in the crystal structure and re-docked form was analyzed with DockRMSD web server produced by Zhang Lab (Bell & Zhang, 2019). As such, the SAM was exported from the retrieved pdb file, and both conformations were imported to the server in mol2. format. A total of 27 atoms were aligned by server and RMSD value pointing the sufficiency of the study was analyzed. In addition, a novel Inhibitors Library composed of 16 commercially available inhibitors of RelA, Licochalcone D, Stachydrine, Sauchinone, Neferine, SC75741, Dihydroartemisinin, 5-Aminosalicylic Acid, Neochlorogenic Acid, Mangiferin, Morusin, Tectochrysin, Sulfasalazine, Tomatitine, Maslinic Acid, Vanillic Acid, and (-)-DHMEQ (Compound CIDs: 10473311, 115244, 11725801, 159654, 23661638, 3000518, 4075, 5280633, 5281647, 5281671, 5281954, 5339, 65576, 73659, 8468, 9881652, respectively) was created by retrieving the ligands from PubChem database. The molecular docking procedure was repeated with this library in order to analyze the efficiencies of the inhibitors.

ADME and Toxicity Properties

Absorption, Distribution, Metabolism, and Excretion (ADME) and toxicity properties of two of the best scored ligands (ZINC000096928979 (Deleobuvir) and ZINC000003974230) with the three best scored inhibitors were analyzed with both the swissADME server (Daina, Michielin, & Zoete, 2017) and OSIRIS Property Explorer tool (Sander, 2022). Therefore, the

ligands' physicochemical, solubility, lipophilicity, pharmacokinetics properties, and toxicity profiles were studied. Since it had been tested and approved by FDA previously, ADME and toxicity analyses were not required for ZINC000012503187 (Conivaptan).

Results and Discussion

Virtual drug screening for the RelA subunit of NF-κB was carried out in order to reveal possible repurposable drugs. Therefore, a drug library consisting of FDA-Approved Drugs, World-not-FDA Approved Drugs, Drugs in Clinical Trials, and Non-human Metabolites datasets were created and a total of 12,111 ligands were docked to the inhibitor binding region of RelA. The results including the binding affinities and the datasets of the 20 best scored ligands as well as the interacting amino acid residues of the receptor with the related ligands are listed in Table 1.

In order to validate the molecular docking strategy, the S-Adenosylmethionine (SAM) inhibitor found in the chemical structure of RelA was exported as a separate file and was re-docked to the same region of the receptor. The re-docked SAM's binding affinity was recorded as -8.8 kcal/mol. Interactions between ligand and receptor in both the SAM re-docking study and the SAM in crystal structure of .pdb file were analyzed in Biovia Discovery Studio software (Figure 1). Accordingly, SAM in crystal structure interacts with ALA 73, TYR 75, TYR 223, ASN 251, HIS 252, TYR 297 residues through conventional hydrogen bonds, TYR 285 and EDO 477 through carbon-hydrogen bonds, VAL 72 through pi-sigma interaction, PHE 299 through pi-pi stacked interaction, ALA 73 through pi-alkyl interaction and the water molecules through water hydrogen bonds. Besides, re-docked SAM interacts with TYR 75, LEU 146, TRP 147, TYR 223, HIS 252, TYR 285 residues through conventional hydrogen bonds, ASN 251 through carbon-hydrogen bonds, ARG 68 through pi-cation interaction, PRO 148 through pi-alkyl interaction, and LEU 146 through unfavorable acceptor-acceptor interaction. Since water molecules had been removed during protein preparation, possible interactions with the ligands could not be analyzed. The RMSD difference between SAM in crystal structure and re-docked form were measured as 1.126 by DockRMSD web server of Zhang Lab. Observing common amino acids, similar interactions, and close RMSD values between re-docked SAM and SAM in retrieved file proves the sufficiency of study's strategy.

In addition, the Inhibitors Library composed of 16 commercially available inhibitors was created and docked to RelA to reveal the binding affinities and the common amino acids that are interacted with the inhibitors. The binding affinities of the Inhibitor Library including re-docked SAM and the interacting amino acids are listed in Table 2. Accordingly, three inhibitors, which are Morusin, SC75741, and Sauchinone have exhibited

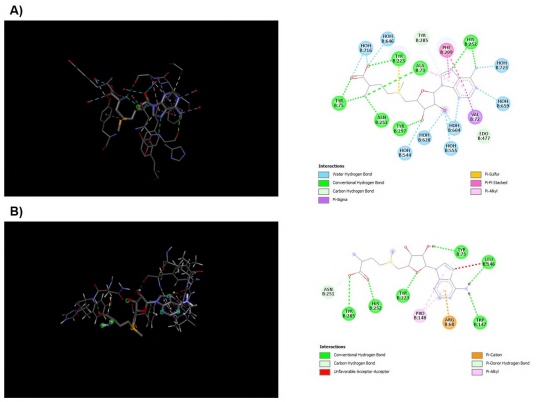


Figure 1. RelA interactions with A) S-Adenosylmethionine (SAM) in crystal structure and B) re-docked S-Adenosylmethionine (SAM).

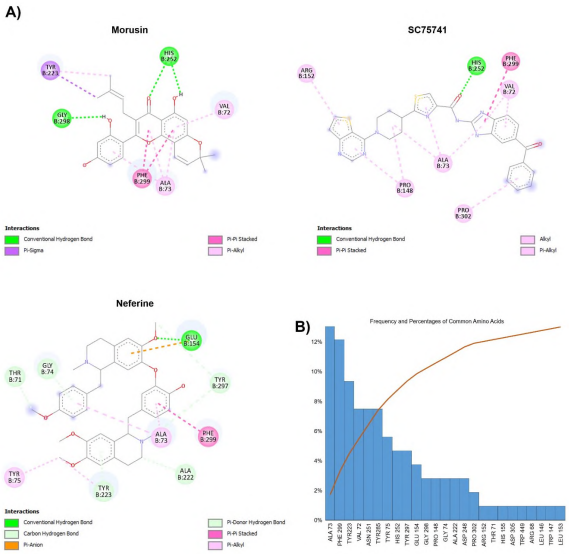


Figure 2. A) RelA interactions with the best scored inhibitors which are Morusin, SC75741, and Neferine, B) Frequency and percentages of common interacting amino acids.

high binding affinities as -10.9 kcal/mol, -10.6 kcal/mol, and -10.2 kcal/mol, respectively. Furthermore, Morusin interacts with HIS 252 and GLY 298 through conventional hydrogen bonds, VAL 72, ALA 73, TYR 223, and PHE 299 through pi-alkyl interactions, TYR 223 through pi-sigma interaction, PHE 299 through pi-pi stacked interactions, SC75741 interacts with HIS 252 through conventional hydrogen bonds, VAL 72, ALA 73, PRO 148, ARG 152, and PRO 302 through alkyl interactions; PHE 299 through pi-pi stacked interaction, VAL 72 and ALA 73; and PRO 148 through pi-alkyl interactions. Sauchinone interacts with GLU 154 through conventional hydrogen bonds, THR 71, ALA 222, and TYR 297 through carbon-hydrogen bonds, GLY 74 and TYR 223 through pi-donor hydrogen bonds, ALA 73, TYR 75, and TYR 223 through pi-alkyl interactions, PHE 299 through pi-pi stacked interaction, and

Table 1. Best Scored 20 Ligands' binding affinities, datasets, and the amino acid residues that interacted with.

30 Best Scored			
Ligand Name	**Score (kcal/mol)**	**Dataset**	**Receptor Residues Interacting with Ligands**
ZINC000096928979	-13.7	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 75, TRP 147, TYR 223, ASP 248, LEU 250, ASN 251, PHE 299, TRP 449
ZINC000012503187	-13.0	FDA-Approved Drugs	ARG 68, ALA 73, TYR 75, LEU 146, PRO 148, GLU 154, ALA 222, TYR 223, PHE 289
ZINC000003974230	-12.9	Drugs in Clinical Trials	VAL 72, ALA 73, PRO 148, GLU 154, TYR 223, TYR 285, PHE 299
ZINC000095618662	-12.8	World-not-FDA Approved Drugs	ARG 68, VAL 72, ALA 73, TYR 75, PRO 148, TYR 223, ASN 251, HIS 252, TYR 285, TYR 297, PHE 299, VAL 300, PRO 302
ZINC000003922429	-12.3	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 223, ASP 248, ASN 251, HIS 252, PHE 299
ZINC000004214612	-12.3	World-not-FDA Approved Drugs	ARG 68, VAL 72, ALA 73, TYR 75, LEU 146, TRP147, PRO 148, GLU 149, LEU 153, TYR 223, ILE 249, HIS 252, TYR 285, PHE 299, VAL 300, TRP 449
ZINC000004215812	-12.3	World-not-FDA Approved Drugs	ARG 68, VAL 72, ALA 73, TYR 75, GLU 154, TYR 223, PHE 299
ZINC000011616852	-12.3	FDA-Approved Drugs	ARG 68, VAL 72, ALA 73, GLY 74, PRO 148, GLU 149, ALA 222, TYR 223, PHE 225, ASN 251, HIS 252, TYR 285, TYR 297, PHE 299, VAL 300, PRO 302, TRP 449
ZINC000100016063	-12.3	Drugs in Clinical Trials	VAL 72, ALA 73, ALA 222, TYR 223, TYR 285, TYR 297, PHE 299
ZINC000043204146	-12.2	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 75, GLU 149, ARG 152, GLU 154, TYR 223, HIS 252, TYR 285, PHE 299, GLU 301, PRO 302
ZINC000052955754	-12.2	FDA-Approved Drugs	ARG 68, VAL 72, ALA 73, GLY 74, TYR 75, PRO 148, TYR 223, PHE 299, VAL 300
ZINC000003924139	-12.1	Drugs in Clinical Trials	ARG 68, VAL 72, ALA 73, TYR 75, LEU 146, PRO 148, TRP 147, TYR 223, GLY 298, PHE 299
ZINC000003926844	-12.1	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 75, PRO 148, ARG 152, LEU 153, GLU 154, ALA 222, TYR 223, HIS 252, TYR 285, PHE 299
ZINC000003978005	-12.1	FDA-Approved Drugs	ARG 68, VAL 72, ALA 73, TYR 75, PRO 148, ILE 249, PHE 299
ZINC000003985678	-12.1	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 75, LEU 146, LEU 153, HIS 155, ALA 222, ASP 248, ILE 249, ASN 251, HIS 252, LEU 253, TYR 285, PHE 299
ZINC000006717791	-12.1	Drugs in Clinical Trials	VAL 72, ALA 73, LEU 153, HIS 155, ALA 222, TYR 223, ASP 248, TYR 285, PHE 299
ZINC000030728718	-12.1	Non-human Metabolites	VAL 72, ALA 73, TYR 75, TYR 223, SER 224, ALA 247, ILE 249, ASN 283, TYR 297, PHE 299
ZINC000063933734	-12.1	Drugs in Clinical Trials	VAL 72, ALA 73, ASN 251, ALA 222, TYR 223, ASP 248, TYR 285, TYR 297, GLY 298, PHE 299, TRP 449
ZINC000072190224	-12.1	Drugs in Clinical Trials	VAL 72, ALA 73, TYR 75, LEU 146, TRP 147, PRO 148, LEU 153, GLU 154, ALA 222, TYR 223, PHE 225, ASN 251, HIS 252, TYR 285, ASP 248, PHE 299
ZINC000095618690	-12.1	World-not-FDA Approved Drugs	ALA 73, TYR 223, ASP 248, ILE 249, PHE 299

GLU 154 through pi-anion interaction (Figure 2-A). The frequency and percentages of common amino acids interacting with inhibitors are shown in Figure 2-B. Accordingly, common interacting amino acids are VAL 72, ALA 73, GLY 74, TYR 75, PRO148, GLU 154, ALA 222, TYR 223, ASP 248, ASN 251, HIS 252, TYR 285, TYR 297, GLY 298 and PHE 299.

Virtual drug screening findings demonstrate that the three best scored ligands, ZINC000096928979 (Deleobuvir), ZINC000012503187 (Conivaptan), and ZINC000003974230 have binding affinities of 13.7 kcal/mol, 13.0 kcal/mol, and 12.9 kcal/mol, respectively, and possess high potential to be used in the treatment of RelA based diseases. The interactions of these ligands with RelA protein are demonstrated in Figure 3. The analysis put forward that ZINC000096928979 (Deleobuvir), which is used in the treatment of Hepatitis C (HCV) through inhibiting the NS5B polymerase (Larrey et al., 2013), might create conventional hydrogen bonds with ASN 251, carbon-hydrogen bonds with TRP 147, ASP 248, and LEU 250, pi-donor interactions with TYR 223, pi-sigma interactions with TYR 75, pi-pi stacked and pi-pi T-shaped interactions with TRP 449, and PHE 299, alkyl interactions with TRP 449, and pi-alkyl interactions with VAL 72, ALA 73, and TYR 223 residues. ZINC000012503187 (Conivaptan) which is used in hypervolemic and euvolemic hyponatremia (Zeltser, Rosansky, Van Rensburg, Verbalis, & Smith, 2007) as Vasopressin receptor inhibitor (Ali, Raufi, Washington, & Ghali, 2007) creates conventional hydrogen bonds with LEU 146, and TYR 223, pi-cation and pi-anion interactions with ARG 68, and GLU 154, pi-donor hydrogen bonds with TYR 75, pi-pi stacked interactions with PHE 299, pi-alkyl interactions with ALA 73, TYR 75, PRO 148, ALA 222, and TYR 223 residues. The ZINC000003974230 ligand, whose unknown activity creates conventional hydrogen bonds with TYR 223, has unfavorable acceptor-acceptor interactions with TYR 285, pi-anion interactions with GLU 154, pi-pi stacked interactions with TYR 223, TYR 285, and PHE 299, alkyl and pi-alkyl interactions with VAL 72, ALA 73, PRO 148, and PHE 299 residues. Since the interacting amino acids and interaction types exhibit similarity with the inhibitors, HCV inhibitor ZINC000096928979 (Deleobuvir), Vasoppressin receptor inhibitor ZINC000012503187 (Conivaptan), and ZINC000003974230 ligands are considered as repurposable in the treatment of stress based diseases progressed by RelA activation. Chemical structures of the best scored ligands and the inhibitors are shown in Figure 4.

ADME and possible toxicity properties of the best scored ligands, which are ZINC000096928979 (Deleobuvir), and ZINC000003974230, were carried out with the OSIRIS Property Explorer tool and swissADME server. In order to compare the potential of these ligands, the three best scored inhibitors were analyzed by the same strategy as well (Table 3). Since ZINC000012503187 (Conivaptan) had been approved by the FDA, it does not require analysis for ADME and toxicity properties. Findings demonstrate that ZINC000096928979

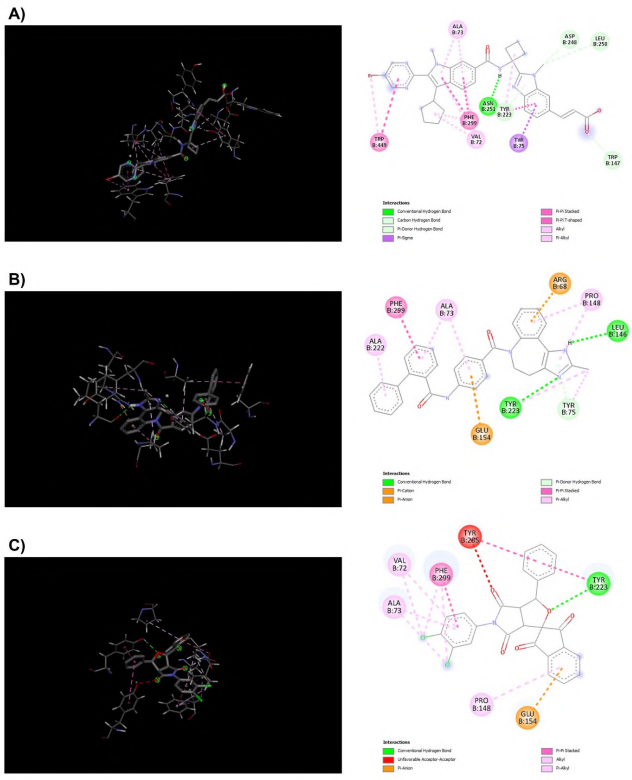


Figure 3. RelA interactions with the best scored ligands; A) ZINC000096928979 (Deleobuvir), B) ZINC000012503187 (Conivaptan), and C) ZINC000003974230.

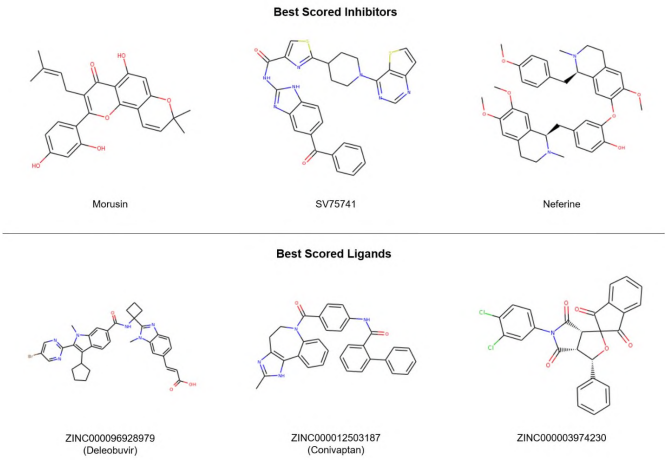


Figure 4. Chemical structures of the three best scored inhibitors and ligands from virtual screening.

(Deleobuvir) is poorly soluble, has low gastrointestinal (GI) absorption, no CYP isoform inhibition activity except CYP2C19 and CYP2D6, and no toxicity potential. ZINC000003974230 is moderately soluble, has high GI absorption, no CYP isoform inhibition activity except CYP2C19 and CYP2C9, and possible mutagenicity and reproductive effects. It has been demonstrated

Table 2. Inhibitor Library results including re-docked SAM.

Inhibitors Including Re-doc ed Ben amidine		
Ligand Name	**Binding Affinity (kcal/mol)**	**Receptor Residues Interacting with Ligand**
Morusin	-10.9	VAL 72, ALA 73, TYR 223, HIS 252, GLY 298, PHE 299
SC75741	-10.6	VAL 72, ALA 73, PRO 148, ARG 152, HIS 252, PHE 299, PRO 302
Ne erine	-10.2	THR 71, ALA 73, GLY 74, TYR 75, GLU 154, ALA 222, TYR 223, TYR 297, PHE 299
Sauchinon	-9.8	ALA 73, HIS 155, PHE 299
Mangi erin	-9.5	VAL 72, ALA 73, GLU 154, TYR 223, ASN 251, TYR 285, TYR 297, GLY 298, PHE 299
Sul asala ine	-9.4	VAL 72, ALA 73, TYR 223, ASN 251, TYR 285, PHE 299, ASP 305
Licochalcone D	-9.2	VAL 72, ALA 73, GLY 74, TYR 75, PRO 148, TYR 223, ASP 248, HIS 252, TYR 285, PHE 299, TRP 449
Tectochr sin	-8.9	VAL 72, ALA 73, ASN 251, HIS 252, TYR 285, PHE 299
S-Adenos lmethionine SAM	-8.8	ARG 68, TYR 75, LEU 146, TRP 147, PRO 148, TYR 223, ASN 251, HIS 252, TYR 285
Tomatidine	-8.8	ALA 73, TYR 297, PHE 299, PRO 302
Maslinic Acid	-8.7	GLU 154, ALA 222, TYR 223, TYR 285
Neochlorogenic Acid	-8.6	VAL 72, ALA 73, GLY 74, TYR 75, ASP 248, ASN 251, PHE 299
Dih droartemisinin	-8.2	ALA 73, PHE 299
- -DHME	-8.1	VAL 72, ALA 73, LEU 153, GLU 154, ASN 251, PHE 299
Vanillic Acid	-5.8	ALA 73, ALA 222, TYR 223, TYR 285, TYR 297, GLY 298, PHE 299
5-Aminosalic lic Acid	-5.7	ALA 73, TYR 75, TYR 223, ASP 248, ASN 251
Stach drine	-4.9	TYR 223, ASN 251, TYR 285, TYR 297

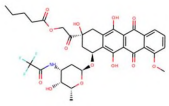
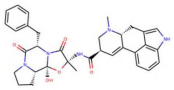
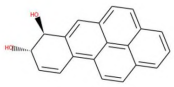
that these best scored ligands exhibit similarity with inhibitors about several parameters such as drug-scores, CYP inhibitory activities, and toxicity. In particular, since ZINC000096928979 (Deleobuvir) has no toxicity effects and a rather favorable drug-score, and ZINC000003974230 has low molecular weight compared to the inhibitors, these ligands and ZINC000012503187 (Conivaptan) might be considered as promising repurposable drugs.

One of the novel approaches to develop inhibitor molecules against target proteins is based on designing ligands by referencing the structures with potential. In order to reveal potential structure scaffolds, the 30 best scored ligands from four datasets were analyzed. Among 120 ligands, 16, 5, and 11 ligands share structurally similarity with ZINC000011616852 (Valstar), ZINC000052955754 (Ergotamine), and ZINC000002019693 (Benzo[a]pyrene-7,8-Diol), respectively. The scaffold structures and the ligands sharing similarity are listed in Table 4. The scaffold of Valstar composes five benzene rings and long Carbon (C) chain carrying Oxygen (O) and hydroxyl (OH) groups. In addition, Ergotamine structure composes five benzene rings connected with three cyclopropane via carbon atom. Lastly, Benzo[a]pyrene-7, an 8-Diol structure, composes five strictly connected benzene rings. While these scaffolds share common structures such as five benzene rings, accordingly, these structures might be considered as a template in structure based drug design studies for RelA inhibition.

Table 3. ADME and toxicity properties of the three best scored inhibitors, ZINC000096928979 (Deleobuvir), and ZINC000003974230 ligands.

DM roperties and o icit ro iles						
roperties		**est Scored Inhi itors**			**est Scored Ligands**	
h sico chemical properties	**Ligand ame**	Morusin	SC75741	Ne erine	ZINC00009692897 9 Deleobuvir	ZINC00000397423 0
	ormula	C25H24O6	C29H23N7O2S2	C38H44N2O6	C34H33BrN6O3	C26H15Cl2NO5
	Molecular eight (g/mol)	420.45	565.67	624.77	653.57	492.31
	Molar Re racti it	121.83	160.76	188.02	174.90	127.60
	S (topological polar sur ace area)	100 .13	173.24	72.86	114.93	80.75
Lipophilicit	**Log o/w (iL)**	3.77	3.18	5.21	3.93	3.13
	Log o/w (L)	5.52	5.48	6.70	5.64	4.29
	Log o/w (L)	5.16	5.32	5.35	6.63	3.98
	Log o/w (ML)	2.09	2.69	3.46	3.73	3.27
	Log o/w (SILI S I)	5.18	6.26	6.64	5.84	4.82
	onsensus Log o/w	4.35	4.59	5.47	5.15	3.90
Solu ilit	**Log (SILI S I)**	-6.11	-9.74	-10.74	-9.40	-8.63
	SILI S I Solu ilit (mg/ml)	3.22e-04	1.02e-07	1.12e-08	2.57e-07	1.14e-06
	SILI S I Solu ilit (mol/l)	7.79e-07	1.80e-10	1.80e-11	3.94e-010	2.32e-09
	Solu ilit lass	Poorl Soluble	Poorl Soluble	Insoluble	Poorl soluble	Moderatel soluble
Druglikeness	**Druglikeness**	-0.78	7.33	5.45	1.89	0.73
	Drug score	0.29	0.15	0.23	0.21	0.12
harmacokinetics	**I a sorption**	High	Lo	High	Lo	High
	permeant	No	No	No	No	No
	gp su strate	No	No	No	No	No
	inhi itor	No	No	No	No	No
	inhi itor	Yes	Yes	No	Yes	Yes
	inhi itor	Yes	Yes	No	No	Yes
	D inhi itor	No	No	No	Yes	No
	inhi itor	No	Yes	No	No	No
o icit	**Mutagenicit**	No	No	No	No	Yes
	umorigenicit	No	No	No	No	No
	Irritant ects	No	No	No	No	No
	Reproducti e ects	No	Yes	No	No	Yes

Table 4. Structurally similar ligands with Valstar, Ergotamine, and Benzo[a]pyrene-7, 8-Diol observed during screening.

Structurall Similar Ligands			
Chemical Structure	**Ligand ame**	**inding init (kcal/mol)**	**Dataset ame**
 ZINC000011616852 Valstar	ZINC000095618662	-12.8	World-not-FDA Approved Drugs
	ZINC000004214612	-12.3	World-not-FDA Approved Drugs
	ZINC000011616852	-12.3	FDA Approved Drugs
	ZINC000028232755	-12.0	FDA Approved Drugs
	ZINC000150339052	-12.0	World-not-FDA Approved Drugs
	ZINC000068205977	-11.9	Drugs in Clinical Trials
	ZINC000150338912	-11.8	World-not-FDA Approved Drugs
	ZINC000150339055	-11.8	World-not-FDA Approved Drugs
	ZINC000256630457	-11.8	World-not-FDA Approved Drugs
	ZINC000163535243	-11.7	World-not-FDA Approved Drugs
	ZINC000245224599	-11.7	World-not-FDA Approved Drugs
	ZINC000049783788	-11.4	FDA Approved Drugs
	ZINC000049918329	-11.4	World-not-FDA Approved Drugs
	ZINC000256630463	-11.4	World-not-FDA Approved Drugs
 ZINC000052955754 Ergotamine	ZINC000028232750	-11.3	FDA Approved Drugs
	ZINC000049918330	-11.3	World-not-FDA Approved Drugs
	ZINC000004215812	-12.3	World-not-FDA Approved Drugs
	ZINC000052955754	-12.2	FDA Approved Drugs
	ZINC000003978005	-12.1	FDA Approved Drugs
	ZINC000053683151	-11.7	FDA Approved Drugs
	ZINC000003995616	-11.4	World-not-FDA Approved Drugs
 ZINC000002019693 Ben o a p rene-7,8-Diol	ZINC000030728718	-12.1	Non-human Metabolites
	ZINC000030728728	-12.0	Non-human Metabolites
	ZINC000030728723	-11.6	Non-human Metabolites
	ZINC000030728707	-11.5	Non-human Metabolites
	ZINC000030728712	-11.4	Non-human Metabolites
	ZINC000002019693	-11.2	Non-human Metabolites
	ZINC000002019694	-11.2	Non-human Metabolites
	ZINC000002019692	-11.1	Non-human Metabolites
	ZINC000002019691	-11.0	Non-human Metabolites
	ZINC000030728694	-10.7	Non-human Metabolites
	ZINC000030728703	-10.4	Non-human Metabolites

Conclusion

Due to the fact that activation of the RelA subunit of NF-κB might be induced under various stresses, and it's responsible for regulation of the proliferation, apoptosis, and inflammatory genes, a strong connection between the activation of RelA and many proliferative, inflammatory, and muscle tissue diseases have been reported in the literature. As such, repurposable drugs and design novel inhibitors against RelA have potential to treat such diseases. Therefore, a novel Drug Library including 12,111 ligands was created and screened for the RelA protein. In addition, 16 commercially available inhibitors and the S-Adenosylmethionin (SAM) ligand found in chemical structure of the protein were analyzed with the same strategy. Results show that ZINC000096928979 (Deleobuvir), ZINC000012503187 (Conivaptan), and ZINC000003974230 ligands might be repurposed to stress based diseases progressed by RelA activation since they have high binding affinity through interactions with common amino acids recognized by the inhibitors, sufficient ADME properties and toxicity properties. Furthermore, 16 structurally similar ligands with Valstar, 5 structurally similar ligands with Ergotamine, and 11 structurally similar ligands with Benzo[a]pyrene-7,8-Diol were discovered. These findings demonstrate that the structures of the ligands might be utilized as scaffolding in further structure based drug design studies. Therefore, the ligands with high potential to be

used in the treatment of RelA based diseases should be tested both in vitro and in vivo applications, and the stabilities of the ligands should be verified with further moleculer dynamics (MD) simulation studies.

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Original Article

Pro-inflammatory 'M1 macrophage' vs anti-inflammatory 'Hydrocortisone' a new approach to wound healing in HaCaT cells

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ABSTRACT

Background and Aims: Wound healing is a process of repairing the skin that has lost its integrity through inflammation, proliferation, and remodeling. Macrophages exhibit adaptability, transitioning from a pro-inflammatory "M1" to an anti-inflammatory "M2" phenotype throughout wound healing for optimal outcomes. Hydrocortisone's M2c polarization makes it a key agent for balancing M1/M2 polarization. In this study, we specifically explored the effects of M1 macrophages and hydrocortisone on cell migration and wound healing in HaCaT keratinocytes.

Methods: To better understand how macrophages contribute to wound healing, we created a co-culture scratch assay model of HaCaT cells using M1-polarized macrophages derived from THP-1 cells. In addition, we administered hydrocortisone, 'an anti-inflammatory drug', to our experimental groups to compare the effects. We determined the proliferation effects of different concentrations of hydrocortisone and PMA on HaCaT cells. Then, we evaluated the effects of polarized M1 macrophages and hydrocortisone on the wound healing of HaCaT cells by scratch assay and COL1A1 mRNA gene expression levels.

Results: As a result, it was determined that 100 µM hydrocortisone increased HaCaT cell migration and *COL1A1* mRNA gene expression compared to control, while M1 polarized macrophages decreased these effects negatively.

Conclusion: To understand the macrophages responsible for the mechanisms of wound healing, much more study is required. Macrophages are a vital component in the healing process for wounds, and the shifting of M1/M2 in the treatment of wounds can potentially lead to the enlargement of novel treatment methods.

Keywords: HaCaT, hydrocortisone, macrophage, wound healing

INTRODUCTION

The process of wound healing holds significant importance in enhancing the quality of life of patients as it facilitates the restoration of skin integrity, the preservation of underlying tissues, and safeguarding against potential risks of infection and dehydration (Rahmannia, Amini, Chien, & Bayat, 2022). Collagen type I, which is responsible for fiber formation, is predominantly present in the skin and is encoded by the COL1A1 gene. It constitutes approximately 90% of the total collagen in the skin (Öztürk, Çevikelli, Tilki, Güven, & Kıyan, 2023).

The pivotal role of macrophages in the regulation of wound healing is widely acknowledged. The cells demonstrate notable adaptability and an altering phenotype, shifting from a pro-inflammatory or "M1" phenotype to an anti-inflammatory "M2" phenotype during the distinct phases of the wound healing process to facilitate ideal healing outcomes (Sharifiaghdam et al., 2022). M1 macrophages, along with other cells in the microenvironment, play a role in wound healing. This has a negative impact on wound closure, migration, and collagen expression, particularly in diabetic wounds (Miao et al., 2012). Limiting the M1 macrophage to the processes of bacteriophage, necrotic cell removal, and proinflammatory cytokine secretion in the wound microenvironment might be helpful in the wound healing process (Basu Mallik, Jayashree, & Shenoy, 2018). Establishing the M1/M2 polarization balance in the wound microenvironment will contribute to wound healing studies because the effects of M2 macrophages on preventing tissue damage are known (Louiselle, Niemiec, Zgheib, & Liechty, 2021).

Studies on macrophages are divided into different functional categories: proinflammatory and anti-inflammatory responses; M1 and M2 macrophages, respectively (Bashir, Sharma, Elahi, & Khan, 2016). M1 macrophages can become polarized in the combination of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) (Engür-Öztürk & Dikmen, 2022; Orecchioni,

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Ghosheh, Pramod, & Ley, 2019; Chanput, Mes, Savelkoul, & Wichers, 2013). Although M1 macrophages have potent antimicrobial activity, they can also exert the effect of mediating ROS-induced tissue damage and inhibiting tissue regeneration and wound healing (Huang, Li, Fu, & Xin, 2018). The anti-inflammatory activity of M2 macrophages inhibits the chronic inflammatory response to prevent this tissue damage (Shapouri-Moghaddam, 2018). M2 macrophages, particularly M2c macrophages, are polarized by glucocorticoids, which are anti-inflammatory agents (Engür-Öztürk & Dikmen, 2022; Foey, 2014; Tu et al., 2017; Huang et al., 2018).

Wound healing is a process of tissue regeneration that includes inflammation as an initial step. If the physiological inflammatory response during wound healing is prolonged or intensified, it results in a delay in the subsequent stages of appropriate wound healing (Öztürk et al., 2023). For these reasons as well as to promote M2c macrophage polarization, anti-inflammatory agents are required.

In this scope of study, to better understand how macrophages contribute to wound healing, we created a co-culture assay model of HaCaT cells using pro-inflammatory M1-polarized macrophages derived from THP-1 cells. In addition, we administered hydrocortisone, 'an anti-inflammatory drug' provides M2c polarization. We determined the proliferation effects of different concentrations of hydrocortisone and PMA on HaCaT cells. Then, we evaluated the effects of polarized M1 macrophages and hydrocortisone on the wound healing of HaCaT cells by scratch assay and *COL1A1* mRNA gene expression levels.

MATERIALS AND METHODS

Cell culture and treatment

Human skin keratinocytes HaCaT cells (CLS No: 300493, Germany) and human monocyte THP-1 cells (ATCC®TIB-202™, USA) were grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a humidified incubator with a 5% CO2 atmosphere.Hydrocortisone and phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich, USA) were dissolved in dimethyl sulfoxide (DMSO) as a stock solution.

Cell proliferation assay

HaCaT keratinocyte cell viability and proliferation were evaluated by the MTT (3,4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide) method. HaCaT cells were inoculated into 96-well plates at densities of 5 x 10^3 cells/well. After 24h, they were treated with various concentrations of PMA and hydrocortisone for 48h. After incubation, MTT solution was added to reach a final concentration of 0.5 mg/mL and incubated for 3 hours in the incubator. Then, crystals of MTT-formazan were dissolved by adding 100 µL of DMSO to each well. At 540 nm, absorbances were measured using a Cytation 3 cell imaging multi-mode reader (Bio-Tek).

Polarization of M1 subtype macrophages

In our previous study, differentiation of THP-1 monocyte cells to M0 macrophages and then polarization into M1 macrophages were described (Engür-Öztürk & Dikmen, 2022). Briefly, PMA was used to induce macrophage-like (M0) differentiation in THP-1 cells for 24 hours. M0 macrophages were polarized into M1 macrophages after 24 hours of exposure to 20 ng/mL LPS + IFN-γ.

Wound healing with scratch assay in a co-culture model

A co-culture model was established to investigate the effects of M1-polarized macrophages on HaCaT cell proliferation and wound healing. Using transwell inserts, M1-polarized macrophages were co-cultured with HaCaT cells. The HaCaT cells and M1 macrophages were co-cultured using six-well plate cell culture inserts with a 0.4-µm porous membrane dividing the upper and lower chambers. Briefly, THP-1 monocytes were seeded in the transwell apparatus' upper chamber and stimulated to differentiate into M1 polarized macrophages with PMA, IFN-*gamma* and LPS. HaCaT cells were seeded 1×10^6 cells per well 24 h before M1 macrophage polarization ended. Before the lower and upper chambers were assembled, a scratch assay was performed to determine how the cytokines released by M1 macrophages affected the ability of HaCaT cells to proliferate and migrate (Engür-Öztürk & Dikmen, 2022). After removing the medium, a 100 µL sterile plastic pipette tip was used to create a linear wound in the monolayer. The upper chambers, which contained M1 macrophages, were then positioned directly on cover of the HaCaT cells in the plates. For 48 h, HaCaT and M1 macrophage cells were incubated together. In addition, HaCaT cells were incubated for 48 h at 100 µM hydrocortisone to determine its effect on HaCaT cell proliferation during the wound healing experiment. At the end of the incubation periods, the wound was visualized with a Leica DM 300 light microscope for analysis of diameter change(Yuksel, Dikmen, & Canturk, 2021).

RT-PCR analysis

RNA was isolated from HaCaT cells treated with hydrocortisone or co-cultured with M1 polarized macrophages. Total RNA isolation was performed on the MagNA Pure LC 2.0 system (Roche, Germany), 500 ng total RNA was used for cDNA synthesis from each RNA population and cDNA synthesis was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Catalog no: 05091284001, Roche, Germany) according to the manufacturer's instructions. The total mRNA amounts of the

samples were measured at 260 and 280 nm in the NanoDrop 2000® (Thermo Fisher, USA) spectrophotometer.

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the mRNA levels of collagen type I alpha 1 chain (*COL1A1*) gene expression in relation to wound healing. As an internal positive control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used. Probe–primer pairs for target genes were supplied from Roche Diagnostic as real-time ready catalog assays. The primer sequences were *COL1A1* forward: 5-GCA AGA CAG TGA TTG AAT ACA AAA CCA-3, reverse: 5- ATC AAA GGA GCG GAT CGA GTG GTC-3 and GAPDH forward: 5-CTCTGCTCCTC CTGTTCGAC-3, reverse: 5- ACGACCAAATCCGTTGACTC-3. The real time PCR mix kit (LightCycler® 480 Probes Master, Catalog no: 04707494001, Roche, Germany), containing 10 µL 2x LightCycler® 480 Probes Master, 1 µL of each primer (Real Time Ready Assay, Roche), 4 µL PCR grade water, and 5 µL of cDNA were prepared. The cycling conditions included an initial incubation step at 95°C for 10 min, followed by 45 cycles of amplification with 10 s at 95°C, 30 s at 60°C and 1s at 72°C. The final cooling step was holding at 40°C for 30s. Results were analyzed by advanced relative quantification with the LightCycler® 480 System's software (version 1.5.0.39) (Öztürk et al., 2023).

Statistical analysis

GraphPad Prism 8.0 software was used for one-way ANOVA and Tukey's post hoc test. P values represent the significance of the results compared to the control group (P<0.0001****, P<0.001***, P<0.01**, P<0.05* and P>0.05 n.s) (± standard deviation).

RESULTS

Proliferative effects of hydrocortisone were assessed using the MTT assay

HaCaT cells were exposed to various concentrations of PMA and hydrocortisone for 48h, and the proliferative effect of the cells was determined by the MTT method. As a result, in comparison to the control group, PMA concentrations had no effect on cell proliferation (**Figure 1**). However, 100 µM hydrocortisone showed significant proliferative effects on the cells (****p<0.0001) (**Figure 2**).

M1 subtype polarization of THP-1-derived macrophages

In this study, THP-1 cells were exposed to PMA concentrations for 24h in order to differentiate them into M0 macrophages. These M0 cells were then polarized to M1 macrophages by incubating them for 24 h with 20 ng/mL LPS+IFN-γ. As a result,

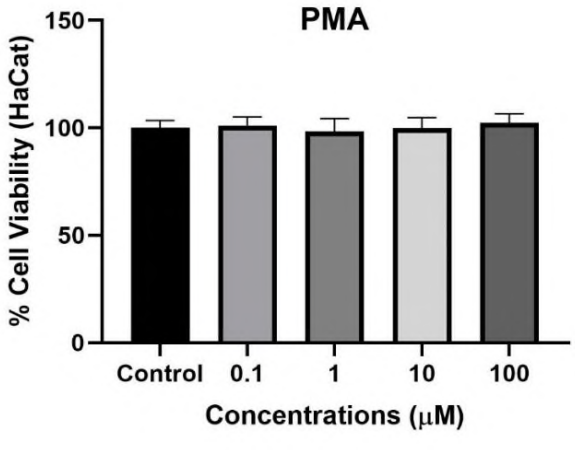

Figure 1. The HaCaT cells were treated with different concentrations of PMA for 48 h and percentage cell viability was determined from MTT results.

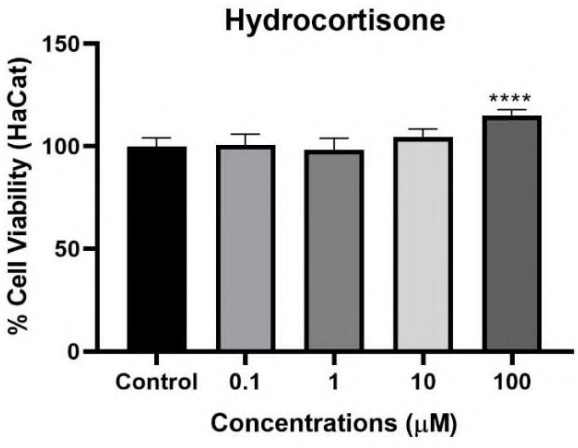

Figure 2. The HaCaT cells were treated with different concentrations of hydrocortisone for 48 h and percentage cell viability was determined from MTT results.

THP-1 cells, M0 macrophages, and M1 polarized macrophage cells were photographed with a microscope (Leica DM 300) (**Figure 3**).

Assessment of wound healing

Using a wound healing assay that measures cell population growth, the spread and migratory capacities of HaCaT cells were examined. The wound diameter change data, expressed in graphics, was obtained with the measurement program (The LAS Image Analysis Application) of the microscope (Leica DM) on the photographs, an example of which is shown in **Figure 4**. Each of the 48-h control and hydrocortisone groups had smaller wounds compared to the 0h control group. In addition, when a comparison was made with the control group at 48h, no significant difference was found between the hydrocortisone

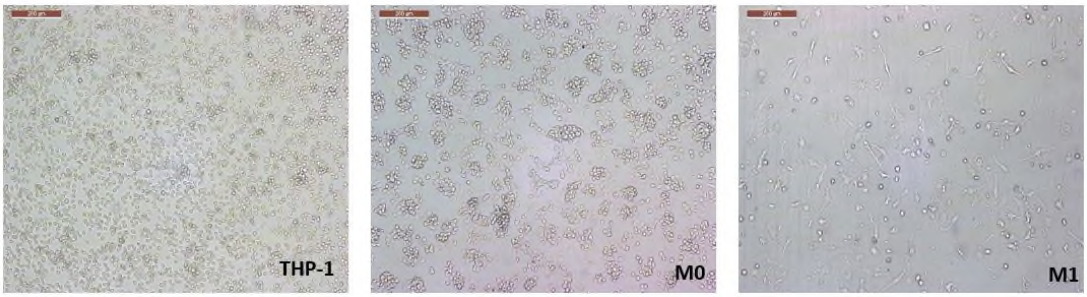

Figure 3. Microscope image of THP-1 cells (A), THP-1 derived macrophages (M0) and M1 polarized macrophage 10X objective.

and the control. As a result, the presence of M1 macrophages negatively affected the migration of HaCaT cells and therefore wound closure, while 100 µM hydrocortisone was effective in cell proliferation but did not show a significant effect in terms of wound closure (Figure 5).

Evaluation of *COL1A1* mRNA expression levels by RT-PCR analysis

HaCaT cells were treated with hydrocortisone (100 µM) and co-cultured with M1 macrophages, and mRNA expression levels of the *COL1A1* gene were determined using RT-PCR. Expression levels of the *COL1A1* gene were decreased in response to M1 macrophage co-culture ($P<0.05$*). Contrarily, in HaCaT cells treated with 100 µM hydrocortisone, the expression levels of the *COL1A1* gene increased approximately 1.5-fold ($P<0.01$**) (**Figure 6**).

DISCUSSION

The complex multicellular process of wound healing involves keratinocytes, fibroblasts, endothelial cells, and inflammatory cells (Leibovich & Ross, 1975; Loots et al., 1998; Huang et al., 2019). For a very long time, fibroblasts and keratinocytes have been the focus of studies on the repair of skin wounds. Therefore, current research is aimed at evaluating intracellular wound healing functions, and the focus of this evaluation is on these cell types (Calabrese, Dhawan, Kapoor, Agathokleous, & Calabrese, 2022). Therefore, the HaCaT keratinocyte cells used in our study were a viable alternative for a modeling of the process of healing wounds. During wound healing, macrophages play important roles, and a delayed healing period is associated with an ongoing inflammatory response (Huang et al., 2019). Recent research indicates that wound-healing macrophages exist in a variety of phenotypic states and may have a significant influence on the healing of wounds (Koh & DiPietro, 2011). The classically M1 and alternatively M2 polarized macrophages are defined (Engür-Öztürk & Dikmen, 2022). M1 macrophages are pro-inflammatory and eliminate damaged tissue, and they are crucial in the eradication of necrotic cells from the damage and other debris during the initial periods of the inflammatory phase (Calabrese et al., 2022;Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011) Similar to the findings that we obtained, Huang et al. demonstrated that the ability of cultured keratinocytes to migrate was inhibited when M1 macrophages were present in the environment (Huang et al., 2019).

In all wounds, macrophages are crucial, from contributing to inflammation to killing pathogens to resolving inflammation and initiating tissue remodelling and regeneration M2 macrophages are important players in tissue repair (Kim & Nair, 2019). M2 macrophages, particularly M2c macrophages, are polarized by the presence of glucocorticoids (Engür-Öztürk & Dikmen, 2022). It is widely known for its ability to reduce inflammation; a glucocorticoid (e.g., hydrocortisone) controls the increase of keratinocyte cells and regulates the dermal process (Terao & Katayama, 2016). Also, topical applications with anti-inflammatory drugs (such as corticosteroids) are one of numerous treatments used to promote gingival wound healing (Kongkadee, Wisuitiprot, Ingkaninan, & Waranuch, 2022).

Collagen I is one of the dermal extracellular matrix proteins in the skin (Krieg & Aumailley, 2011). Specifically, collagen type I, which is the product of the *COL1A1* gene, represents the predominant form of collagen within the skin, comprising approximately 90% of the total collagen content (Gelse, Pöschl, Aigner, 2003). Therefore, the effects on *COL1A1* gene expression levels related to wound healing have been tested in our study. The increased *COL1A1* mRNA expression levels detected in HaCAT cells treated to hydrocortisone can be attributed to pharmacologically induced anti-inflammatory properties, which is consistent with previous research findings (Wu et al., 2017).

Our study findings indicate that the utilization of hydrocortisone leads to an elevation in keratinocyte cell proliferation, facilitates cell migration, and enhances collagen expression levels in the environment of wound healing. Further research may establish hydrocortisone as a valuable therapeutic agent for M1/M2c conversion in the scope of wound healing treatment.

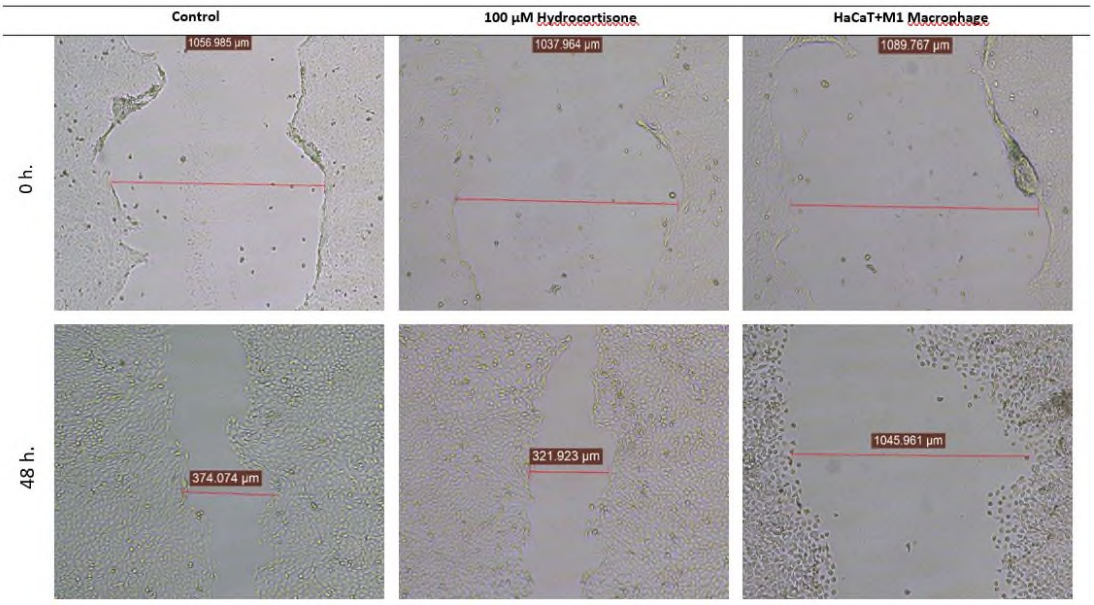

Figure 4. Scratch assay, photographs of HaCaT cells at 0 and 48. hours and wound diameter (A representative result for each group from two independent replicates is shown, 10X objective).

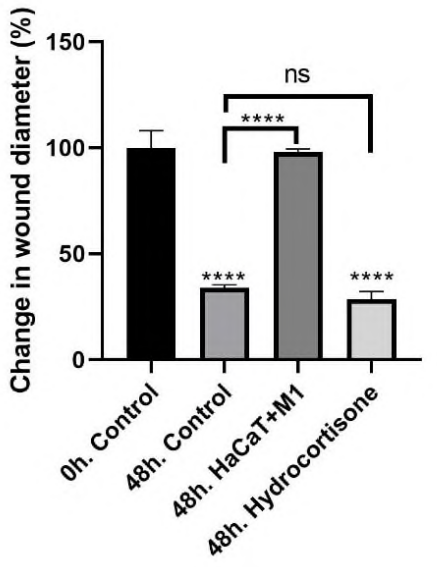

Figure 5. Effects of wound healing effects of M1 macrophage co-culture and 100 µM hydrocortisone on HaCaT cells on wound diameter change at 0 and 48 hours (±Sd., n=3, ns: not significant, p<0.0001**** compared to the 0 h. control group).

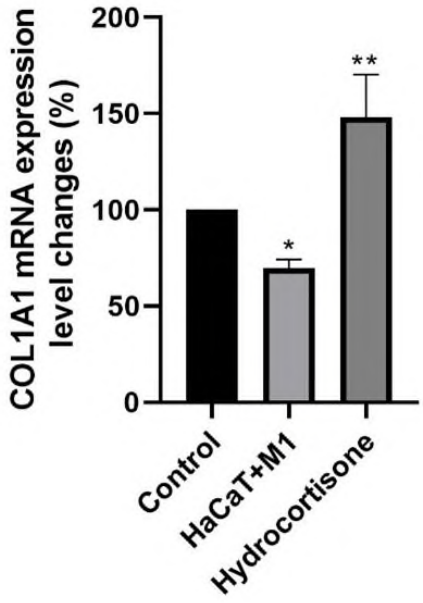

Figure 6. Changes in the mRNA expression levels (%) of wound healing related *COL1A1* gene. The error bars represent the standard deviations (n = 4, P<0.05*, P<0.01**).

CONCLUSION

Understanding the role of macrophages in the mechanisms of wound healing will require extensive research. Macrophages are a critical component of this dysregulation, and studies have revealed that macrophage polarization and timing are becoming increasingly important for both knowledge of disease progression and potential novel treatment methods.

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Original Article

The investigation of drug repurposing for HDAC1 inhibitory effects by *in silico* and *in vitro* methods

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ABSTRACT

Background and Aims: Histone deacetylases (HDACs) modulate chromatin structure and regulate gene expression. The imbalance in chromatin acetylation and dysregulation of histone deacetylases are challenging in many pathologies, ranging from cancer to neurodegeneration. Computer-based *in silico* methods are becoming increasingly important in the determination of therapeutic targets and the development of personalized treatment approaches. This study aimed to investigate the HDAC1 inhibitory effects of chronic prescription drugs using *in silico* and *in vitro* methods.

Methods: Five chronically used prescription drugs were chosen: ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine. Molecular docking was performed for each of the chosen drugs as well as the known inhibitor Trichostatin A on HDAC1. The binding pose with the best scores was saved for each compound and analyzed for its interaction with the protein. An HDAC1 inhibitor screening assay kit was used to determine the IC_{50} value for each drug.

Results: The IC_{50} values for HDAC1 inhibition by ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine were found to be 352.10 µM, 255.70 µM, 219.80 µM, 289.50 µM, and 132.70 µM, respectively, whereas the value for the positive control Trichostatin A was 36.13 nM. GraphPad Prism 5 was used to conduct statistical analyses.

Conclusion: In this study, the in vitro HDAC1 inhibitory effect of ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine is shown for the first time. *In silico* and *in vitro* methodologies used to show HDAC1 inhibitory activity in marketed drugs can provide insight into new drug discovery studies against cancer or neurodegenerative diseases.

Keywords: Docking, drug repurposing, HDAC1, *in silico*, molecular modeling

INTRODUCTION

Epigenetics is a research area that focuses on the study of modifications that occur in gene expression and function without a change in the genetic code. Histone modifications, DNA methylation, hydroxymethylation, and regulation of gene expression by non-coding RNAs are examples of epigenetic mechanisms. All these mechanisms mediate the effects of aging and environmental factors on the genome and play a crucial role in the development of disorders (Cacabelos & Torrellas, 2014; Lardenoije et al., 2015). Post-translational modification of specific amino acids in histone proteins causes changes in chromatin structure. Chromatin architecture, which is modulated by the antagonistic activity between histone deacetylases (HDACs) and histone acetyltransferases (HATs), plays a decisive role in transcriptional regulation. HDACs suppress transcription through chromatin condensation by deacetylating both histone and non-histone proteins, whereas HATs activate transcription through chromatin decondensation (Ganai, Abdullah, Rashid, & Altaf, 2017).

The HDAC enzymes take the acetyl group off of lysine residues in the N-terminal histone tails. According to their structural characteristics, eighteen human HDACs are divided into four classes and assigned numbers based on the chronology in which they were discovered: Class I (HDAC1, 2, 3, and 8), Class II (HDAC4, 5, 6, 7, 9, and 10), Class III (sirtuins), Class IV (HDAC11) (Park & Kim, 2020). The yeast Rpd3 (reduced potassium dependence 3) protein and the Class I proteins share sequence similarities. In 1996, HDAC1 the first histone deacetylase was discovered and cloned (Taunton, Hassig, &

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Schreiber, 1996). HDAC1 has >80% homology with HDAC2 (sister protein) and their embryonic knockout is lethal (Dovey, Foster, & Cowley, 2010). HDAC1 and HDAC2 play a major role in the deacetylation of histone as well as many other nuclear proteins involved in transcriptional regulation (Serebryannyy, Cruz, & de Lanerolle, 2016). HDAC1 has been proven to be involved in the pathogenesis of several diseases. It has been reported to play an important role in carcinogenesis (Müller et al., 2013). Moreover, previous studies have shown an association between HDAC1 overexpression and schizophrenia (Bahari-Javan et al., 2017). The development of novel HDAC1 inhibitors is, therefore, a promising approach to the treatment of such diseases (Johnstone, 2002).

Drug repurposing (drug repositioning or re-tasking) is a new approach in drug design that reduces the high costs and attrition rates in clinical studies and speeds up the drug development process (Pushpakom et al., 2019). For example, Nelfinavir, an HIV-1 protease inhibitor, has been used to block the AKT pathway in cancer cells and is, therefore, a successful example of drug repurposing (Li & Jones, 2012; Guan, Fousek, & Chow, 2012). Molecular docking simulation predicts a ligand's pose within a macromolecular target's binding site and its binding affinity using a scoring system. The structure-based virtual screening's ranking of predicted ligand conformations is an essential component (Kitchen, Decornez, Furr, & Bajorath, 2004). This approach is used in many drug discovery studies, including those for novel HDAC inhibitors (Park, Kim, Kim, & Lim, 2010).

In this study, we aimed to identify drug candidates for repurposing as HDAC1 inhibitors. Furthermore, we intended to validate the ability of molecular docking to predict the activities of HDAC1 inhibitors. Through a preliminary docking study with 21 compounds, we selected five chronically used drugs with the highest Chemscore values from different pharmacological groups and studied them with the HDAC1 enzyme (Table 1). The drugs were: ipratropium bromide, used to treat chronic obstructive pulmonary disease and asthma; metoprolol, a selective β1 receptor blocker used in the treatment of high blood pressure; leflunomide, an immunosuppressive drug used in rheumatoid arthritis and psoriatic arthritis; nateglinide, a drug for the treatment of type 2 diabetes; and levothyroxine, used to treat thyroid hormone deficiency (hypothyroidism) and thyroid tumors. We then examined the in vitro HDAC1 inhibitory activities of the selected drugs. With this study, the HDAC1 enzyme inhibitory activities of different drugs that are used chronically are reported for the first time in the literature.

MATERIALS AND METHODS

In silico studies: Molecular docking

The structure of the HDAC1 protein was downloaded in PDB format from the Protein Data Bank (ID 1C3R) (https://www.rcsb.org) and processed with UCSF Chimera software (https://www.rbvi.ucsf.edu/chimera). The bound inhibitor, Trichostatin A, was extracted and saved in a separate PDB file. The Flare 6.0 software (Cresset, UK) was then used to load and process the PDB file. The best ionization states were assigned for each residue after the addition of hydrogens. The chemical structures of the tested compounds were downloaded in SDF format from Pubchem (https://pubchem.ncbi.nlm.nih.gov). To determine the best binding pose at the predicted binding pocket, molecular docking was performed with Gold and Flare software. Each compound in SDF file format was loaded into the program and processed using the default settings. The grid was selected to include the binding site of Trichostatin A. The binding pose with the best scores was saved for each compound and analyzed for its interaction with the protein. To validate the performance of the molecular docking experiments, we superimposed and compared the original extracted and docked poses of Trichostatin A.

In vitro HDAC1 Inhibitor Activity Studies

The inhibitory actions of the chosen drugs were evaluated using the HDAC1 inhibitor screening assay kit (Cayman Chemical, Item No. 10011564). The HDAC1 enzyme was first treated with an acetylated lysine substrate. Deacetylation makes the substrate sensitive enough that the second step's HDAC developer treatment results in the release of a fluorescent product. Using the CLARIOstar Plus microplate reader (BMG LABTECH, Ortenberg, Germany) and excitation and emission wavelengths of 340–360 nm and 440–465 nm, the fluorophore was examined.

For background fluorescence, 10 µL of solvent was added to 150 µL of buffer solution. For initial activity, 10 µL of diluted HDAC1 and 10 µL of solvent were added to 140 µL of buffer solution. A positive control was prepared by adding 10 µL of diluted HDAC1 and 10 µL of Trichostatin A to 140 µL of buffer solution. For inhibitory fluorescence, 10 µL of diluted HDAC1 and 10 µL of synthetic or naturally sourced active substances in different concentrations were added to 140 µL of buffer solution.

All samples received 10 µL of HDAC substrate before the reaction could begin. After that, samples were incubated for 30 mins at 37°C. 40 µL of HDAC developer was added at the end of the incubation period, and the mixture was then incubated for an additional 15 mins at room temperature. Fluorescence was finally detected at the designated emission wavelengths. According to the following formula, the HDAC1 percent inhibition values of the drugs under investigation were determined:

$$\%Inhibition = \left[\frac{InitialActivity - Sample}{InitialActivity}\right] x100 \quad (1)$$

Table 1. Pharmacological properties of selected active substances **(Source: DrugBank; https://go.drugbank.com/drugs)**

Drug	Indication	Mechanism o ction	o icit	argets
Ipratropium romide	Anticholinergic drug used in the control o s mptoms related to bronchospasm in chronic obstructive pulmonar disease COPD .	Antagonist o the muscarinic acet lcholine receptor	LD_{50} 1500 mg g in mice, oral administration	Muscarinic acet lcholine receptor M1, Muscarinic acet lcholine receptor M2, Muscarinic acet lcholine receptor M3
Metoprolol	Beta-bloc er used in the treatment o h pertension and angina, and used to reduce mortalit due to m ocardial in arction.	Metoprolol is a beta-1-adrenergic receptor inhibitor speci ic to cardiac cells ith negligible e ect on beta-2 receptors.	LD_{50} in the range o 3090 to 4670 mg g in rats, oral administration	Beta-1 adrenergic receptor inhibitor
Le lunomide	P rimidine s nthesis inhibitor indicated to treat rheumatoid arthritis.	Le lunomide is a prodrug that is rapidl and almost completel metaboli ed ollo ing oral administration to its pharmacologicall active metabolite. The mechanism o action o le lunomide has not been ull determined, but appears to primaril involve regulation o autoimmune l mphoc tes.	LD_{50} 100-250 mg g in rats, oral administration	Mitochondrial dih droorotate deh drogenase inhibitor.
ateglinide	For the treatment o non-insulin dependent-diabetes mellitus in con unction ith diet and e ercise.	Nateglinide activit is dependent on the presence o unctioning cells and glucose. The insulinotropic e ects o nateglinide are highest at intermediate glucose levels and it does not increase insulin release alread stimulated b high glucose concentrations.	LD_{50} 2000 mg g in mice, oral administration	ATP-binding cassette sub- amil C member 8 inhibitor
Le oth ro ine	Levoth ro ine is indicated as replacement therap in primar th roidal , secondar pituitar and tertiar h pothalamic congenital or ac uired h poth roidism	Levoth ro ine is a s ntheticall prepared levo-isomer o the th roid hormone th ro ine T4, a tetra-iodinated t rosine derivative that acts as a replacement in de icienc s ndromes such as h poth roidism	LD_{50} 20 mg g in rats, oral administration .	Integrin alpha-V Integrin beta-3 Th roid hormone receptor alpha agonist Th roid hormone receptor beta agonist

RESULTS

In silico studies: Molecular docking

As shown in Table 2, the molecular docking results for both Flare and Gold software were comparable. However, the correlation between the docking scores and IC_{50} values, shown in Table 3, was poor. This implies that these docking scores are not sufficient to predict the activities of HDAC1 inhibitors and that other parameters involved in binding should be considered. Such parameters include solvation and desolvation parameters as well as the flexibility of residues in the binding site.

Ipratropium (Figure 1A) formed two hydrogen bonds (GLY 128 and MET 130) and fourteen hydrophobic interactions (PHE 141, CYS 142, LEU 23, and TYR 17). Metoprolol (Figure 1B) formed five hydrogen bonds (ARG 27, GLY 294, MET 130, ALA 127, and TYR 297), an ion-dipole interaction with zinc, one aromatic-aromatic interaction (HIS 131), and nine hydrophobic interactions (ALA 106, PHE 141, TYR 17, and CYS 142). Leflunomide (Figure 1C) formed four hydrogen bonds (HIS 131, HIS 132, GLY 140, and GLY 295), an ion-dipole interaction with zinc, three aromatic-aromatic interactions (PHE 141, HIS 170, and HIS 132), and seven hydrophobic interactions (PHE 141, PHE 198, CYS 142, and LEU 265). Nateglinide (Figure 1D) formed four hydrogen bonds (HIS 131, HIS 132, GLY 295, TYR 297, and GLY 140), an ion-dipole interaction with zinc, two aromatic-aromatic interactions (HIS 131 and HIS 132), and twenty hydrophobic interactions (PHE 141, LEU 23, CYS 142 and PHE 198). Levothyroxine (Figure 1E) formed five hydrogen bonds (HIS 131, HIS 132, TYR 297, and GLY 140), an ion-dipole interaction with zinc, five aromatic-aromatic interactions (LEU 265, HIS170, PHE 141, HIS 131 and HIS 132), and five hydrophobic interactions (PHE 141 and PHE 198). Trichostatin A (Figure 1F) formed two hydrogen

Table 2. Docking scores and interacting residues of selected compounds and positive control Trichostatin A.

Drug	old Score	irtual Score (lare)	Interacting residues
Ipratropium bromide	48.8	-7.7	MET130, GLY128, GLY294, TYR297, PHE141, CYS142, LEU23, TYR17
Metoprolol	56.2	-10.2	HIS131, CYS142, ARG27, GLY294, ALA106, ALA127, TYR17, MET130, PHE141, TYR297
Le lunomide	59.1	-8.6	LEU265, HIS170, GLY140, TYR297, GLY129, GLY295, HIS132, CYS142, HIS131, PHE141, PHE141, PHE198,
Nateglinide	65.2	-13.6	PHE198, ASP168, GLY295, HIS131, HIS132, LEU23, CYS142, GLY140, TYR297, PHE141
Levoth ro ine	64.6	-13.8	LEU265, HIS170, PHE198, HIS132, HIS131, TYR297, GLY140, PHE 141
Trichostatin A	62.1	-11.2	HIS131, HIS132, CYS142, LEU23, TYR17, MET130, PHE141

Table 3. IC_{50} values for HDAC1 inhibition by ipratropium bromide, metoprolol, leflunomide, nateglinide, levothyroxine, and Trichostatin A.

Drug	D I (M	SD	S	RSD
Ipratropium bromide	352.10	0.80	0.46	0.23
Metoprolol	255.70	0.83	0.48	0.32
Le lunomide	219.80	0.94	0.54	0.43
Nateglinide	289.50	0.72	0.42	0.25
Levoth ro ine	132.70	0.58	0.34	0.44
Trichostatin A	36.13	1.51	0.87	4.18

nm concentration

bonds (HIS 131 and HIS 132), an ion-dipole interaction with zinc, an aromatic-aromatic interaction (TYR 17), and nine hydrophobic interactions (MET 130, CYS 142, and LEU 23). As shown in Figure 2, the binding poses of the predicted binding pose after redocking are very similar to the original binding pose extracted from the complex from the Protein Data Bank.

In vitro HDAC1 Inhibitor Activity Studies

IC_{50} values for HDAC1 inhibition by Trichostatin A, ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine were found to be 36.13 nM, 352.10 µM, 255.70 µM, 219.80 µM, 289.50 µM, and 132.70 µM, respectively (Table 3). The data were representative of three independent experiments. The values of mean, standard deviation (SD), and standard error (SE) were analyzed with GraphPad Prism 5. In our study, the IC_{50} value for HDAC1 inhibition by the reference Trichostatin A was lower than the IC_{50} values of the selected drugs. Daily-administered doses of ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine can achieve values above the IC_{50}.

DISCUSSION

Epigenetic modifications are effective in almost all pathways in tumor development. HDAC inhibitors have been shown to prevent proliferation by inducing apoptosis and differentiation in many transformed or cancerous cell types by arresting the cell cycle in G1 or G2 (Lindemann, Gabrielli,& Johnstone, 2004; Marks, Miller, & Richon, 2003). It has been reported that HDAC1 is overexpressed in pancreatic, prostate, colorectal, gastric, and hepatocellular cancers, which correlates with a poor prognosis (Spiegel, Milstien, &Grant, 2012). The pathogenesis of neurodegenerative diseases is mediated by the epigenetic mechanisms involved in neuronal development. HDAC inhibitors have been demonstrated to prevent neuronal injury by lowering excitotoxicity, oxidative stress, and inflammation in various in vitro and in vivo models of cerebral ischemia (Wang, Yu, Tan, Jiang, & Tan, 2013; Hirata et al., 2018).

The IC50 values for HDAC1 inhibition by ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine were found to be 352.10 µM, 255.70 µM, 219.80 µM, 289.50 µM, and 132.70 µM, respectively. There are no previous stud-

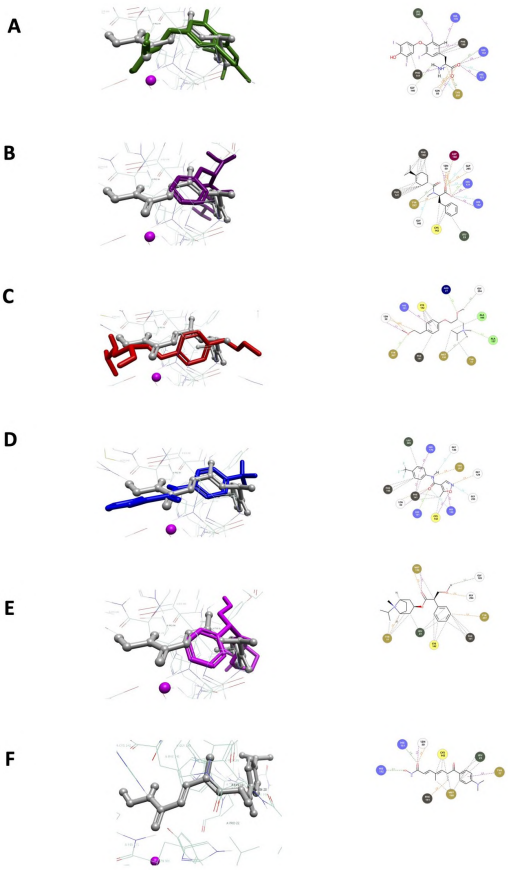

Figure 1. Docking poses of each selected compound and Trichostatin A in binding sites of HDAC1 proteins (PDB 1C3R): A. Ipratropium bromide, B. Metoprolol, C. Leflunomide, D. Nateglinide, E, Levothyroxine. and F. Trichostatin A. The panels on the left side show the docking poses of the compounds superimposed with the positive control Trichostatin A (grey). The panels on the right side show the 2D representations of the interactions, including strong (light green), average (dark green), and weak (cyan) H-bonds; hydrophobic contacts (grey); aromatic and ionic interactions (purple); and steric clashes (orange)

ies in the literature on the effects of these drugs on HDAC1. Our molecular docking study suggests that interactions with several residues are important for the inhibitory activity against HDAC1. These include the formation of hydrogen bonds with HIS 131 and HIS 132 and the ion-dipole interaction with zinc. These interactions were found in Trichostatin A and were common to all the investigated drugs except ipratropium bromide, which showed the lowest *in vitro* activity. Furthermore, the predicted binding pose of Trichostatin A was very similar to the original one, which implies that the molecular docking experiments were performed with good accuracy. Therefore, these interactions should be taken into consideration in the future design of novel HDAC1 inhibitors.

Ipratropium bromide, a derivative of the alkaloid atropine, is used in the treatment of asthma and chronic obstructive pulmonary disease. It acts as a muscarinic acetylcholine receptor

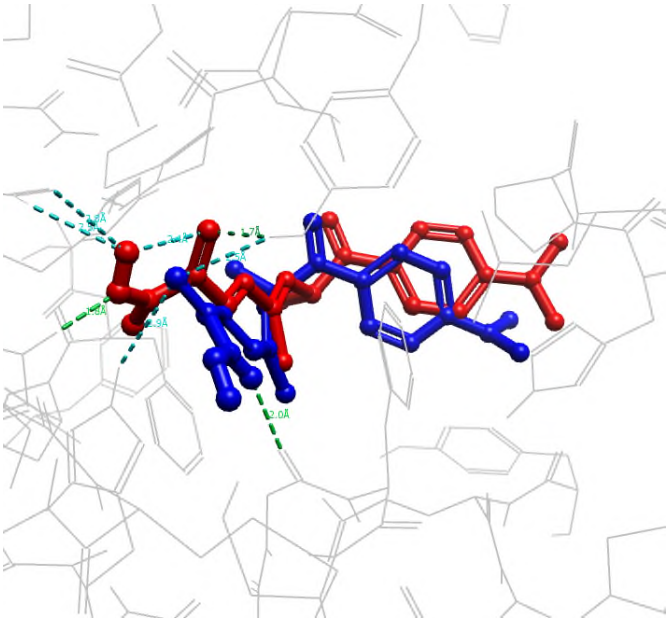

Figure 2. Representation of Trichostatin A in original binding pose (red) extracted from the original complex from the Protein Data bank (ID: 1C3R) superimposed with the predicted pose (blue) after redocking. Strong and weak hydrogen bonds are shown as green and cyan dotted lines, respectively.

antagonist to dilate bronchial smooth muscles and open the lungs' airways (Kazi, Reddy, &Singh, 2021). Metoprolol, a selective β1-blocker, is used to treat hypertension, chest pain, heart failure, palpitations, and arrhythmias, and to prevent migraine attacks (Grassi, 2018). However, metoprolol has been shown to increase the levels of Sirt1, a histone deacetylase that is nicotinamide adenine dinucleotide (NAD+) dependent, and to have a cardioprotective effect in the vasopressin-induced cellular aging model in cardiomyocytes (Li et al., 2022). Leflunomide is used in the treatment of rheumatoid arthritis and psoriatic arthritis when the disease cannot be controlled with other disease-modifying drugs. It acts by inhibiting the dihydroorotate dehydrogenase enzyme, decreasing intracellular pyrimidine levels, and the activity of tumor necrosis factor-α (Boyd, 2012). Leflunomide has been found to block UVB-induced Fyn kinase, which in turn blocks histone H3 phosphorylation. However, studies are reporting that HDAC inhibitors may be effective in the treatment of chronic inflammatory and autoimmune diseases (Vishwakarma et al., 2013). Nateglinide is an oral hypoglycemic agent that can be used alone or in combination with metformin in the treatment of type 2 diabetes. It is a derivative of D-phenylalanine, which stimulates insulin release from pancreatic beta cells (Halas, 2001). In an *in silico* simulation and drug repositioning study by Gao et al., nateglinide has been reported to have an inhibitory effect on the HDAC2 enzyme, which is an important therapeutic target in cancer and neurodegeneration (Gao, Yao, Wang, Yao, & Zhang, 2022). Levothyroxine is used in the treatment of hypothyroidism (Ianiro et al., 2014). In a study conducted by Cordeiro et al., it was demonstrated that

thyroid hormones controlled the expression of Sirt1 in mice subjected to calorie restriction (Cordeiro et al., 2013).

All tested drugs were found to show weaker *in vitro* activities than the reference standard agent, Trichostatin A. However, the tested concentrations of the studied drugs were extremely low compared to their daily recommended doses for regular treatment, where 1 mg/day, 200 mg/day, 20 mg/day, 360 mg/day, and 100 µg/day are the common doses for ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine, respectively. Hence, with the regular use of these drugs for the treatment of chronic diseases, concomitant inhibitory effects against HDAC1 activity may also be clinically observed.

Due to regular or lifelong use of these drugs, possible concomitant activity against HDAC1 is of great importance. To the best of our knowledge, this study provides the first demonstration of the HDAC1 inhibitory action of ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine using *in vitro* and *in silico* techniques.

CONCLUSION

Drug repurposing is a strategy for finding new indications for clinically-used drugs. In this study, we proposed that repurposing of lifelong used and FDA-approved drugs may also be effective on the HDAC1 enzyme. Our results showed that the chronically used drugs ipratropium bromide, metoprolol, leflunomide, nateglinide, and levothyroxine showed moderate inhibitory effects against the HDAC1 enzyme, which is an important therapeutic target in cancer and neurodegenerative diseases. We suggest that these drugs can be repurposed for the treatment of cancer and neurodegenerative diseases, concurrently with the indications for which they are used.

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Original Article

Synthesis, characterization and antimicrobial activity of some novel 4-amino-5- phenyl-4H-1,2,4-triazole-3-thiol derivatives

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ABSTRACT

Background and aims: The discovery of new antifungals and antimicrobials to overcome resistance has always been a crucial topic for sustainable world health. Since sulfur-containing triazole heterocycles derivatives have shown greater interest due to their valuable applications, we reported herein, the synthesis of some mercaptotriazole derivatives to discover underlying structural requirements for antimicrobial and antifungal activity.

Methods: Firstly, the benzoic acid hydrazide was synthesized. Then it was reacted with carbon disulfide in the solution of alkali ethanol to give potassium dithiocarbazinate salt. Then the basic nucleus 4-amino-5-phenyl-1-4H-1,2,4-triazole-3-thiol was prepared by cyclization of potassium salt with hydrazine hydrate. After that, a condensation reaction with different aldehydes was conducted to synthesize Schiff bases, which were cyclized by treating with thioglycolic acid to prepare desired compounds.

Results: All the synthesized compounds were confirmed by their melting point, FTIR, 1H-NMR, and 13C-NMR spectra, elemental analysis was determined for their antimicrobial activity by using a simple susceptibility screening test with agar-well diffusion. Few compounds showed promising activity against bacteria and yeast-like fungi.

Conclusion: 4-amino-5-phenyl-4H-1,2,4-triazole-3-thiol derivatives proved promising antimicrobial activities.

Keywords: Triazole-3-thiol, antimicrobial activity, Schiff base, thiazolidenon derivative, triflucan

INTRODUCTION

Synthesis and development of new and safe therapeutic values containing chemical compounds, to avoid resistance as well as to increase selective effectiveness are taking the attention of worldwide researchers and scientists, particularly nitrogen-containing heterocyclic are mostly found in significant therapeutic agents. In this regard, the synthesis of some mercaptotriazole derivatives to discover underlying structural requirements for antimicrobial and antifungal activity was conducted.

The usage of most antimicrobial agents is now very limited, mainly because of rapidly developing drug resistance but also because of the unsatisfactory result of present bacterial and fungal infection treatments and side effects (Fidler, 1998). In the last few decades, great consideration has been dedicated to the synthesis of 1,2,4-triazole derivatives possessing such comprehensive bioactivities as antibacterial, antifungal (Karabasanagouda, Adhikari, & Shetty, 2006; Sztanke, Pasternak, Rzymowska, Sztanke, & Kandefer-Szerszeń, 2008), antimycobacterial (Klimesova, Zahajska, Waisser, Kaustova, & Mollmann, 2004), anti-inflammatory (Mullican et al., 1993), analgesic (Tozkoparan, Kupeli, Ozalp, & Ertan, 2005), anticancer (Demirbas, Ugurluoglu, & Demirbas, 2002) antihypertensive (Wright et al., 1986), anticonvulsant (Küçükgüzel et at., 2004), antiasthmatic (Youichiro et al., 1996), antiviral (El-Essawy, El-Sayed, El-Kafrawy, Morshedy, & Abdel-Rahman, 2008) diuretic (Shah, Mhasalkar, Patki, Deliwala, & Sheth, 1969), antidepressant (Kane, Dudley, Sorensen, & Miller, 1988) and hypoglycemic (Blank, Nichols, & Vaidya, 1972) activities. Although both, imidazole and triazole, are five-membered ring heterocycles, imidazole contains two ring nitrogen atoms, while triazoles have three. Nevertheless, when compared with imidazole (clotrimazole, ketoconazole, miconazole), triazoles are less susceptible to metabolic degradation and have much greater target specificity, increased potency, and an expanded spectrum of activity.

Sulfur containing triazole heterocycles are also very attractive to scientist because of their significant practical applications, particularly mercapto-and thione-substituted 1, 2, 4-triazoles are well known important compound (Sobhi et al.,

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2022; Shcherbyna et al., 2022; Karpun & Polishchuk, 2021; Sameliuk, Zedan, & Kaplaushenko, 2021; Desai et al., 2021).

Triazole derivatives are also taking attention due to its valuable application in medicine (Kazeminejad et al., 2022; Zveaghintseva et al., 2021; Zazharskyi et al., 2021; Vagish, Sudeep, Jayadevappa, & Ajay Kumar, 2020; Benhammadi, Salimairaten, & Othman, 2016; Kumari et al., 2021; Mohamed, Sheha, Hassan, Abdel-Hafez, & Omar, 2018; Cavusoglu, Yurttas, & Canturk, 2018; Popiołek, Paruch, Patrejko, Biernasiuk, & Wujec, 2016; Sekhar et al., 2018; Xie et al., 2017; Wu et al., 2018), agriculture (Subhas, Sindhu, & Sreeveena, 2019; Shang et al., 2012; Yang, He, & Zhu, 2006; Howatt, 2005; Zhang, Damu, Cai, & Zhou, 2014) and, industry (Nazarov, Miroshnichenko, Ivakh, & Pyshyev, 2023; Yan, Jinchao, Yang, & Cheng, 2022; Popova et al., 2021; Shevtsov et al., 2020; Yin et al., 2009; Ueda & Nagasawa, 2009; Yeung & Farkas, 2005; Huntsman & Balsells, 2005; Zhou &Wang, 2012). Furthermore, some triazoles are recognized and used as analytical reagents (Seebunrueng, Tamuang, Ruangchai, Sansuk & Srijaranai, 2021; Liu et al., 2021; Wang, He, Chen, & Hu, 2020; Li, He, Chen, & Hu, 2019), dyes (Tkach et al., 2023; Diogo et al., 2023; Ma et al., 2023; Bakr, Abdel-Wahab, Bekheit, Mashaly, & Fahmy, 2023) and photographic chemicals (Ahmed et al., 2022; Koparir, Parlak, Karatepe, & Omar, 2022; Shimada, Ito, Maeta, Matsuoka, & Sato, 2006) and in the polymers preparation (Li et al., 2023; Sloop, 2023).

MATERIALS AND METHODS

General

The reagents used in the reactions were purchased commercially from Sigma Aldrich and Merck. Melting points were examined on the Barnstead Electro-thermal melting point device. ^{1}H-NMR and ^{13}C-NMR spectra (δ, ppm) were observed on a Varian Mercury 200 MHz spectrophotometer as a standard substance using tetramethyl silane.

Match constants (J values) were given as Hertz. NMR coefficients are truncated as follows: s= singlet, d= doublet, t= triplet, m= multiplet signal. The IR spectra (υ, cm^{-1}) were viewed with a Perkin-Elmer 1600 FTIR spectrometer in KBr pellets. Compounds **(1-5)** were synthesized benefiting a published method (Selvaraj et al., 2011, Čačić et al., 2010) (scheme-1). Elemental analysis was performed on a Fisons - EA-1108 CHNS-O Element Analyzer (Table 1).

All test microorganisms were received from the Refik Saydam Hıfzıssıhha Institute (Ankara, Turkey). Those are *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 60193, *Candida tropicalis* ATCC 13803 and *Bacillus cereus* 709 ROMA. All chemicals have been weighed and then dissolved in dimethyl sulfoxide (DMSO), as solvent, to prepare extract stock solutions of 10 mg/mL (Table 2).

The ager-well diffusion method was used for the simple susceptibility screening test (Mullican et al., 1993) as adapted in the previously reported method (Tozkopran et al., 2005). All microorganisms were suspended in Mueller-Hinton (Difco, Detroit, MI, USA) broth and diluted to ca. 10^6 colony-forming units (CFU) per mL. They were flood-inoculated on the surface of Mueller Hinton agar and Sabouraud dextrose agar (SDA) (Difco), then they were dried. SDA was used for *C. albicans* and *C. tropicalis.* 5-mm diameter wells were cut using a sterile cork-borer from the agar and 500 μg/50 μL (10 mg/mL) of the chemical substances were transferred into the wells. The plates were then incubated for about 18 h at 35 ºC. The antimicrobial activity was determined by measuring the inhibition zone against the test organism. Ampicillin (10 μg/50 μL) was used as the control antibiotic. Triflucan (5 μg/50 μL) was used as control fungicide. DMSO was used as the control solvent. The results are shown in Table 2.

Table 1. Compounds and R groups

ompound o	**R group**
4a, 5a	Br
4b, 5b	Cl
4c, 5c	OCH_3

Synthesis of benzoic acid hydrazide (1)

Methyl benzoate (0.01 mole, 1.63g, 15 mL) with hydrazine hydrate (0.01 mole, 0.6g, 0.58 mL) was refluxed (reflux time was 1 hour, later 40 mL absolute ethanol was added then reflux was continued for 3 more hours. After cooling the solution, white crystals were formed which were then recrystallized by ethanol. Yield was 1.03g, and 75.73%. melting point (M.p), 112-114°C (Selvaraj et al., 2011)

Synthesis of potassium dithiocarbazinate (2)

Potassium hydroxide (0.03 mole, 1.68 g) and acid hydrazide, which is 1, (0.01 mole, 1.36g) mixture was dissolved in absolute ethyl alcohol (15 mL). The solution was then cooled in an ice bath and carbon disulfide (0.05 mole, 3mL) was added in small portions with continued stirring. Then the reaction mixture was allowed to continue stirring at room temperature for 18 hours. Dry ether (10mL) was then added to the solution, which resulted in forming a yellow precipitate, which was filtered and washed

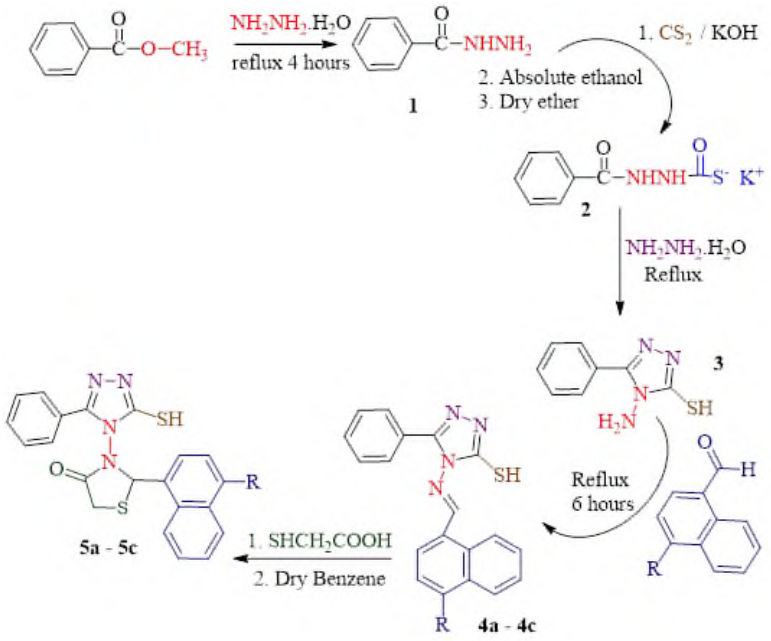

Figure 1. The Steps for Synthesis of Compounds (1-5)

using ether to obtain dried potassium salt (2) which was used in the next step without further purification. Yield was 1.68 g, and67.20%., M.p. 186-188°C (Selvaraj et al., 2011).

Synthesis of 4-amino-5-phenyl-4H-1, 2, 4-triazole -3-thiol (3)

5g (0.02 mole) of potassium salt (2) was dissolved in 40 mL of water and hydrazine hydrate (2mL, 0.04 mole) was added to the suspension, the color of the reaction mixture went from yellow to green, after the mixture was refluxed until the evaluation of hydrogen sulfide was observed and which was ceased by lead acetate paper. Then the reaction mixture was allowed to cool at room temperature and diluted with 30 mL of cold water. Upon acidification by HCl, white powder was obtained as a precipitate, which was then recrystallized by ethanol. Yield was 1.25 g and 65.10%., M.p. 198-200°C (Selvaraj et al., 2011).

Synthesis of Schiff bases (4a-c)

A mixture of compound (3) (0.01mole) and respective aromatic aldehyde (0.01mole) was refluxed for 4 to 6 hours in absolute ethanol (25 mL) and a few drops of glacial acetic acid. The reaction mixture was then cooled, and the precipitate formation occurred which was filtered and recrystallized by using ethanol. (Selvaraj et al., 2011; Čačić, Molnar, Šarkanj, Has-Schön, & Rajković, 2010)

(E)-4-(4-bromonaphthalen-1-yl methylene amino)-5-phenyl-4H-1,2,4-triazole-3-thiol (4a)

Yield 2.91g, 71.15%. M.p. 173-175 °C; IR (KBr) cm^{-1} 3109 (ν aromatic C-H), 2928 (ν aliphatic C-H), 2740 (ν S-H), 1616 (ν C=N); ^{1}H-NMR (DMSO-d_6) δ (ppm) Ar-H [7.28 (d, 1H, j= 8.83 Hz), 7.40 (d, 1H, j= 8.83 Hz), 7.50-7.75 (m, 5H), 7.90 (d, 1H, j= 8.65 Hz), 7.94-8.10 (m, 2H), 8.16 (d, 1H, j= 8.65 Hz)], 9.31 (s, 1H, N=CH), 12.80 (s, 1H, SH); ^{13}C-NMR (DMSO-d_6) δ (ppm) 171.52 (N=CH), 152.07, 150.76 (2C, triazole C_3, C_5), Ar-C [150.20 (2CH), 148.72 (C), 139.48 (C), 133.93 (C), 129.19 (2CH), 128.60 (2CH), 125.35 (CH), 123.34 (C), 122.46 (C), 121.19 (2CH), 115.90 (CH), 110.86 (CH)]; Elemental analysis ($C_{19}H_{13}BrN_4S$); calcd. C, 55.75; H, 3.20; N, 13.68; S, 7.83; Found C, 55.62; H, 3.27; N, 13.41; S, 8.06%.

(E)-4-(4-chloronaphthalen-1-yl methylene amino)-5-phenyl-4H-1,2,4-triazole-3-thiol (4b)

Yield 2.48g, 7.94%). M.p. 197-199 °C; IR (KBr) cm^{-1} 3112 (ν aromatic C-H), 2933 (ν aliphatic C-H), 2744 (ν S-H), 1621 (ν C=N); ^{1}H-NMR (DMSO$_6$) δ (ppm) Ar–H [7.45 (d, 2H, j= 8.65 Hz), 7.60-7.70 (m, 2H), 7.75-7.88 (m, 4H), 7.90-8.00 (m, 3H)], 8.80 (s, 1H, N=CH), 12.74 (s, 1H, SH); ^{13}C-NMR (DMSO-d_6) δ (ppm) 169.97 (N=CH), 150.00, 149.80 (2C, triazole C_3, C_5), Ar-C [140.11 (C), 139.98 (C), 130.22 (C), 130.09 (CH), 129.85 (2CH), 129.63 (2CH), 129.00 (2CH), 127.38 (2CH), 126.41 (C), 124.00 (2CH), 123.56 (C)]; Elemental analysis ($C_{19}H_{13}ClN_4S$); calcd. C, 62.54; H, 3.59; N, 15.33; S, 8.78; Found C, 62.71; H, 3.52; N, 15.41; S, 8.14%.

(E)-4-(4-methoxynaphthalen-1-yl) methylene amino)-5-phenyl-4H-1,2,4-triazole-3-tiol (4c)

Yield 2.62g, 72.77%. M.p. 227-229 °C; IR (KBr) cm^{-1} 3107 (ν aromatic C-H), 2968 (ν aliphatic C-H), 2740 (ν S-H), 1616 (ν C=N); ^{1}H-NMR (DMSO-d6) δ (ppm) 3.78 (s, 3H, OCH_3), Ar–H [6.80 (d, 1H, j= 8.83 Hz), 7.14 (d, 1H, j= 8.83 Hz), 7.20-7.40 (m, 2H), 7.45-7.55 (m, 3H), 7.60-7.70 (m, 2H), 7.72-7.85 (m, 2H), 8.44 (s, 1H, N=CH), 13.02 (s, 1H, SH); ^{13}C-NMR (DMSO−6) δ (ppm) 171.92 (N=CH), 150.66, 150.17 (2C, triazole C_3, C_5), Ar–C [148.33 (C), 139.21 (C), 129.57 (CH), 129.31 (2CH), 128.89 (2CH), 127.96 (2CH), 127.18 (2CH), 126.60 (C), 125.75 (CH), 123.12 (C), 122.72 (C), 115.79 (CH)], 55.46 (OCH_3); Elemental analysis ($C_{20}H_{16}N_4SO$); calcd. C, 66.65; H, 4.47; N, 15.54; S, 8.89; Found C, 66.72; H, 4.39; N, 15.59; S, 8.47%.

Synthesis of thiazolidenon derivatives (5a-c)

Schiff bases (4a-c) (0.002 mole) mixture with thioglycolic acid (0.04 mole, 0.26 mL) in the presence of dry benzene (30 mL) refluxed for 10 hours. Then, the mixture was concentrated and recrystallized with ethanol (Selvaraj et al., 2011).

2-(4-bromonaphthalen-1-yl)-3-(3-mercapto-5-phenyl-4H-1,2,4-triazol-4-yl)thiazolid- in-4-one (5a)

Yield 3.61g, 77.28%. M.p. 162-164°C; IR (KBr) cm^{-1} 3018 (ν aromatic C-H), 2913 (ν aliphatic C-H), 2748 (ν S-H), 1718 (C=O), 1609 (ν C=N), 694 (C-S-C); 1H-NMR (DMSO-d_6) δ (ppm) 3.91 (s, 2H, CH_2), 5.48 (s, ^{1}H, CH), Ar–H [7.30 (d, 2H, j= 8.65 Hz), 7.40-7.60 (m, 2H), 7.65-7.70 (m, 2H), 7.84-7.91 (m, 1H), 8.20- 8.45 (m, 4H)], 14.03 (s, 1H, SH); ^{13}C-NMR (DMSO-d_6) δ (ppm) 165.14 (C=O), 149.37, 148.32 (2C, triazole C3, C5), Ar–C [161.96 (2C), 139.25 (2C), 133.13 (2CH), 132.96 (2CH), 131.10 (2CH), 129.41 (2CH), 127.06 (2CH), 124.65 (C),113.41 (CH)], 55.26 (CH), 45.87 (CH_2); Elemental analysis ($C_{20}H_{15}BrN_4S_2O$); calcd. C, 50.96; H, 3.20; N, 11.88; S, 13.60; O, 3.39; Found C, 50.72; H, 3.22; N, 11.51; S, 13.35; O, 3.81%.

2-(4-chloronaphthalen-1-yl)-3-(3-mercapto-5-phenyl-4H-1,2,4-triazol-4 yl)thiazolidi-n-4-one (5b)

Yield 2.80g, 65.57%. M.p. 181-183°C; IR (KBr) cm^{-1} 3010 (ν aromatic C-H), 2945 (ν aliphatic C-H), 2698 (ν S-H), 1702 (C=O), 1614 (ν C=N), 672 (C-S-C); 1H-NMR (DMSO-d_6) δ (ppm) 3.80 (s, 2H, CH_2), 5.69 (s, 1H, CH), Ar–H [6.60 (d, 1H, j= 8.83 Hz), 7.28 (bs, 1H), 7.60-7.80 (m, 5H), 8.00-8.20 (m, 4H)], 13.67 (s, 1H, SH); ^{13}C-NMR (DMSO-d_6) δ (ppm) 158.68 (C=O), 151.12, 149.78 (2C, triazole C_3, C_5), Ar–C [139.08 (2C), 134.01 (CH), 133.82 (CH), 130.23 (2CH), 129.65 (2CH), 127.82 (2CH), 124.66 (C), 121.95 (CH), 118.04 (2C), 115.10 (2CH)], 58.92 (CH), 45.18 (CH_2); Elemental analysis ($C_{20}H_{15}ClN_4S_2O$); calcd. C, 56.26; H, 3.54; N, 13.12; S, 15.01; O, 3.74; Found C, 56.12; H, 3.46; N, 13.85; S, 15.18; O, 3.65%.

3-(3-mercapto-5-phenyl-4H-1,2,4-triazol-4-yl)-2-(4-methoxynaphthalen-1-yl)thiazol-idin-4-one (5c)

Yield 3.40g, 80.57%). M.p. 204-206°C; IR (KBr) cm^{-1}3032(ν aromatic C-H), 2957 (ν aliphatic C-H), 2679 (ν S-H), 1715 (C=O), 1597 (ν C=N), 671 (C-S-C); ^{1}H-NMR (DMSO-d_6) δ (ppm) 3.65 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2), 5.31 (s, 1H, CH), Ar–H [6.91 (d, 1H, j= 8.83 Hz), 7.24, (d, 1H, j= 8.83 Hz), 7.38-7.70 (m, 5H), 7.80-8.00 (m, 4H)], 13.18 (s, 1H, SH); ^{13}C-NMR (DMSO-d_6) δ (ppm) 164.05 (C=O), 151.05 149.82 (2C, triazole C_3, C_5), Ar–C [139.83 (2C), 131.84 (2CH), 130.56 (2CH), 130.17 (CH), 129.98 (2CH), 129.41 (2CH), 128.53 (2CH), 127.00 (C), 124.27 (C), 124.05 (C)], 59.06 (CH)], 55.82 (OCH3), 45.12 (CH_2); Elemental analysis ($C_{21}H_{18}N_4S_2O$); calcd. C, 59.69; H, 4.29; N, 13.25; S, 15.17; O, 7.57; Found C, 59.47; H, 4.24; N, 13.29; S, 15.23; O, 7.64%.

RESULTS AND DISCUSSION

The synthesis of basic 4-amino-5-phenyl-4H-1, 2, 4-triazole-3-thiol **(3)** nucleus was carried out as in the literature method (Fedotov & Hotsulia). Then compound **(3)** was used for the synthesis of Schiff bases **(4a-c)**, which showed confirmation by the absence of NH_2 peak in IR spectra and the presence of peaks at (8.92-9.20) ppm due to N=CH in 1HNMR spectra and singlet as expected in all three compounds. The proton bound to the azomethine group is generally resonance in the range of δ=8-9 ppm. IR spectra showed the C=N bands of **(4a–c)** in the 1616-1621 cm−1 area. Peaks of imine carbons are seen in the 13C-NMR spectrum between δ=164-168 ppm. Imine peak emerged as a singlet. It was observed that the NMR results supported the formation of the compound and were consistent with the literature (Fedotov & Hotsulia, 2023; Valicsek & Badea, 2021; Klimesova, Zahajska, Waisser, Kaustova, & Mollmann, 2004).

The reaction of Schiff bases **(4a-c)** with thioglycolic acid in dry benzene resulted in the formation of thiazolidenone derivatives **(5a-c) (Figure 1)**. The FTIR spectrum of compounds (5a-c) confirmed by the presence of stretching band between 1718-1702 $cm^{-1}$for C=O of thiazolidinone ring and absorption bands at 671-694 cm^{-1} due to C-S-C, 3010-3032 cm^{-1} for C-H aromatic, 2913-2957 cm^{-1} For C-H aliphatic, 2679-2748 for S-H group and 1614-1597 cm^{-1} for C=N of triazole ring.

^{1}HNMR spectrum shows the disappearance of the azomethine group (CH=N) and the appearance of a signal at 3.42-3.87 ppm due to the methylene group ($COCH_2S$) singlet as expected in all three compounds. Singlet signal at 5.31-5.64 ppm for CH (SCHN), singlet signal at 13-14 ppm for S-H group, and

Table 2. Antimicrobial activity screening result for the selected compounds dissolved in dimethyl sulfoxide (DMSO) as solvent (10 mg/mL)

Compound no.	**Microorganisms and inhibition zone (mm)**								
	Ec	Pa	Yp	Kp	Ef	Sa	Bc	Ca	Ct
4a	5	14	5	13	5	5	5	5	15
4b	5	23	5	18	5	5	5	22	20
4c	5	5	5	5	5	10	5	25	17
5a	5	13	5	5	5	13	5	5	5
5b	5	5	5	5	5	12	5	5	5
5c	5	5	5	5	5	5	5	5	11
DMSO	5	5	5	5	5	5	5	5	5
Ampicillin	8	5	5	5	12	16	13	5	5
Triflucan								25	25

-: Results were concluded based on the inhibition zone diameter (5 mm: no antimicrobial activity; > 5 mm: positive antimicrobial activity). Ec: *c eric ia co i* ATCC 25922; Pa: *e domona aer ino a* ATCC 10145; Yp: *er inia e dot erc o i* ATCC 911; Kp: *e ie a ne monia* ATCC 13883; Ef: *nterococc aeca i* ATCC29212; Sa: *ta ococc a re* ATCC 25923; Bc: *aci cere* 709 ROMA; Ca: *andida a ican* ATCC 60193; Ct: *andida tro ica i* ATCC 13803.

signal 3.76 ppm for (OCH_3) group as compound 5c. The peaks of carbonyl, CH_2, and CH carbons are seen in the ^{13}C- NMR spectrum between δ = 156-157, 5.31-5.64, 3.42-3.87 ppm respectively. It was observed that the NMR results supported the formation of the compound and were consistent with the literature (Fedotov &Hotsulia, 2023; Valicsek & Badea, 2021; Klimesova et al., 2004).

Compound **5c** showed good antifungal activity only against yeast-like fungi, while compound **4a-c** showed antimicrobial activity against bacteria and yeast-like fungi. Compound **5b** was only found effective on the gram-positive bacteria, *S. aureus* ATCC 25923. The highest activity was observed against *P. aeruginosa* ATCC 10145 by **4b**. Compound **5a** was found to be effective on both, *S. aureus* ATCC 25923 as well as *P. aeruginosa* ATCC 10145. Compound **4c** showed the highest activity against *Candida albicans* ATCC 60193.

CONCLUSION

In summary, compounds (4a-c, 5a-c) were successfully synthesized and characterized quantitatively and qualitatively by using FTIR, 1HNMR, 13CNMR, and elemental analysis. 4-amino-5-phenyl-4H-1,2,4-triazole-3-thiol derivatives and their promising antimicrobial activities were proved.

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Original Article

Ameliorative effect of cranberry on erectile function in diabetic rats

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ABSTRACT

Background and Aims: Cranberry products are beneficial in erectile dysfunction (ED). Therefore, we assessed the impact of Cranberry fruit extract (Cranberry-E) on *in vivo* erectile response and *in vitro* relaxant responses in the corpus cavernosum (CC).

Methods: Rats (n=10) were divided into control and diabetic groups. In vivo erectile function was measured following intracavernosal injection of Cranberry-E. The relaxation responses to Cranberry-E were obtained after pre-contraction with phenylephrine (Phe, 10 μM) and KCl (60 mM). Cranberry-E caused relaxant responses in the incubation with nitric oxide synthase (NOS) blocker (L-NAME, 100 μM) and soluble guanylate cyclase (sGC) blocker (ODQ, 30 μM), and relaxation responses of cavernosal tissue were calculated before and after the incubation with Cranberry-E.

Results: Erectile responses were significantly reduced in diabetic animals as compared to controls ($p<0.001$), which was normalized after the intracavernous administration of Cranberry-E. There was no difference in the relaxation responses to Cranberry-E between the control and diabetic groups. Cranberry-E induced the relaxation of cavernosal tissue, which remained unaltered in the presence of L-NAME and ODQ. Relaxation responses to Cranberry decreased after KCl-induced precontraction ($p<0.001$). The relaxation of cavernosal tissue increased after Cranberry-E incubation.

Conclusion: Cranberry-E improved diabetes-induced ED and induced relaxation of cavernosal tissue via a nitric oxide-independent mechanism. Thus, cranberry consumption is likely to be effective as a potential strategy to prevent diabetes-induced ED.

Keywords: Cranberry-E, Corpus cavernosum, diabetes, erectile function, ericaceae, *Vaccinium oxycoccos* L.

INTRODUCTION

Diabetes is a significant reason for erectile dysfunction (ED), negatively affecting quality of life (Mazzilli et al., 2015). ED is observed at a younger age and more frequently in the diabetic population compared to the general population (Johannes et al., 2000). Multifactorial mechanisms play a role in diabetic ED with a weak response to oral phosphodiesterase type 5 (PDE-5) inhibitors (Ruan et al., 2016). Plant and plant-derived drugs have long been investigated in treating ED patients (Shin et al., 2015; Stasiak, Zarlok, & Tomaszewski, 2016). The widespread plant-based options for ED are *Epimedium sagitatum, Pausynstalia yohimbe, Eurycoma longifolia, Panax ginseng, Tribulus terrestris*, and *Gingko biloba* (Karakaya et al., 2019; Shin et al., 2015; Petre et al., 2023). Alternative or complementary therapies for diabetic ED may be referred to as herbal medicines or phytomedicines.

Plant-based compounds can help to treat or prevent atherosclerosis, hypertension, cancer, and infectious diseases (such as gastric mucosa, urinary tract, and oral cavity infections) through their potential activities regarding antioxidant properties (Liska, Kern, & Maki, 2016; Olas, 2017; Vidlar et al., 2010). The berry fruits of the Ericaceae family represent essential sources of active compounds, for instance, proanthocyanidins, anthocyanins, phenolic acids, terpenes, and flavonoids (Blumberg et al., 2013). Earlier data have shown that the fruits have strong antioxidant properties and include exotic flavors (Jeszka-Skowron, Zgola-Grzeskowiak, Stanisz, & Waskiewicz, 2017; Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). The *Vaccinium* genus includes more than 450 species in Europe, Central America, North America, Japan, Africa, Asia, and Madagascar. The most popular of these species are cranberry (*Vaccinium macrocarpon* Aiton, *Vaccinium oxycoccos* L.), bilberry (*Vaccinuim myrtillus*), blueberry (*Vaccinium angustifolium* Aiton, *Vaccinium ashei*, *Vaccinium corymbosum* L.), lingonberry (*Vaccinium vitis*) and huckleberry (*Vaccinium ovatum, Vaccinium parvifolium*). European cranberry, *Vaccinium oxycoccos* also known as “small cranberry” or “bog

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cranberry", is found in Europe, Asia, and North America (Jurikova, Skrovankova, Mlcek, Balla, & Snopek, 2018). The polyphenol-rich extract from cranberry is a potentially powerful tool to protect against obesity-induced metabolic disorders in obese mice (Anhe et al., 2015). In addition, Cranberry fruit extract (Cranberry-E) has been traditionally used to treat bladder and kidney ailments (Mojaverrostami, Bojnordi, Ghasemi-Kasman, Ebrahimzadeh, & Hamidabadi, 2018). It is the most popular herbal medicine for urinary tract infections (UTI) in the United States (Bukhari et al., 2015; Rossi, Porta, & Canovi, 2010; Yarnell, 2002). Furthermore, a prospective clinical study has shown the beneficial effects of a mix of cranberry, soy germ, pumpkin seed extract, and isoflavonoids on lower urinary tract symptoms and erectile function (Nemr et al., 2020).

In the present study, we evaluated the potential favorable effects of Cranberry-E on streptozotocin (STZ)-induced diabetic ED and in vitro relaxation responses in the penile tissue.

MATERIALS AND METHODS

Sample preparation

The sample was supplied from Spring Valley®, a dietary supplement for Urinary Tract Health. Each capsule (highly concentrated) contained 500 mg of cranberry fruit extract (*Vaccinium oxycoccos* L., European cranberry, small cranberry) that was dissolved in water (10 mL) and applied to the tissues. The stock solution concentration was 50 mg/mL.

The induction of diabetes

Sprague-Dawley rats (n=10) were divided into two groups: control and diabetic rats. In a temperature-controlled room (22±1°C), the rats were held in individual cages with food and water *ad libitum*. Diabetes was induced by a single intraperitoneal injection with STZ (50 mg/kg, i.p.) in a citrate buffer (pH:5.5). Seventy-two hours after the STZ injection, diabetes was confirmed by the assessment of blood glucose levels higher than 250 mg/dL with a glucometer (Roche Diagnostics, Indianapolis, IN). The experimental animal procedure was accepted by the Institutional Animal Care and Use Committee of Ankara University (2019-12-117).

In vivo assessment of erectile function

Eight weeks after the induction of diabetes, the intracavernosal pressure (ICP, mmHg) and the main arterial pressure (MAP, mmHg) were estimated using polyethylene-50 tubing for cannulation of the crura and carotid artery in anesthetized rats. The right crura were cannulated to measure ICP using the transducer (Statham, CA, USA) with a data acquisition system (Biopac MP 100 System). After the determination of the cavernous nerve (CN) and the major right pelvic ganglion, the CN was induced (2.5, 5, and 7.5 V, 15 Hz, 30-s pulse width) with a stainless-steel bipolar hook electrode and a square pulse stimulator (Grass Instruments, MA, USA). The measurements were repeated after intracavernosal administration of Cranberry-E (5mg/mL) in the control and diabetic rats. A rest period of 5 min. before each measurement was given in order to allow a return to baseline (Onder et al., 2019; Karakaya et al., 2019; Yilmaz et al., 2014).

In vitro organ bath studies

Following the *in vivo* studies, isolated corpus cavernosum (CC) strips (1 × 1 × 8 mm) were transferred in an organ bath under an initial isometric tension (1 g) within Krebs solution with a mixture of O_2/CO_2 (95% / 5%). The CC strips were equilibrated for 1 hour, and the solution was changed every 15 minutes. All changes in tension were recorded using an isometric force transducer connected to a computer-based data acquisition system (Biopac Systems). Cranberry-E-induced relaxant responses were obtained after precontraction with phenylephrine (Phe, 10 μM) and KCl (60 mM) (Onder et al., 2019; Salahdeen, Idowu, Yemitan, Murtala, & Alada, 2015). After precontraction with Phe (10 μM), Cranberry-E-induced relaxant responses were obtained before and after the incubation (20 min) with nitric oxide synthase (NOS) blocker, L-N(G)- nitroarginine methyl ester (L-NAME, 100 μM); soluble guanylate cyclase (sGC) blocker, 1H-[1,2,4]-oxadiazolo[4,3-a] quinoxaline-1-one (ODQ, 30 μM).

In the second series of trials, acetylcholine (ACh, 10 μM), electrical field stimulation (EFS, duration: 15 seconds, amplitude: 40 V, frequency: 10 Hz, pulse width: 5 ms) and sodium nitroprusside (SNP, 0.01 μM)-induced relaxation responses were measured before and after the incubation (20 min) with Cranberry-E (1,2 mg/mL).

Chemicals and reagents

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

All measurements were displayed as mean±standard error of the mean (SEM). Statistical differences were determined by one-way analysis of variance (ANOVA) with repeated measures followed by a Bonferroni post-test performed using Prism 4 (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered to be significant.

RESULTS

Body weight and blood glucose levels in animals

The body weight in the diabetic animals declined compared to the controls (Figure 1). The blood glucose level in the diabetic rats was significantly greater than in the controls (Figure 1).

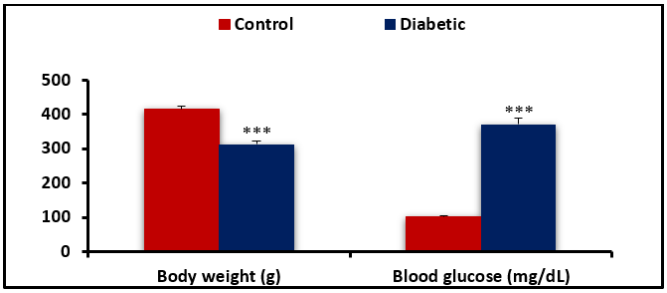

Figure 1. Body weight and blood glucose levels in the groups. Results displayed the mean ± SEM of 4-5 observations. ***p<.001 vs. controls.

Effects of intracavernosal Cranberry-E on *in vivo* erectile functions

Figure 2 shows ICP/MAP (A) and total ICP (B) values in the control and diabetic rats. *In vivo* erectile responses in the diabetic animals were less than in the controls (P<.001), which improved following intracavernosal injection of Cranberry-E (5 mg/mL) (Figure 2). Furthermore, erectile responses in the control rats increased after injection of Cranberry-E, except at 7.5V (Figure 2).

Relaxant responses of the CC strips

The maximum relaxation to Cranberry-E in the control rats was 74.4 ± 3.6%, which was not different for diabetic rats (73.3 ± 2.3%; Figure 3).

The relaxant responses to Cranberry-E were not altered after incubating with the NOS inhibitor, L-NAME (61.0 ± 5.0%, Figure 4A). In addition, ODQ (65.0 ± 4.0%) did not change the relaxation responses (Figure 4A). Cranberry-E caused 10% relaxation in the CC obtained from the control rats at 2.4 mg/mL (P <.001) after pre-contraction with KCl, which was 85% lower than after pre-contraction with Phe (Figure 4B).

The relaxant response to EFS at 10 Hz in the CC obtained from the control rats was significantly increased after incubating Cranberry-E at 1.2 mg/mL (P< .01, Figure 5). The relaxant response to ACh at 10 μM in the controls was raised in the presence of Cranberry-E at 1.2 mg/mL (P <.01, Figure 5). The relaxant response to SNP was enhanced after incubating with Cranberry-E at 1.2 mg/mL (P <.01, Figure 5).

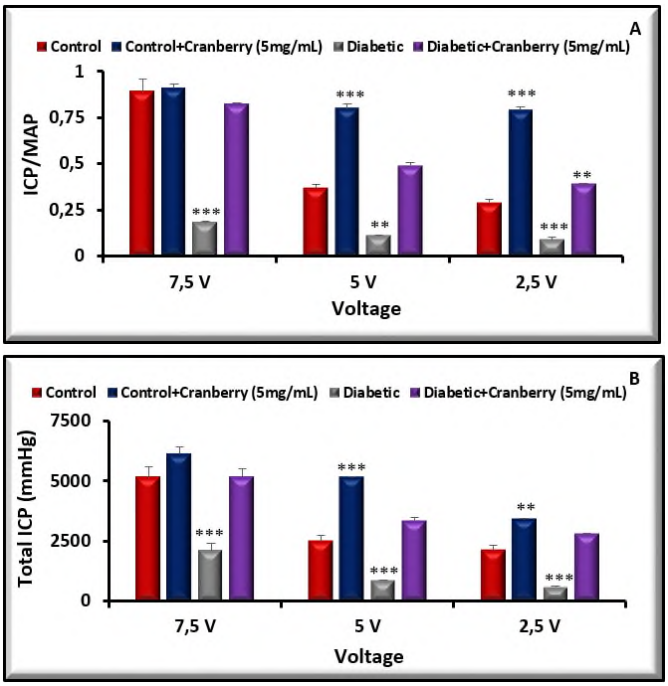

Figure 2. *In vivo* intracavernosal effect of Cranberry-E on ICP/MAP and Total ICP values in control and diabetic rats. Results displayed the mean ± SEM of 4-5 observations. **p<.01, ***p<.001 vs. corresponding controls.

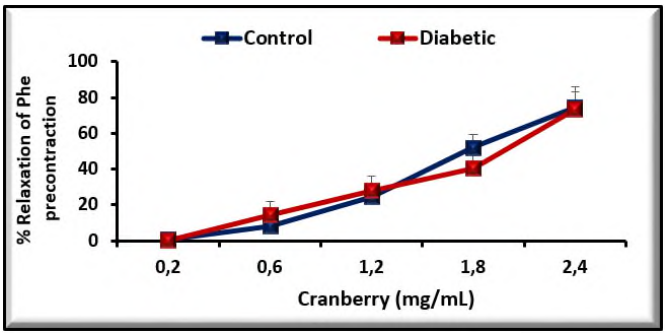

Figure 3. Concentration-response curves to Cranberry-E (0.2-2.4 mg/mL) after pre-contraction with Phe (10^{-5} M) in the control and diabetic rat CC. Data represent the mean ± SEM of 4-5 observations.

DISCUSSION

The present results exhibit that (a) Cranberry-E increases erectile function in control and diabetic rats; (b) Cranberry-E induces relaxant responses in the CC from both groups; (c) Cranberry-E-caused relaxant response is independent of NO pathway while it is likely to depend on K+ channels; (d) the relaxant responses in CC from the control animals were considerably increased after incubation with Cranberry-E.

In the present study, ICP/MAP and total ICP values in diabetic rats were diminished. Both groups' erectile responses dramatically increased after receiving cranberry intracavernosal injections. Also, we showed an increased neurogenic relaxant response in the CC from controls after cranberry incuba-

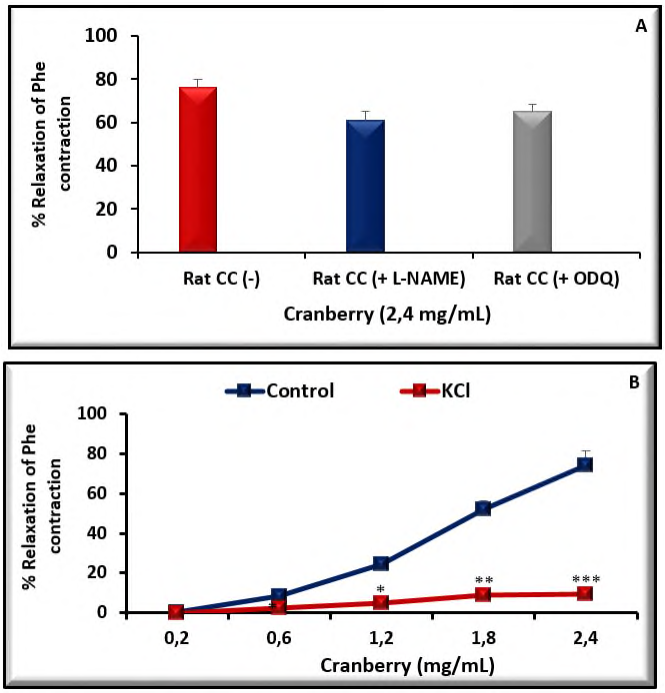

Figure 4. The bar graph shows relaxation responses to Cranberry-E at 2.4 mg/mL incubation with L-NAME (100 µM) and ODQ (30 µM) in control rat CC. Concentration-response curves to Cranberry-E (0.2-2.4 mg/mL) after pre-contraction with KCl (60mM, B) in relaxation in the CC obtained from the control rats. Data represent the mean ± SEM of 4-5 observations. *p<.05, **p<.01, ***p<.001 vs. controls.

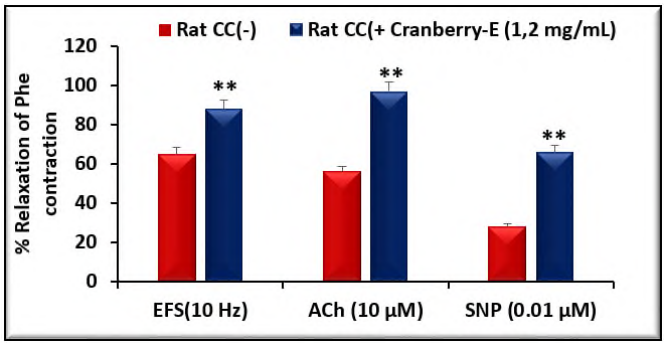

Figure 5. The bar graph shows relaxation responses to EFS (10Hz), ACh (10μM) and SNP (0.01 μM) in the absence and presence of Cranberry-E 1.2 mg/mL in the CC obtained from the control rats. Data represent the mean ± SEM of 4-5 observations. **p<.01, vs. controls.

tion. In addition, Cranberry-E relaxed both control and diabetic CC following Phe, α1-adrenergic receptor agonist-induced pre-contraction. There are no earlier studies that assess the effects of Cranberry-E on erectile function. An earlier prospective multi-center study revealed that cranberry was administered for three months and was beneficial to erectile function in men (Nemr et al., 2020). Cranberries are a rich source of polyphenols such as proanthocyanidins, phenolic acids, flavonoids and anthocyanins with antioxidant properties (Nemzer, Al-Taher, Yashin, Revelsky, & Yashin, 2022). Previous clinical trials displayed that the consumption of cranberry considerably decreased glycated hemoglobin and fasting blood glucose levels. Cranberry consumption also changed oxidative stress and proinflammatory markers in patients with diabetes and obesity (Delpino, Figueiredo, Goncalves da Silva, & Flores, 2022; Hsia, Zhang, Beyl, Greenway, & Khoo, 2020). Furthermore, Shukitt-Hale et al. demonstrated that 16 weeks of cranberry supplementation improved motor functions, neural function, and neuroprotective responses in aged rats (Shukitt-Hale et al., 2005). According to the available research, cranberries can treat oxidative stress from hyperglycemia, reducing diabetes-related ED.

The current results show that cranberry caused relaxation independent of the NO-cGMP pathway. The relaxing mechanism of cranberries in the cavernosal smooth muscle has not been studied previously. However, a conflicting result indicated that cranberry juice induced vasodilation in rat aorta, and the relaxant response was inhibited after incubating with L-NAME (Maher, Mataczynski, Stefaniak, & Wilson, 2000). In our study, pre-contraction of the cavernosal tissues with 60 mmol KCl significantly decreased cranberry-induced relaxations compared to pre-contraction with Phe. The contraction induced by KCl is generated due to membrane depolarization (Ebeigbe & Aloamaka, 1987). Our findings show that high K+ concentration inhibited the relaxation response induced by cranberry in rat cavernosal tissue suggesting that K+ conductance channels are probably responsible for this reduction. Based on the current result, understanding the relaxant mechanisms of cranberry in the penile erection mechanism is necessary for additional research.

Our findings show that Cranberry-E incubation boosted the isolated CC from the control group's endothelium-dependent ACh and endothelium-independent SNP relaxant responses. Additionally, in the porcine coronary artery, juice from various berries can cause endothelium-dependent relaxations involving endothelium-derived NO and endothelium-derived hyperpolarizing factors (Auger et al., 2011). Similarly, earlier data demonstrated that Cranberry juice enhanced endothelium-dependent relaxation in the aorta from ovariectomized rats via repairing endothelial NO synthase (Yung et al., 2013).

CONCLUSION

According to the present research, cranberries may have an impact on diabetes-related ED that is independent of the NO/sGC/cGMP pathway. Additionally, our *in vivo* and *in vitro* investigations suggest that consuming Cranberry-E may be appropriate and result in an alluring novel technique for avoiding and treating ED in diabetic male patients.

Peer Review: Externally peer-reviewed.

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Original Article

Synthesis, characterization and in vitro cytotoxic activity of platinum(II) oxalato complexes involving 2-substitutedimidazole or 2-substitutedbenzimidazole derivatives as carrier ligands

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ABSTRACT

Background and Aims: Cisplatin is currently one of the most widely used anticancer drugs in the world. However, its clinical usefulness has frequently been limited by severe side effects, such as nephrotoxicity, ototoxicity and neurotoxicity. Therefore, platinum(II) oxalato complexes with substitute imidazole or benzimidazole carrier ligands were synthesized and their cytotoxic effects were investigated against non-small cell lung cancer (H1299) and human colon adenocarcinoma (CaCo-2), and mouse fibroblast cells lines (L929).

Methods: Four platinum(II) complexes, [Pt(**L1-L4**)$_2$(oxalate)] were synthesized and characterized by FT-IR, 1H NMR and elemental analyses. The MTT method was used to determine the potential antiproliferative effect of synthesized platinum(II) complexes and positive controls.

Results: In this study, the cytotoxic activity of platinum(II) complexes against tested cell lines was assessed, with moderate IC_{50} values. According to IC_{50} values, **Complex 5** with 2-ethylbenzimidazole ligand was found to be the most active complex against H1299 and CaCo-2 cell lines. In general, the compounds are also promising drug candidates for H1299 cell lines with very low activity against the CaCo-2 cell lines.

Conclusion: Further modification and development of **Complex 4** and **5** derivatives and *in vitro* cytotoxic activity studies against different cancer cell lines may lead to the emergence of new anticancer agents in the near future.

Keywords: Cytotoxic activity, 2-ethylbenzimidazole, 2-methylbenzimidazole, 2-phenylimidazole, platinum(II) complexes

INTRODUCTION

Cancer is characterized by uncontrolled cell division and can spread throughout the body via metastasis, which makes it a disease that causes the second-highest mortality rate in the world (Sung et al., 2021). In our clinic, cancer patients are currently treated with chemotherapeutic drugs alone or in combination with radiotherapy and surgery if necessary. In chemotherapeutic treatment, the immediate aim is to inhibit the growth of tumor tissue, avoid metastasis or trigger cytotoxic activity to eliminate the cancerous cells if possible (Dasari & Tchounwou, 2014). Since cancer comes in various forms and has widespread diagnosis and a high mortality rate, novel chemotherapeutic drugs are being throughly researched for the effective treatment of various types of cancer. (Diamond et al., 2015).

Cisplatin, the pioneer of platinum complex-based anticancer drug, has been used successfully for the treatment of many cancers. Although it is a highly effective and widely used chemotherapeutic agent against tumors, due to the development of resistance and side effects such as nephrotoxicity, neurotoxicity, ototoxicity and bone marrow toxicity, the development of new platinum complexes continues intensively (Peng, Liang, Liu, & Mao, 2021).

The need for cisplatin analogs with fewer toxic side effects and a broader spectrum of activity has led to the synthesis of numerous platinum(II) complexes over the last four decades. Second and third-generation platinum complexes are obtained by replacing the leaving groups with carboxylate groups, which are very slowly activated and significantly less toxic. These

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platinum-based compounds, namely cisplatin and its second or third-generation derivatives carboplatin and oxaliplatin, act as cytotoxic drugs through the formation of intrastrand or interstrand platinum-DNA adducts. These interactions are known to inhibit transcription and thus trigger apoptosis which eventually causes cell death (Ho, Woodward, & Coward, 2016; Deo et al., 2018).

Carboplatin is effective against cancers sensitive to cisplatin, but carboplatin has far fewer side effects. Similar to carboplatin, the less severe side effects of oxaliplatin compared to cisplatin are related to the cleavage of the dicarboxylate group, which again slows the production of reactive metabolites. Furthermore, the two ammine ligands in cisplatin were replaced by a single bidentate ligand (1R,2R)-cyclohexane1,2-diamine) in the oxaliplatin. Oxaliplatin is thought to overcome cisplatin resistance through different adducts formed with DNA (Burger et al., 2011; Perego & Robert, 2016).

The efficacy and broad range of activity of platinum(II) complexes can be changed through modifications to the carrier ligands, as is well known. The use of sterically demanding heterocyclic amines as carrier ligands for alternative compounds to cisplatin are slow or block repair enzymes (Deo et al., 2018).

Imidazole and benzimidazole are bioactive heteroaromatic compounds that exhibit different pharmacological activities. They involve biologically important histamine, histidine amino acid, iron-heme system, various metalloproteins and vitamin B12 derivatives (Iakovidis & Hadjiliadis, 1994; Sundberg & Martin, 1974). Furthermore, in organisms, histidine residue is involved in metal-binding regions to bind metal atoms in the active sites of many different enzymes (Živković, Rajković, & Djuran, 2008; Szulmanowicz, Zawartka, Gniewek, & Trzeciak, 2010). Also, as a biologically recognized heteroaromatic ring system, imidazole and benzimidazole possess ligand properties for various transition metals. Because of their low toxicity, high stability, interactions with metals, and electronic or steric properties, these two heteroaromatic rings are crucial for medicinal chemists (Salahuddin, Shaharyar & Mazumder, 2017; Ali, Lone, & Aboul-Enein, 2017).

Platinum compounds containing N-donor ligands such as substituted imidazole or benzimidazole derivatives show better biological activity with less toxicity. According to data in the literature, bulky or lipophilic substituted benz(imidazole)s at the C2 position have activity in various cancer cell types (Gümüş et al., 2003; Gümüş et al., 2009; Boğatarkan, Utku, & Acik, 2015). In our previous studies, with the consideration that variations in the chemical structure of the ammine groups of cisplatin might have a significant effect on the cytotoxic activity of platinum complexes and for the purpose of determining the role of the substituents on position 2 of the benzimidazole carrier ligands of platinum(II) complexes on cytotoxic properties, we synthesized some Pt(II) complexes with 2-substituted imidazole and 2-substituted benzimidazole carrier, thus leaving chloride and oxalate ligands (Figure 1) (Boğatarkan, Utku, & Acik, 2015; Gümüş et al., 2003; Gözelle et al., 2019; Özçelik et al., 2012; Utku et al., 2014; Utku, Topal, Döğen, & Serin, 2010). Based on in vitro cytotoxic tests against HeLa, MCF-7 and MDA-MB 231 cell lines, it was found that several of these [Pt(carrierligands)$_2$X (X=Cl$_2$ or oxalate] complexes possessed cytotoxic activity comparable to cisplatin or oxaliplatin.

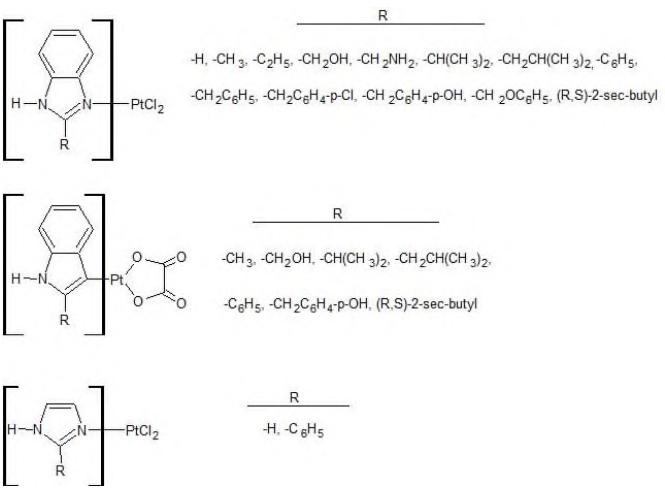

Figure 1. Platinum compounds bearing 2-substituted imidazole and benzimidazole ligands.

In this study, as an extension of our investigation on the probable anticancer activity of platinum complexes with 2-substituted imidazole or benzimidazole ligands, four platinum(II) complexes with bulky or/and planar carrier ligands, including imidazole **(L1)**, 2-phenylimidazole **(L2)**, 2-methylbenzimidazole **(L3)** and 2-methylbenzimidazole **(L4)**, were evaluated for their in vitro cytotoxic activities against H1299 and CaCo-2 cell lines using the MTT method.

MATERIAL AND METHODS

Chemistry

The starting materials were provided by Sigma-Aldrich. The elemental (C, H, N) analyses were run on a Leco-932 Elemental Analyzer. The IR spectra of **L1-L4** and **Complex 1-5** were obtained using Perkin Elmer Spectrum FT-IR/NIR Spectrometer between 4000-600 cm^{-1}. ^{1}H NMR spectra of carrier ligands **L1-L4** and **Complex 2-5** were recorded on a Varian 400 MHz FT NMR Spectrometer using a deuterium dimethyl sulfoxide (DMSO−d$_6$) solution.

General synthesis of carrier ligands (L3, L4)

2-substituted benzimidazole derivatives **L3** and **L4** used as carrier ligands were prepared according to the Phillips method (Phillips, 1928).

2-Methylbenzimidazole (L3)

Yield 44.66 %, mp: 174°C (175-176 °C), ^{1}H NMR (400 MHz, DMSO−d$_6$): δ 12.12 (s, 1H, N-H), 7.44-7.40 (m, 2H, ArH),

7.10-7.06 (m, 2H, ArH), 2.46 (s, 3H, -CH_3); IR (υ cm^{-1}, KBr): 3176-2536 (N-H, =C-H, -C-H), 1622-1270 (C=N, C=C, C-H), 731 (substituted benzene =C-H) (Rabiger & Joullié, 1964).

2-Ethylbenzimidazole (L4)

Yield 46.71%, mp: 174°C (172-173°C); ^{1}H NMR (400 MHz, DMSO−d_6): δ 12.14 (s, 1H, N-H), 7.46-7.43 (m, 2H, ArH), 7.12-7.08 (m, 2H, ArH), 2.84-2.79 (q, 2H, -CH_2-), 1.33-1.29 (t, 3H, -CH_3); IR (υ cm^1, KBr): 3152-2632 (N-H, =C-H, -C-H), 1621-1270 (C=N and C=C and C-H), 738 (substituted benzene =C-H) (Rabiger & Joullié, 1964).

Synthesis of potassium bis(oxalato)platinate(II) dihydrate K_2[Pt(oxalate)$_2$].2H_2O (Complex 1)

Complex 1 was obtained similarly to a previously published approach as follows: 12 mmol potassium oxalate monohydrate was added to a solution of 2.41 mmol potassium tetrachloroplatinate in 10 mL of hot distilled water. The mixture was heated at 70 °C for 3 days. The light green product was filtered and washed in hot and then in cold water, and finally recrystallized from hot water. Green needle-like crystals of K_2[Pt(oxalate)$_2$].2H_2O which formed were filtered off and washed with cold water and ethanol. Yield 74.35%, IR (υ cm^{-1}, KBr): 3559 and 3476 (O-H, (H_2O)), 1696 and 1668 (C=O), 1234 (C-O), 565 (Pt-O)

General synthesis of platinum(II) complexes

To a solution of **L1-L4** (0.90 mmol) in ethanol/isopropanol at 50-60 °C, a solution of **Complex 1** (0.5 mmol) in distilled water at 50-60 °C was added dropwise and stirred for 4-6 days at 50-60 °C until complexation was finished. The precipitate was filtered and the crude product was washed with hot water, cold water, hot ethanol and cold ethanol.

Oxalato-di(imidazole)platinum(II) 0.5 H_2O (Complex 2)

Yield 53.64%, mp: >400°C. ^1H NMR (400 MHz, DMSO−d_6): 8.06 (s, 2H, 2x imidazole H), 7.32 (s, 2H, 2x imidazole H), 6.93 (s, 2H, 2x imidazole H); IR (υ cm^{-1}, KBr): 3135-2821 (N-H, =C-H and O-H), 1699 (C=O) 1653-1490 (C=N, C=C and C-O), 560 (Pt-O). Anal. Calcd. for [$C_8H_8N_4O_4$Pt. H_2O]: C, 21.97; H, 2.31; N, 12.81%; Found: C, 21.18; H, 2.44; N, 13.35%.

Oxalato-di(2-phenylimidazole)platinum(II) (Complex 3)

Yield 81.4 %, mp: > 400 C. ^1H NMR (400 MHz, DMSO−d_6): 8.67-8.56 (m, 2H, ArH), 8.29-8.16 (m, 2H, ArH), 7.55-7.46 (m, 4H, 2x ArH), 7.40-7.33 (m, 2H, 2x ArH and 4H 2x imidazole H); IR (υ cm^{-1}, KBr): 3140-2757 (N-H, =C-H), 1696 (C=O), 1651-1472 (C=N, C=C and C-O), 535 (Pt-O). Anal. Calcd. for [$C_{20}H_{16}N_4O_4$Pt]: C, 42.04; H, 2.82; N, 9.80%; Found: C, 42.16; H, 3.19; N, 10.25%.

Oxalato-di(2-methylbenzimidazole)platinum(II) (Complex 4)

Yield 26.56%, mp: > 400 °C. ^1H NMR (400 MHz, DMSO−d_6): δ 7.76-7.74 (m, 2H, 2x ArH), 7.46-7.44 (m, 2H, 2x ArH), 7.24-7.21 (m, 4H, 2x ArH), 2.69 (s, 6H, 2x -CH_3); IR (υ cm^{-1}, KBr): 3188-2781 (N-H, =C-H, -C-H), 1700 (C=O), 1645-1284 (C=N, C=C, C-H and C-O), 565 (Pt-O). Anal. Calcd. for $C_{18}H_{16}N_4O_4$Pt: C, 39.49; H, 2.95; N, 10.23 %; Found: C, 39.69; H, 2.52; N, 10.47% (Gözelle et al., 2019).

Oxalato-di(2-ethylbenzimidazole)platinum(II).H_2O (Complex 5)

Yield 25.13%, mp: > 400 °C. ^1H NMR (400 MHz, DMSO−d_6) δ 13.41 (s, 2H, 2x N-H), 7.92-7.90 (m, 2H, 2x ArH), 7.52-7.50 (m, 2H, 2x ArH), 7.33-7.29 (m, 4H, 2x ArH), 3.14-3.10 (q, 4H, 2x -CH2-), 1.33-1.31 (t, 6H, 2x -CH_3); IR (υ cm^{-1}, KBr): 3118-2744 (N-H, =C-H, -C-H), 1694 (C=O), 1645-1278 (C=N and C=C and C-H), 747 (substituted benzene =C-H). Anal. Calcd. for $C_{20}H_{20}N_4O_4$Pt.H2O: C, 40.47; H, 3.74; N, 9.44; Found: C, 40.59; H, 3.43; N, 9.56

MTT cell viability assay

H1299 (non-small-cell lung cancer), CaCo-2 (An1/human adenocarcinoma) and L929 (mouse fibroblast, An2 Mouse C3), cell lines were obtained from the Foot and Mouth Disease Institute (Ankara, Turkiye). L929 and H1299 cells in 10% bovine serum, 100 IU/mL penicillin/streptomycin with 4 μM glutamine DMEM liquid broth and CaCo-2 cells in 10% bovine serum, 100 IU/mL penicillin/streptomycin with 4 μM glutamine EMEM broth were incubated in an atmosphere containing 5% CO_2 at 37°C. 1.0 x 10^4 cells were seeded into each well of a 96-well cell culture plate and incubated for 24 h at 37°C and 5% CO_2 in a humidified incubator. **Complex 2-5** were then added to the cells at seven different concentrations. After 48 h incubation, 50 μl MTT (1 mg/mL) was added to each well and after an incubation period of 2 h at 37 °C, 100 μl isopropanol was added to the wells (Wang, Wang, Tao, & Cheng, 2012). A cell viability assay was run in a 96-well plate with measuring absorbance at 570 nm. Each compound was studied in three independent experiments. The amount of DMSO used as solvent did not exceed 1%. Cisplatin and oxaliplatin were used as positive controls and cell broth was used as blank.

RESULTS AND DISCUSSION

Chemistry

Complex 1, a yellow-colored compound with needle-like crystals, was determined via IR through its OH vibration from H_2O between 3559-3476 cm^{-1} and Pt-O vibration at 565 cm^{-1}. The spectral data and physical properties found in the literature are in agreement with our analyses (Štarha, Trávníček, & Popa, 2010).

Complex 2-5 were synthesized through the addition of L_1−L_4 solutions in ethanol/isopropanol into the aqueous solution of Complex1 (Figure 1).

Structural analyses of **Complex 2-5** were elucidated using elemental analysis, FT-IR and ^{1}H NMR spectra. Elemental analysis of **Complex 2-5** shows that monodentate **L1-L4** ligands react with **Complex 1** with a ratio of 1:2 metal:ligand (Grimmett, 1970; Manocha, Wakode, Kaur, Anand, & Kumar, 2016; Wright, 1951).

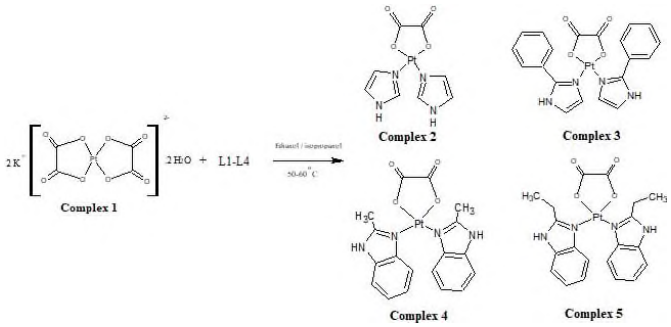

Figure 2. Synthesis of **Complexes 2-5**

The ^{1}H NMR spectra of **Complex 2-5** were obtained by dissolving in DMSO−d_6 due to the insolubility of complexes in other NMR solvents. In general, related to complexation, the aromatic or/and aliphatic proton peaks of **Complex 2-5** shifted to low areas compared to **L1-L4**. In addition, because of ½ spin-quant number and 33% isotope abundance of ^{195}Pt isotope, peak diversion was observed as a result of ^{195}Pt-^{1}H spin-spin coupling. Complexation-related ligand protons' peak shift to high ppm values is in agreement with the literature data (Navarro-Ranninger, Zamora, Alfonson Martínez-Cruz, Isea, & Masaguer, 1996).

Biological Evaluation

Complexes 2-5 were tested for their cytotoxic activity on H1299, CaCo-2, and L929 cell lines using the MTT method. The results of this experiment and IC_{50} values of compounds are presented in Table 1.

An evaluation of **Complex 2-5** using IC_{50} values revealed that cytotoxic activity enhances if substitution exists at position 2 or if the size of substitution is increased. **Complex 5** bearing 2-ethylimidazole is the most potent complex on H1299 and CaCo-2 cell lines compared to other complexes. Based on MTT results, IC_{50} values of tested complexes are less active compared to cisplatin and oxaliplatin.

Platinum(II) complexes bearing dicarboxylate or chloride leaving ligands have previously been tested for their cytotoxic activities on various cell lines. These tests revealed that depending on the substituent groups in the carrier ligands of these platinum(II) complexes, there are differences in the intracellular entry, their binding to DNA and also in their cytotoxic activity values (Gözelle et al., 2019; Özçelik et al., 2012; Özçelik, Gümüş, Sagkan, & Musabak, 2015; Özçelik, Kılıç Süloğlu, Selmanoğlu, & Gümüş, 2019; Tarı, Gümüş, Açık, & Aydın, 2017; Utku et al., 2014). In these studies, it was observed that the cytotoxicity of compounds increased as the substituent's size expanded. In this present study, **Complex 4** and **Complex 5** bearing methyl and ethyl substituents at position 2 of benzimidazole, respectively, were found to be the most potent compounds among the synthesized complexes. These results are in agreement with the literature (Spingler, Whittington, & Lippard, 2001; Wu et al., 2004; Todd & Lippard, 2009).

Table 1. IC_{50} (μM) values of **Complex 2-5**, cisplatin and oxaliplatin by using the MTT test in cancerous and healthy cells

Complexs No	H1299		CaCo-2		L-929
	IC_{50}[a]	SI[b]	IC_{50}[a]	SI[b]	IC_{50}[a]
2 [Pt(L1)$_2$oxalate]	168.84 ± 9.87	1.14	281.25 ± 4.37	0.68	192.90 ± 5.03
3 [Pt(L2)$_2$oxalate]	132.31 ± 8.89	1.05	273.75 ± 5.79	0.51	139.49 ± 6.14
4 [Pt(L3)$_2$oxalate]	110.48 ± 5.42	1.42	286.95 ± 7.14	0.55	157.78 ± 3.67
5 [Pt(L4)$_2$oxalate]	101.24 ± 6.47	1.44	270.36 ± 9.94	0.54	145.43 ± 7.48
Cisplatin	50.97 ± 7.55	1.25	64.51 ± 14.32	0.99	63.66 ± 9.37
Oxaliplatin	27.21 ± 12.78	2.10	53.58 ± 6.47	1.06	57.04 ± 5.36

[a] IC_{50} = 50% cytotoxic concentration against in vitro tested cells. Data are presented as mean ± SD.
[b] SI = Selectivity Index—IC_{50} value relative to a healthy cell.

CONCLUSION

In summary, this work is based on the synthesis, characterization and in vitro cytotoxic of oxalato platinum(II) complexes. **Complexes 2-5** were investigated for their potential anticancer activity against H1299 and CaCo-2 cell lines using the MTT method. Among all the synthesized complexes tested, Complex 4 and Complex 5, which have methyl and ethyl substituents at the second positions of the carrier ligand, were found to be the most effective platinum(II) complexes. It is also likely that novel molecules to be designed by development and modification of **Complex 4** and **Complex 5** derivatives will exhibit selective inhibitor activity against different cancer cell lines.

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Original Article

Inhibitory potentials of *Moringa oleifera* on activities of neuraminidase, xanthine oxidase and adenosine deaminase

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ABSTRACT

Background and Aims: The use of *Moringa oleifera* as nutraceuticals in alternative medicine has received tremendous attention in recent years. Its diverse bioactive composition, multipurpose benefits and ease of cultivation give it a superior advantage over other herbs.

Methods: Fresh leaves and roots were obtained from *M. oleifera* grown in northwestern Nigeria. The inhibitory effect of *M. oleifera* extracts on the activities of neuraminidase, xanthine oxidase, and adenosine deaminase were determined.

Results:: The present study explored the aqueous, methanol, and hexane extract of *M. oleifera* leaves and roots for the inhibition of neuraminidase, xanthine oxidase, and adenosine deaminase. In comparison to quercetin (Half maximum inhibitory concentration (IC_{50}) = 14.28 ± 2.30 µg/mL), aqueous (IC_{50}= 0.12 ± 0.01 µg/mL) and methanol (IC_{50} = 0.57 ± 0.13 µg/mL) the extract of the moringa root strongly inhibited neuraminidase activity. The enzyme was moderately inhibited by aqueous (IC_{50} = 89.56 ± 9.77 µg/mL) and hexane (IC_{50} = 104.33 ± 3.39 µg/mL) extracts of the plant leaf. The inhibition of xanthine oxidase by aqueous (IC_{50} = 7543.86 ± 1127.19 µg/mL), and methanol (IC_{50} = 1779.48 ± 126.50 µg/mL) leaf extracts were far below that of a standard inhibitor - allopurinol (IC_{50} = 0.88 ± 0.01 µg/mL). Amongst the extracts used, only the hexane extract of the moringa leaf (IC_{50} = 4580.38 ± 75.69 µg/mL) inhibited adenosine deaminase and was less effective than erythro-9-(2-Hydroxy-3-nonyl)-adenine hydrochloride (EHNA) (IC_{50} = 53.00 ± 1.83 µg/mL).

Conclusion: The findings suggest that moringa roots and leaves can be an excellent source of agents against microbial infection and viral induced respiratory syndrome. The extracts may also attenuate influenza A infection, the progression of oxidative stress, cancer, inflammation, diabetes, cardiac failure, and coronary artery disease, since they have an effect on neuraminidase, xanthine oxidase, adenosine deaminase, and possibly superoxide levels.

Keywords: *Moringa oleifera,* Neuraminidase, Xanthine oxidase, Adenosine deaminase, Inhibition.

INTRODUCTION

Many drugs exhibit biochemical and clinical effects via hindering the activity of enzymes (biological catalysts), either by directly blocking the binding of substrates to enzyme (thereby preventing enzyme-complex formation), or by retarding the catalytic activity of an enzyme rate (i.e., rate of product formation) upon binding to a regulatory/allosteric site. Therefore, many drugs function as inhibitors of enzymes. Thus, enzyme inhibition is amongst the principal techniques employed for drug discovery. This technique and regimen have paramount importance in both therapeutics and pharmacognosy. This is due to the widespread use of plants (as alternative or folk medicine) in the treatment or management of metabolic diseases, metabolic disorders, infectious diseases, drug resistant strain of microbes, and pathogens, in addition to their use as nutraceuticals and food additives (Hodas, Zorzenon, & Milani, 2021).

Neuraminidases are a group of glycoside hydrolases that catalyze the hydrolysis of the glycosidic bonds of neuraminic acid and/or its derivative (sialic acids). These enzymes play a significant role in microbial pathogenesis and virulence (Rothe, Rothe, Roggentin, & Schauer, 1991). They are believed to modulate motility, virion aggregation, elution of virion progeny as well as the interaction between pathogens and host cell receptors (McAuley et al., 2017). Therefore, inhibition of these group of enzymes confers great advantage to host organisms against the virulent and infectious agents as earlier demonstrated by Gulati et al. (2013).

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Xanthine oxidase is an important xanthine oxidoreductase involved in purine metabolism. It oxidizes hypoxanthine to xanthine, and then to uric acid. This reaction is accompanied by the generation of superoxide radicals (Rechreche, Abbes & Iovanna, 2020), thus assuming great significance in the antioxidant system. The activity of xanthine oxidase in reported to increase in several disease conditions. These include oxidative stress, diabetes, cardiac failure, coronary arteries disease, and in influenza A infection (Penislusshiyan, Chitra, Ancy, Kumaradhas, & Palvannan, 2020). Therefore, inhibition of this enzyme is of great significance in attenuating the accumulation of a reactive oxygen species and pathogenesis/progression of many disease conditions.

The irreversible deamination adenosine to inosine in purine metabolism is catalyzed by adenosine deaminase. This same enzyme has demonstrated to be critical for immune responses, transmission of nerve impulses, pregnancy, and the differentiation of epithelial cells (Moriwaki, Yamamoto, & Higashino, 1999). Elevated levels of adenosine deaminase are reported in arthritis, psoriasis, sarcoidosis, cancer, ischemia, haemolytic anemia, and AIDS (Blackburn & Kellems, 2005). Therefore, food-based extracts/compounds capable of diminishing adenosine deaminase activity will play a vital role in the management of diseases and their accompanying symptoms.

In recent years, *Moringa oleifera* received a tremendous amount of attention in the field of alternative medicine, either as nutraceuticals, food supplements, or herbs (in the form of tea or spices) due to its diverse bioactive composition, benefits, and ease of cultivation. A review by Pandey et al., (2012) and more recently by Khor, Lim, Moses & Abdul Samad, 2018, advocates that *M. oleifera* exhibited several medicinal properties. These reports indicated that the plant exhibited antidiabetic, antihypertensive, anticancer, antioxidant, anti-inflammatory, antipyretic, antiplasmodial, and antimicrobial effects, in addition to chemoprotective and radioprotective action properties. In addition, the extracts are cytotoxic to a diverse type of cancer cells but had minimal toxicity to normal cell and experimental animals. The plethora of multifarious therapeutic effects of *M. oleifera* is attributed to its disparate and assorted chemical or phytochemical composition (Ajagun-Ogunleye & Ebuehi, 2020). The aim of the present study is to investigate the inhibitory potentials of *M. oleifera* leaves and roots extracts on neuraminidase, xanthine oxidase, and adenosine deaminase.

MATERIALS AND METHODS

Sample collection and preparation

The fresh leaves and roots of *M. oleifera* were obtained from Northwest Nigeria. The plant was authenticated by a Taxonomist (Umar Abdullahi, PhD), at the Botany Unit of Biological Sciences Department, Usmanu Danfodiyo University Sokoto. This was followed by deposition of a voucher specimen (Voucher Number: UDUS/VS/2011/31) in the University herbarium. The plant samples were processed, and extracts prepared according to the method of Magaji, Sacan, & Yanardag, (2020).

Enzyme inhibition assay

The inhibitory effect of *M. oleifera* extracts on the activities of neuraminidase, xanthine oxidase, and adenosine deaminase were determined according to the method of Myers et al., (1980), Abdullahi et al., (2012) and Blum & Schwedt, (1998), respectively. Quercetin, allopurinol, and erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride (EHNA) were used as standard inhibitors of neuraminidase, xanthine oxidase, and adenosine deaminase. The results are presented as mean ± standard deviation of triplicate values. Half maximum inhibitory concentration (IC_{50}) were calculated from % enzyme inhibition activities using regression analysis data. The IC_{50} values are inversely correlated to inhibition.

RESULTS AND DISCUSSION

The inhibitory activities of aqueous, methanol, and hexane extracts of the moringa leaf and root on neuraminidase are presented in Table 1. The outcome of the present study indicates that both the aqueous and methanol root extracts of moringa exhibited strong neuraminidase inhibitory activity (with IC_{50} values corresponding to 0.12 ± 0.01 µg/mL and 0.57 ± 0.13 µg/mL, respectively). Their inhibitory effect was higher than that of quercetin (IC_{50} = 14.28 ± 2.30 µg/mL), which was used as a standard inhibitor. Moderate inhibition was exhibited by an aqueous extract (IC_{50} = 89.56 ± 9.77 µg/mL) and hexane extract (IC_{50} = 104.33 ± 3.39 µg/mL) of the plant leaf. However, the methanol leaf extract and hexane root extract did not exhibit neuraminidase at the tested concentrations. Fouad, Abu Alnaga, & Kandil, (2019) demonstrated that the moringa leaf extract had a strong antibacterial effect on pyogenic bacteria isolated from the abscess of a dromedary camel. The microorganisms inhibited are *Escherichia coli, Staphylococcus aureus, Micrococcus spp., Citrobacter spp., Corynebacterium pseudotuberculosis, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Corynebacterium ulcerans,* and *Staphylococcus epidermidis*. In another study by Dahot (1998), fractions of moringa extracts were shown to inhibit bacteria *(E. coli, Klebsiella aerogenes, K. pneumoniae and Bacillus subtilis)* and *Aspergillus niger* (a fungus). In comparison to amoxicillin, the moringa leaf extracts were shown to be a better antibiotic candidate against Bacillus spp. (Kilany, 2016). The antimicrobial studies by Elgamily et al. (2016) revealed that both root and leaf extracts of the moringa significantly inhibited the growth of *S. aureus* and *Streptococcus mutans*, but had no effect on *Candida albicans* growth. These reports agree with the present findings, where the leaves and roots demonstrated anti-neuraminidase

Table 1. Inhibitory effect of *M. oleifera* extract on neuraminidase activity.

n me	tract/ Standard	oncentration (g/mL)	Inhi ition ( )	I (g/mL)
Neuraminidase	A ueous lea e tract	400.00	79.61 4.87	89.56 9.77
		200.00	70.62 0.48	
		100.00	53.44 0.74	
		10.00	35.29 1.47	
	Methanol lea e tract		ND	
	He ane lea e tract	100.00	45.12 1.41	104.33 3.39
		50.00	38.36 0.59	
		30.00	21.80 2.56	
		20.00	12.89 1.86	
	A ueous root e tract	0.10	43.58 2.39	0.12 0.01
		0.08	34.74 0.09	
		0.05	11.62 1.59	
		0.01	5.40 0.18	
	Methanol root e tract	1.00	70.72 1.63	0.57 0.13
		0.75	58.43 2.83	
		0.05	14.24 1.43	
		0.01	6.44 2.36	
	He ane root e tract		ND	
	uercetin	70.00	91.62 0.63	14.28 2.30
		40.00	77.05 1.09	
		20.00	55.56 2.28	
		10.00	42.23 2.42	

Mean SD o triplicate values ND Activit not detected.

activity (an enzyme necessary for microbial pathogenesis, virulence, and motility). The stronger neuraminidase inhibition by the moringa root extract may be attributed to its elevated levels of 4-(alpha-lrhamnopyranosyloxy) benzylglucosinolate and benzyl glucosinolate than was reported in the leaves (Bennett et al., 2003). Quercetin, kaempferol, and myricetin are flavonoids found in moringa leaves (Athira Nair & James, 2020). These compounds and their derivatives were demonstrated to inhibit both 3-chymotrypsin-like protease (3CLpro) and papain-like protease (PLpro) (Athira Nair & James, 2020; Jo, Kim, Shin, & Kim, 2020) – the two main protease enzymes critical for the virulence of several viruses including severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV). A recent review revealed that quercetin (a compound chiefly available in the moringa) was one of the most potent compounds with anti-CoV activity (Solnier & Fladerer, 2020). Thus, moringa extracts can be indispensable antibacterial and antiviral agents due to their neuraminidase, 3CLpro and PLpro inhibition properties.

As seen in Table 2, only aqueous (IC_{50} = 7543.86 ± 1127.19 µg/mL), and methanol (IC_{50}= 1779.48 ± 126.50 µg/mL) leaf extracts of the moringa had an inhibitory effect on xanthine oxidase in the present study. The effect of the extracts was much lesser than that of allopurinol (IC_{50} = 0.88 ± 0.01 µg/mL). Yumita, Suganda, & Sukandar, (2014) reported that the root of the moringa exhibited xanthine oxidase. This contrasts with the findings of the present study where only the leaf extracts had xanthine oxidase inhibition.

As shown in Table 3, the inhibitory effect of the hexane extract of the moringa leaf (IC_{50} = 4580.38 ± 75.69 µg/mL) on adenosine deaminase activities was low as compared to that of EHNA (IC_{50} = 53.00 ± 1.83 µg/mL). Though not many reports are available on the effect of moringa on adenosine deaminase activity, the aqueous extract of plants such as Urtica dioica have been shown to strongly inhibit adenosine deaminase of prostate tissue (Durak, Biri, Devrim, Sozen, & Avci, 2004). What is more, several studies have shown that moringa extracts

Table 2. Inhibitory effect of *M. oleifera* extracts on xanthine oxidase activity.

n me	tract/ Standard	oncentration (g/mL)	Inhi ition ( )	I (g/mL)
anthine o idase	A ueous lea e tract	4000.00	31.88 2.66	7543.86 1127.19
		3000.00	25.18 3.91	
		2000.00	18.94 1.25	
		1000.00	15.69 0.59	
	Methanol lea e tract	3000.00	73.80 3.78	1779.48 126.50
		2000.00	51.75 0.78	
		500.00	29.05 3.26	
		250.00	20.10 2.14	
	He ane lea e tract		ND	
	A ueous root e tract		ND	
	Methanol root e tract		ND	
	He ane root e tract		ND	
	Allopurinol	2.00	98.79 0.24	0.88 0.01
		1.00	82.50 1.59	
		0.50	25.23 1.82	
		0.25	5.87 1.58	

Mean SD o triplicate values ND Activit not detected.

Table 3. Inhibitory effect of *M. oleifera* extract on adenosine deaminase activity.

n me	tract/ Standard	oncentration (g/mL)	Inhi ition ( )	I (g/mL)
Adenosine deaminase	A ueous lea e tract		ND	
	Methanol lea e tract		ND	
	He ane lea e tract	5000.00	54.54 1.53	4580.38 75.69
		4000.00	41.94 0.28	
		3000.00	34.17 1.27	
		2000.00	15.56 1.11	
	A ueous root e tract		ND	
	Methanol root e tract		ND	
	He ane root e tract		ND	
	EHNA	60.00	52.78 0.80	53.00 1.83
		40.00	44.63 0.20	
		20.00	39.48 1.14	
		10.00	34.94 0.49	

Mean SD o triplicate values ND Activit not detected.

have anticancer and cytotoxic effects (Jung, 2014; Khor et al., 2018). Thus, the inhibition of this enzyme, coupled with the antioxidant activity of the moringa extract may not be unrelated with its anticancer, anti-inflammatory as well as tissue protective effects.

CONCLUSION

The outcome of the present study suggests that root and leaf extracts of the *M. oleifera* have promising anti-neuraminidase, and are a suitable candidate for new and effective antimicrobial agents including influenza A and corona viruses. The inhibition of xanthine oxidase and adenosine deaminase by leaf extracts suggest that the plants can be a source of compounds that can be used to manage or attenuate the progression of oxidative stress, cancer, inflammation, diabetes, cardiac failure, coronary arteries disease, and viral induced respiratory syndrome disease.

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Original Article

A chemometrics-based approach for the determination of thymoquinone from *Nigella sativa* L. (Black Cumin) seeds of different geographical regions using the HPLC technique

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ABSTRACT

Background and Aims: In this study, thymoquinone (TQ) from black cumin will be quantified from several geographical regions, including India, Syria, Saudi Arabia, Iraq, and Turkey. Additionally, to forecast the chromatographic behavior of the analyte in artificial intelligence (AI)-based models, the study used both ensemble machine learning methodologies and chemometrics-based approaches.

Methods: An Agilent Technologies (1200 series, USA) instrument that includes an autosampler, a binary pump, a diode array detector (DAD), and a vacuum degasser was used for the HPLC analysis. Using five different single models—principal component regression (PCR), least square-support vector machine (LSSVM), least square boost (LSQ-BOOST), adaptive neuro-fuzzy inference system (ANFIS), and step-wise linear regression—the HPLC-DAD technique was used to simulate the qualitative and quantitative properties of TQ (SWLR).

Results: The collected results demonstrated that samples from India and Iraq have the highest concentration of TQ. TQ was present in all samples, but in varying amounts; the amounts of TQ in the samples from Iraq, India, Saudi Arabia, Syria, and Turkey, respectively, were 0.031, 0.030, 0.022, 0.005, and 0.001%. According to a comparison of their performances, the four ensemble machine learning techniques can reproduce the chromatographic properties of TQ, PA, and tR with minimum and maximum NSE-values of 0.842 and 0.999 in the training phase and 0.918 and 0.999 in the testing phase, respectively.

Conclusion: The TQ content of each sample of black cumin, which was collected from various geographical locations, was determined quantitatively. The quantity of thymoquinone fluctuates depending on geographic variances, according to HPLC data. Five different AI-based models, including SWLR, PCR, LSSVM, ANFIS, and LSQ-Boost, were used to simulate the chromatographic behavior of TQ information of retention duration and peak area using various independent factors. Additionally, SAE, WAE, NNE, and ANFIS-E are informed by the application of ensemble machine learning to enhance the performance of AI-based models. Comparing the approaches to the individual models, they both demonstrated lower error values in terms of RMSE and MSE.

Keywords: Chemometrics, HPLC, Thymoquinone, Black cumin, geographical regions

INTRODUCTION

Black cumin's major bioactive compound, thymoquinone (TQ), has a wide range of biological effects, including anti-cancer, antioxidant, anti-inflammatory, and hepatoprotective qualities. These effects are hypothesized and supported by evidence from science that illustrates the molecular mechanism of the analyte (Rezai, Işk, Kartal, & Aslan Erdem, 2018). Finding TQ in Black cumin and other medicinal plants is regarded as a challenging and time-consuming task due to the huge quantity of bioactive components that are present in a plant. Depending on the region, the type of plant material, and the objective of the investigation, many methods, such as chemical, microbiological, biological, and chromatographic ones, can be utilized. Although microbiological techniques are the most used, their lack of specificity is making them obsolete (Chen et al., 2019).

Chromatographic techniques like High-performance liquid chromatography (HPLC) are capable of separating various analytes in a plant with a high degree of specificity and sensitivity,

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making them useful for isolating TQ from Black cumin. For appropriate resolution, it's imperative to optimize several chromatographic factors, including the mobile phase concentration, pH of the mobile phase, column temperature, mobile phase flow rate, and column type. For example, achieving appropriate resolution requires optimizing several chromatographic factors, including the mobile phase concentration, mobile phase pH, column temperature, mobile phase flow rate, and column type. The experimental approach is linked to artificial intelligence (AI) in a comprehensive optimization strategy that has recently been put forth (Marrero-Ponce, Barigye, Jorge-Rodrguez, & Tran-Thi-Thu, 2018). Following a proper experimental procedure, chemometrics approaches are used to forecast the outcomes of the experiment using various input and output characteristics, and the coupling is deemed successful. The application of chemometrics and AI-based models in the development of HPLC methods is widely established in the technical literature. For instance, Tham and Agatonovic-Kustrin combined a genetic algorithm with an artificial neural network (ANN) to forecast the retention behavior of phenylthiocarbamyl amino acid derivatives (Tham & Agatonovic-Kustrin, 2002). According to Vasiljevi et al., HPLC method development has been used to optimize ANN for simulating the retention behavior of various analytes. According to their findings (Vasiljevi, Onjia, Okea, & Lauevi, 2004), ANN can be used to estimate retention time in an isocratic elution system for separating mixtures of complex compounds with significant changes in log Kow and pKa values. Support vector machines (SVM) and artificial neural networks (ANN) are used by Golmohammad et al. to analyze the retention times of different peptides using an HPLC system. The top models were characterized using a combination of Genetic Algorithm and Partial Least Squares (GA-PLS) optimization techniques. The findings demonstrated that SVM performed better than all other models in all of the data sets utilized in the study and increased the accuracy of prediction performance (Golmohammadi, Dashtbozorgi, & Vander Heyden, 2015). The application of the heuristic method (HM) and SVM in the creation and evaluation of linear and non-linear models for simulating retention time and molecular predictors of various volatile organic molecules was also covered by Luan et al. The results show that, in terms of mean squared error for the chromatographic prediction of retention index, the non-linear model SVM is superior to the linear model HM (Liu et al., 2019). Shadrin et al. more recently presented their research comparing the uses of ANN and SVM for the prediction of environmental pollutants. The performance standards of the models were validated using the RMSE and the goodness of fit R2. The outcomes demonstrated that both non-linear models can reproduce the physical and biological effects of the samples (Shadrin, Pukalchik, Kovaleva, & Fedorov, 2020).

The use of artificial intelligence (AI)-based models in chemometrics design and chemical process modeling is superior and shows promise, according to prior research. Non-linear models, however, may have several problems, including overfitting, local minima, and generalizability, to mention a few. Numerous scientists claim that no one model is the best in terms of performance indices in the same or different data sets. Finding broadly applicable AI-based methods that may be used at numerous local scales is therefore essential. This study is the first in the technical literature to use the HPLC-DAD technique to measure and analyze the TQ content of black cumin from various geographical regions, including India, Syria, Turkey, Iraq, and Saudi Arabia. This study is also unique in that it is the first to compare the TQ content of black cumin from these regions to that of Saudi Arabia. Second, to the best of the authors' knowledge, this is the first work to show how these ensemble techniques may be used for TQ simulation using the HPLC-DAD technique.

In this study, the HPLC-DAD technique was used to simulate both the qualitative and quantitative properties of TQ using five different single models: principal component regression (PCR), least square-support vector machine (LSSVM), least square boost (LSQ-BOOST), adaptive neuro-fuzzy inference system (ANFIS), and step-wise linear regression (SWLR). Then, four innovative ensemble machine learning techniques—simple average ensemble (SAE), weighted average ensemble (WAE), neural network ensemble (NNE), and adaptive neuro-fuzzy inference system ensemble—were used to enhance the single models' predictive power (ANFIS-E). The mobile phase, which uses an isocratic elution system made up of de-ionized (D.I) water, methanol, and 2-propanol (Mp-A: Mp-B: Mp-C), as well as the concentration of the aqueous standard and flow rate, are thus the independent variables. Retention time (tR), one of the analyte's qualitative chromatographic qualities, and peak area (PA), one of the bioactive compound's quantitative chromatographic properties, are thought of as the study's outcome variables.

This study aims to investigate several models for TQ prediction and to compare and contrast the non-linear AI-based models with the conventional linear model. Additionally, to compare and contrast the ensemble models' performances and illustrate how they improve and boost the performance effectiveness of the individual models.

MATERIALS AND METHODS

Materials

The de-ionized water, methanol, isopropanol, ethanol, and TQ standard utilized in this work were all HPLC-grade chemicals that were purchased from Sigma Aldrich.

Instrumentation

An Agilent Technologies 1200 series HPLC instrument with a diode array detector (DAD) was used for the analysis. An Eclipse XDB-C18 (150 mm 4.6 mm, 5 m) reversed phase column was used to calculate the analyte TQ. For the mobile phase, an isocratic elution system made of de-ionized (D.I) water, methanol, and 2-propanol (Mp-A, Mp-B, and Mp-C) is used. The ideal flow rate was discovered to be 0.9 mL min-1 with an injection volume of 20 mL. 254 nm was chosen as the analytical wavelength and 16 minutes was chosen as the analysis time. The determination of the analyte was done by comparing the retention time of the pure standard with that of the real samples.

Sample preparation

The black cumin seeds were gathered from six distinct geographic areas: India, Syria, Turkey, Saudi Arabia, and Iraq. They were given as gifts by undergraduate students who returned to class in the winter following the summer break. All products are packaged and include the farm information on which they are produced. Particular attention was paid to ensuring that the samples were grown in the countries where they were taken. The seeds were further dried, grounded, powdered, and weighed. The material was then extracted with 100 mL of methanol and agitated for 2 hours using a magnetic stirrer. A rotary evaporator was used to evaporate the obtained extract. After that, the residue was diluted with ethanol and filtered through a solid phase extraction (SPE) cartridge (C8) in preparation for HPLC analysis (Isik, Kartal, & Erdem, 2017).

HPLC Quantification

TMQ solutions with ten distinct concentrations between 1 and 1000 ppm were generated for the quantitative analysis, and the peak areas of these concentrations were used for calibration.

Chemometrics and Models Conceptualization

Chemometrics are applied in two separate circumstances in this study. First, five alternative AI-based models, including SWLR, PCR, LSSVM, ANFIS, and LSQ-Boost employing various independent variables, were used to simulate the chromatographic behavior of TQ informing of retention duration and peak area. Second, SAE, WAE, NNE, and ANFIS-E are informed by the application of ensemble machine learning to enhance the performance of AI-based models.

Using a variety of concentrations of the analyte's standard solution, flow rate, and a mobile phase made up of de-ionized (D.I.) water, methanol, and 2-propanol, the chromatographic behavior of TQ in terms of retention time and peak area was modeled.

Phase 1: Data Acquirement

The experimental studies, which were based on the calibration of a standard TQ solution, produced the entire data set. Additionally, the data points were split into two groups: 30% for the testing stage and 70% for the calibration stage. Following data validation, potential modeling issues like overfitting and underfitting were checked and controlled (Abba et al., 2020).

Phase 2: Data normalization, statistical analysis, and correlation

Based on equation 1, the input and output factors utilized in this investigation were both standardized into a range of 0 to 1. Before modeling, normalization lowers data redundancy and decreases significant numerical errors, which is one of its main benefits.

$$y = \left(\left(\frac{x - x_{min}}{x_{max} - x_{min}}\right)\right) \tag{1}$$

Excel 2016 was used for the statistical analysis and correlation, and a 95% confidence level was used.

Phase 3: Simulation using single models

MATLAB 9.3 was used to run the individual AI-based models (PCR, LSSVM, ANFIS, and LSQ-Boost) as well as the conventional linear regression SWLR (R2020a).

Phase 4: Ensemble machine learning techniques

To increase the performance effectiveness of the individual models, ensemble machine learning approaches (PCR, LSSVM, ANFIS, LSQ-Boost, and SWLR) assemble, add, and integrate both linear and non-linear models.

Phase 5: Performance evaluation metrics

The performance evaluation parameters for any type of data-driven technique are examined using a variety of criteria by contrasting experimental and simulated values. Four separate performance evaluation criteria were used to compare the performance of the individual models and the ensemble machine-learning approaches established in this work during the calibration and verification phases.

Equations 2 through 5 are used to calculate the mean square error (MSE), root mean square error (RMSE), correlation coefficient (CC), and Nash-Sutcliffe efficiency (NSE), respectively.

$$MSE = \frac{1}{N}\Sigma_{i=1}^{N}\left(Y_{obsi} - Y_{comi}\right)^2 \tag{2}$$

$$RMSE = \sqrt{\frac{\Sigma_{i=1}^{N}\left(Y_{obsi} - Y_{comi}\right)^2}{N}} \tag{3}$$

$$CC = \frac{\Sigma_{i=1}^{N}\left(Y_{obs} - \overline{Y}_{obs}\right)\left(Y_{com} - \overline{Y}_{com}\right)}{\sqrt{\Sigma_{i=1}^{N}\left(Y_{obs} - \overline{Y}_{obs}\right)^{2}\Sigma_{i=1}^{N}\left(Y_{com} - Y_{com}\right)^{2}}} \quad (4)$$

$$NSE = 1 - \frac{\Sigma_{j=1}^{N}\left[(Y)_{obs,j} - (Y)_{com,j}\right]^{2}}{\Sigma_{j=1}^{N}\left[(Y)_{obs,j} - \overline{(Y)}_{obs,j}\right]^{2}} \quad (5)$$

Table 1. The amount of thymoquinone in Nigella sativa seed extracts from different regions

Sample Name	Concentration (ppm)	% Thymoquinone amount
Iraq	31.26 ± 0.071	0.031
India	30.35 ± 0.167	0.030
Saudi Arabia	22.15 ± 0.165	0.022
Syria	5.47 ± 0.099	0.005
Turkey	1.39 ± 0.112	0.001

RESULTS

HPLC Linearity Results

By creating a calibration curve, the standard solutions of TQ were prepared between 0 and 1000 ppm concentration.

Our investigation revealed that the samples of black cumin seeds from various geographic locations with the highest concentration of TQ were those from Iraq and India (Figure 1). TQ was present in all samples, but in varying amounts; the amounts of TQ in the samples from Iraq, India, Saudi Arabia, Syria, and Turkey, respectively, were 0.031, 0.030, 0.022, 0.005, and 0.001%. The soil quality, climate changes in the places where the seeds are cultivated, and other factors are thought to be responsible for the variations in the TQ of black cumin seeds. The amount of TQ discovered in *Nigella sativa* seeds obtained from Ankara was discovered to be in the range of 0.010-0.376% due to changes in parameters like those described in a previous study by our team (Isik et al., 2017). The amount of TQ discovered in the seeds of the black cumin plant grown in Kuwait and India ranged from 1039.85 mg/kg to 2940.43 mg/kg (Herlina, Aziz, Kurniawati, & Faridah, 2017). Black cumin seeds were discovered to contain 0.06% TQ by Gholamnezhad et al. (2015) in a study looking into the immunomodulatory and cytotoxic effects of the seeds. Although the TQ range identified in the literature is consistent with the amount of TQ computed within the scope of this study, it can be argued that the amount of TQ is in a very wide range. This vast range of TQ in plants is influenced by genetic abnormalities, harvest period/season, and physiological circumstances (Zribi, Omezzine, & Haouala, 2014). To provide a standard effect and concentration of its main analyte, TQ, it is crucial to ascertain the phytochemical composition of the *Nigella sativa* plant that will be employed for therapeutic purposes.

Using the established calibration equation, the levels of thymoquinone identified in various black cumin seeds from various geographical regions are estimated and summarized in Table 1.

Performance of the chemometrics-based models

The quantitative performance effectiveness of the individual models PCR, LSSVM, ANFIS, LSQ-Boost, and SWLR is displayed in Table 2. The ANFIS model outperforms all the other single models (PCR, LSSVM, LSQ-Boost, and SWLR) in modeling both tR and PA in the training and testing stages, according to the comparative performance of these techniques (see Table 2). According to Nourani et al. 2012, any model must have a minimum Nash-Sutcliffe efficiency (NSE) of 80% to be accepted. (In other words, the model needs to have a minimum R2-value of 0.8 to be considered acceptable) (Nourani, Hakimzadeh, & Amini, 2012). Only ANFIS, according to the performance table, was able to imitate tR, which is mostly attributable to its intricate capacity to model highly non-linear data. This is consistent with the findings of our earlier investigations (Usman, Işik, & Abba, 2021; Abdullahi Garba Usman, Işik, Abba, & Meriçli, 2021a ; and Abdullahi Garba Usman, Isik, Abba, & Mericli, 2021b). In contrast, only SWLR, LSSVM, and ANFIS were able to meet the minimal 80% threshold for a model to be acceptable when it came to the simulation of PA. This demonstrated the necessity for additional methods, such as ensemble machine learning, to improve the performance of the individual models.

The effectiveness of the single models (PCR, LSSVM, ANFIS, LSQ-Boost, and SWLR)

The scatter plot of the comparative performance of the models is illustrated in Figure 2.

According to a comparison of their performances, the four ensemble machine learning techniques can simulate the chromatographic properties of TQ, PA, and tR with minimum and maximum NSE-values of 0.842 and 0.999 in the training phase and 0.918 and 0.999 in the testing phase, respectively. Comparing the approaches to the individual models, they both demonstrated lower error values in terms of RMSE and MSE.

Performance of the Ensemble machine learning techniques

Table 3 provides a summary of the data from ensemble machine learning. To better understand the ensemble machine learning algorithms' exploratory performance for the prediction of tR and PA, scatter plots are used (see Figure 3).

A new graphic design tool dubbed the "Taylor diagram" was used to demonstrate the performance capabilities of the ensemble machine learning technique. Due to its importance, this diagram has been applied to numerous modeling fields, including computer science, computer vision, and climate modeling.

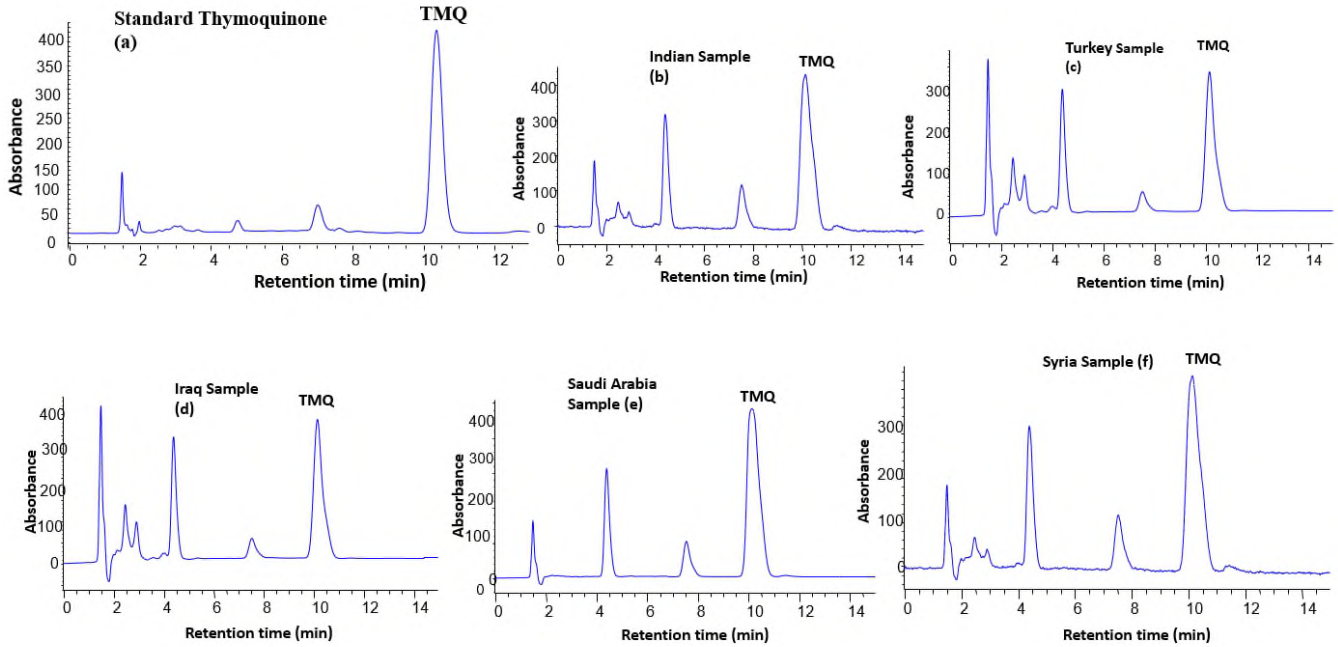

Figure 1. The chromatogram of the a) standard TQ analyte, b) Indian c) Turkey (d) Iraq sample e)Saudi Arabia, and f) Syrian sample

Table 2. Performance table of the single models (PCR, LSSVM, ANFIS, LSQ-Boost, and SWLR)

	raining				esting			
	S		**RMS**	**MS**	**S**		**RMS**	**MS**
S LR tR	0.362	0.601	0.192	0.037	0.220	0.469	0.004	0.000
PCR-tR	0.701	0.837	0.131	0.017	0.586	0.766	0.003	0.000
LSSVM-tR	0.173	0.416	0.218	0.048	0.016	0.128	0.005	0.000
ANFIS-tR	0.999	1.000	0.001	0.000	0.999	1.000	0.000	0.000
LS -Boost-tR	0.613	0.783	0.149	0.022	0.651	0.807	0.003	0.000
SWLR-PA	0.999	1.000	0.005	0.000	0.987	0.994	0.009	0.000
PCR-PA	0.674	0.821	0.126	0.016	0.234	0.483	0.070	0.005
LSSVM-PA	0.923	0.961	0.022	0.000	0.983	0.991	0.029	0.001
ANFIS-PA	0.999	1.000	0.000	0.000	0.999	1.000	0.005	0.000
LS -Boost-PA	0.765	0.875	0.039	0.002	0.567	0.753	0.146	0.021

The graphic is used to demonstrate the study's findings regarding the goodness of fit in terms of CC (see Figure 4).

Table 3. Performance of the Ensemble machine learning techniques (SAE, WAE, NNE, and ANFIS-E)

	Training				Testing			
	NSE	**CC**	**RMSE**	**MSE**	**NSE**	**CC**	**RMSE**	**MSE**
SAE-tr	0.842	0.917	0.087	0.009	0.843	0.918	0.002	0.000
WAE-tr	0.844	0.919	0.086	0.009	0.890	0.944	0.002	0.000
NNE-tr	0.989	0.995	0.025	0.001	0.986	0.993	0.001	0.000
ANFIS-E-tr	0.999	1.000	0.000	0.000	0.999	0.999	0.000	0.000
SAE-PA	0.998	0.999	0.010	0.000	0.987	0.993	0.025	0.001
WAE-PA	0.999	0.999	0.008	0.000	0.998	0.999	0.009	0.000
NNE-PA	0.999	1.000	0.015	0.000	0.999	1.000	0.005	0.000
ANFIS-E-PA	0.999	1.000	0.001	0.000	0.999	0.999	0.004	0.000

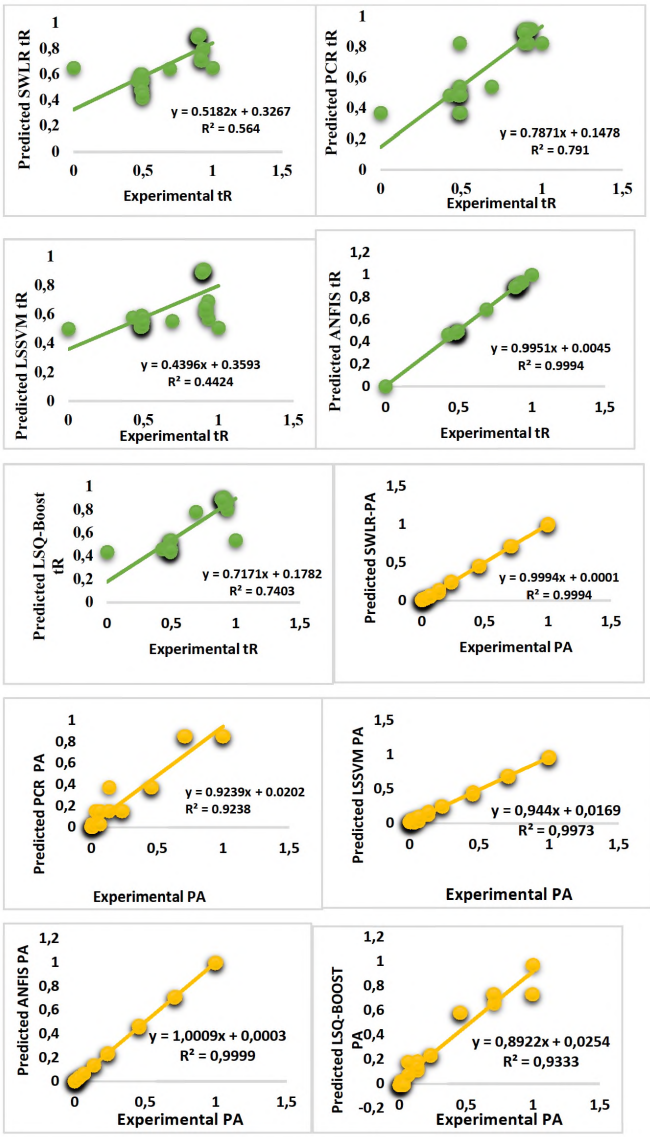

Figure 2. Scatter plots of the single models for their respective retention time (tR) and Peak area (PA).

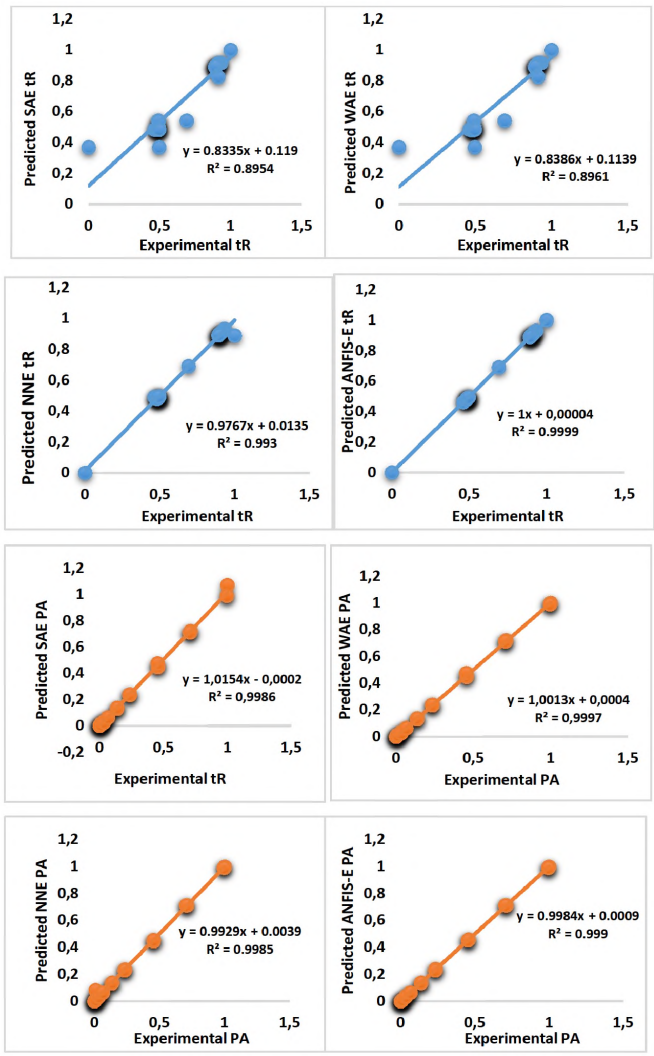

Figure 3. Scatter plots of the ensemble techniques tR and for PA

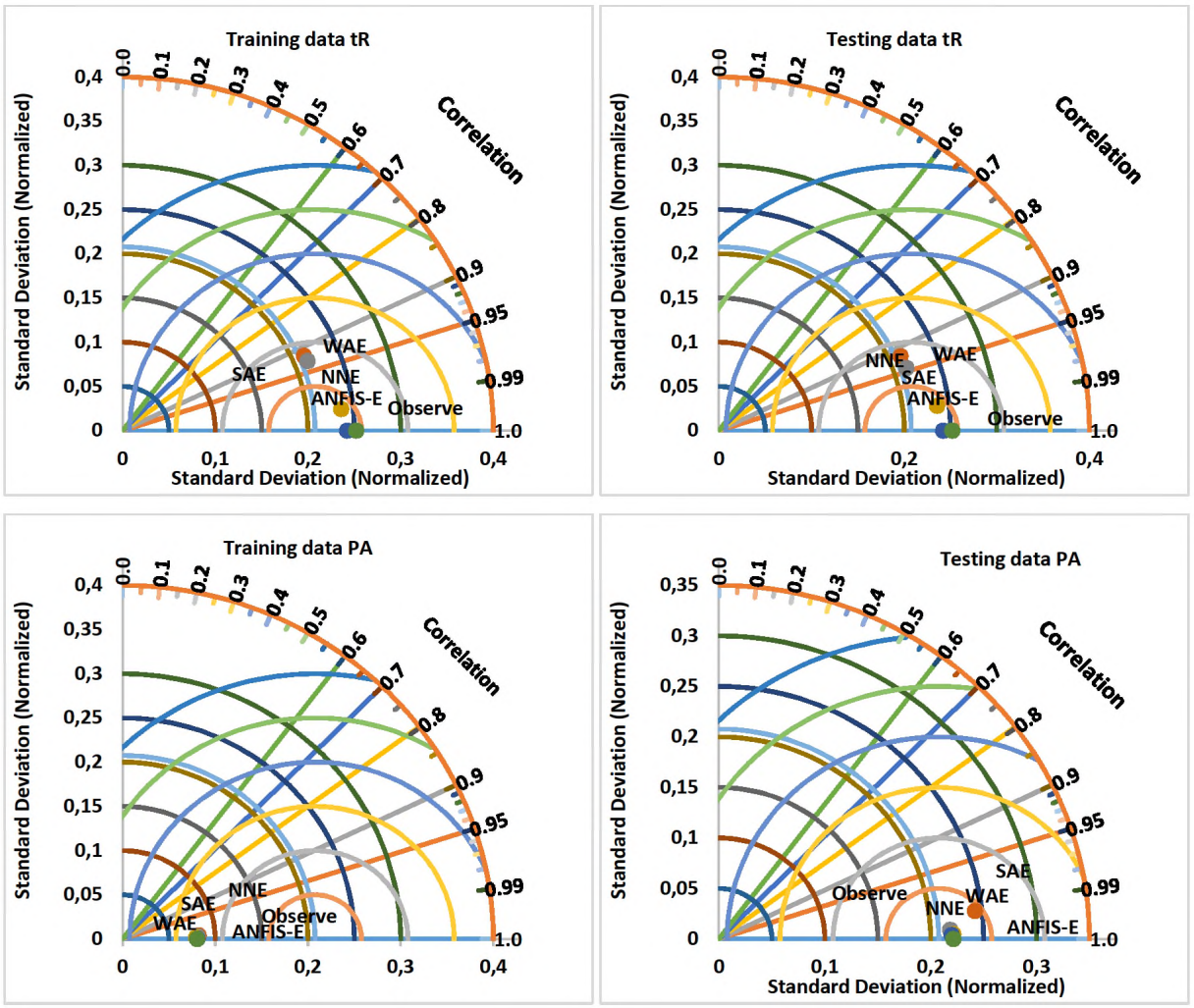

Figure 4. Taylor diagrams of the ensemble techniques tR and for PA

CONCLUSION

To measure the TQ content of each sample of black cumin collected from various geographical locations, a quantitative analysis of each sample was carried out. The overall amount of TQ contained in each sample is influenced by a variety of geographical conditions, including rainfall, seasonal changes, and soil, as mentioned in the literature. The collected results demonstrated that samples from India and Iraq have the highest concentration of TQ. TQ was present in all samples, but in varying amounts; the amounts of TQ in the samples from Iraq, India, Saudi Arabia, Syria, and Turkey, respectively, were 0.031, 0.030, 0.022, 0.005, and 0.001%.

As one of the most modern chemometrics techniques for simulating the chromatographic behavior of various analytes, the study also included the deployment of both single models and ensemble machine-learning methodologies. The chemometrics results show that these models are capable of simulating both the qualitative and quantitative features of TQ.

In addition, additional metaheuristic methods can be employed to simulate the chromatographic characteristics of TQ, such as particle swam optimizations (PSO) and Harris Hawks optimization techniques (HHO).

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Original Article

Analysis of selected steroid hormones in sea of Marmara sediment samples by LC-ESI/MS-MS

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ABSTRACT

Background and Aims: Sediment is the general name given to the muddy structure located at the bottom of aquatic environments such as the sea. In our study, the amounts of steroid hormones were investigated in the sediment samples taken from the Marmara Sea. According to other studies, it has been determined that the excess of the hormone load in the sediments may be an indicator of human/animal sourced pollution, as well as the negative effects of the hormones mixed in the seas with the ecological cycle on the health of humans and animals.

Methods: In our study, 31 selected human/animal, plant, natural and synthetic hormone-steroids were studied using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI/MS-MS). Methanol and QuEChERS were used as extraction methods. Sediment samples were taken from a total of 27 points selected for sampling at the Marmara Sea.

Results: According to the results we found, the androgens: androsterone (24.50-1718.18 ng g^{-1}), testosterone (86.30-1600.32 ng g^{-1}); the estrogens: mestranol (33.73-228.32 ng g^{-1}), equilin (53.44-1232.53 ng g^{-1}); the progestagens; pregnenolone (37.50-374.76 ng g^{-1}), progesterone (39.96-405.60 ng g^{-1}); levonorgestrel (325.25 and 937.93 ng $g^{-}1$); the fecal sterols: cholestanone (57.57-1726.32 ng g^{-1}), coprostanol + epicoprostanol (51.43-1370.33 ng g^{-1}); and the plant sterol; campesterol (35.30-1859.90 ng g^{-1}) were the compounds detected.

Conclusion: Estrogens and progestogens are active components of birth control pills, and cholestanone and coprostanol + epicoprostanol are steroids that are indicative of human/animal pollution. Coprostanol + epicoprostanol and cholestanone, which are indicators of fecal pollution, were detected in all sediment samples. In our study, steroid hormones were detected for the first time in Sea of Marmara sediments and possible environmental risks were evaluated.

Keywords: Marmara Sea, sediment, LC-ESI/MS-MS, steroids, fecal sterols

INTRODUCTION

The Sea of Marmara is a channel between the Black Sea and the Mediterranean, along with the Bosphorus and Dardanelles Straits. The polluting materials are fed into the Sea of Marmara via water by a surface current from the Black Sea and a deep current from the Mediterranean (Kut, Topcuoglu, Esen, Küçükcezzar, & Güven, 2000). The Sea of Marmara forms a link between two large semi-enclosed basins, the Mediterranean and the Black Sea (Erel, 1992). The coastal area of the Sea of Marmara contains 87% of Turkey's entire coastal settlement population (Erel, 1997). Increasing industrial and domestic activities in the Marmara Region mainly affect the coastal and shelf areas of the Marmara Sea. The northern part of the Sea of Marmara is subject to increased human interventions compared to the southern part in the form of industrial (metal, medicine, food, chemical, textile) waste disposal, fishing, dredging, recreation, and port activities. It receives pollution not only from a variety of local land-related sources but also from the densely populated and industrialized Istanbul Metropolitan and maritime transport. Istanbul is the metropolitan region with the densest population and the highest industrialization rate in Turkey. It covers about 15% of Turkey's total population and 40% of its industrial activity (Orhon, Uslu, Meriç, Salihoğlu, & Filibeli 1994). For this reason, it makes the biggest contribution to various pollutions in the Sea of Marmara. In addition to industrial and domestic waste from Istanbul Metropolitan, dissolved and particulate impurities from the Danube are transported to the Bosphorus by coastal currents (Sur, Özsoy, & Ünlüata, 1994; Tuğrul & Polat, 1995). In the coastal areas of densely populated big cities, the anthropogenic component of the sediments predominates. Surface sediments become a source of nourishment

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for biological life, a transport agent for pollutants, and a sink for organic and inorganic sediments. At the same time, the Sea of Marmara is exposed to a very high level of pollution due to the spillage of not only domestic but also industrial wastes (Topçuoğlu, Kırbaşoğlu, & Yılmaz, 2004).

Nowadays, one of the most common environmental issues is water quality (Zhang & Chen 2014). Sediment analysis is also used to understand water quality and detect water pollution. Sediments play an important role in the fate of xenobiotics in aquatic environments. They reflect the existing water system and are used to detect the presence of insoluble contaminants after mixing with surface waters (Chapman, Wang, Janssen, Persoone, & Allen, 1998). One of the substance groups that cause the most pollution in the aquatic environment and are analyzed in sediments is endocrine-disrupting compounds (EDC). At the same time, EDCs are among the most important substances affecting the quality of water. Natural and synthetic hormones (estrogens, progestogens and androgens), phytosterols and some industrial chemical compounds form a group of pollutants called endocrine disruptors. The presence of EDCs in the environment poses a pollution threat due to their effects on ecology and human health (Gutendorf & Westendorf, 2001). Steroid hormones and sterols can cause pollution that affects not only aquatic organisms but also the entire ecosystem and humans through the food chain. Chemical compounds of anthropogenic origin are important factors of contamination in both water and sediments. This causes a potential ecotoxicological risk (Vargas et al., 2001).

Steroid hormones have lipophilic properties. Therefore, they tend to accumulate in solid formations such as sediment (Praveena, Kwan, & Aris, 2012). Aquatic steroids have become a public issue in recent years (Ying, Kookana, & Ru, 2002). Steroids have endocrine-disrupting effects on aquatic organisms, such as adversely affecting fertility, feminization and hermaphroditism, even at low concentrations (1 ng L-1) in target tissues (Fick, Lindberg, Tysklind, & Larsson 2010; Mills & Chichester, 2005; Zeilinger et al., 2009). In one study, it was shown that the presence of ethinylestradiol (5ng L-1) in water seriously affects the reproductive ability of zebrafish (Ryan & Vandenbergh, 2006). Natural and synthetic steroids have been widely detected in a variety of environmental matrices, including surface and groundwater, soil, and sediments (Bradley et al., 2009; Chang, Wan, & Hu, 2009; Liu et al., 2012).

Steroid hormones can be divided into five subgroups depending on their structural features: estrogens, androgens, progestagens, glucocorticoids and mineralocorticoids (Refsdal, 2000). Estrogens and progestogens are widely used as contraceptives and drugs due to their protective properties against various diseases. They are applied in hormone replacement therapy to be used in the treatment of hormonal disorders (Álvarez Sánchez, Capote, Jiménez, & Luque de Castro, 2008; Flor, Lucangioli, Contin, & Tripodi, 2010). Estrogens are primarily used as growth promoters and enhancers in contraception, management of menopausal and postmenopausal syndrome, physiological replacement, and the treatment of prostate cancer (Cleve et al., 2012). For this reason, it is also detected in treated sewage wastewater (Chang & Huang, 2010). Testosterone, androsterone, and many analogs of dihydrotestosterone are used as therapeutic and anabolic agents that promote muscle growth; however, they can cause growth retardation and precocious puberty in children (Lastair, Ood, Arrie, Agatell, & Remner, 1996). Androgens such as testosterone and trenbolone acetate are often preferred in cattle breeding to accelerate growth (Galbraith, 2002). Androgens are thought to be responsible for the masculinization of fish found in rivers where waste from paper mills is dumped (Drysdale & Bortone, 1989; Bortone & Cody, 1999). In studies of androgens, female mosquitofish's anal fin morphometrics modify with androstenedione (Jenkins et al., 2001).

Phytosterols are naturally found in oils, grains, vegetables, and fruits (Froehner, MacEno, & Martins, 2010). They are widely used in the human diet due to their hypocholesterolemic properties, so they have protective properties for cardiovascular diseases (Miettinen, Strandberg, & Gylling, 2000; Sullivan, Brooks, Tindale, Chapman, & Ahmed, 2010; Furtula et al., 2012). In addition, in vitro analyses have shown that phytosterol-rich macroalgae extracts have anti-inflammatory, antibacterial, antifungal, antiulcerative, and antitumor properties (Lopes, Sousa, Valentão, & Andrade, 2013). Furthermore, wastewater from the paper industry often contains high concentrations of phytosterols. One of these plant sterols, β-sitosterol, is considered to be one of the causes of reproductive dysfunction in fish (Maclatchy, Peters, Nickle, & Van Der Kraak, 1997; Orrego, Guchardi, Krause, & Holdway, 2010).

Corticosteroids are divided into glucocorticoids and mineralocorticoids. Drugs in both these corticosteroid groups are used in humans because they reduce inflammation, and suppress allergic reactions and immune system activity (Charman & Williams, 2003).

Fecal sterols, such as coprostanol and epicoprostanol are biomarkers of pollution of coastal areas and urban centers in temperate and tropical regions and result from the anaerobic microbial conversion of cholesterol in the gut of humans and animals (Martins, Fillmann, & Montone, 2007; Bull, Lockheart, Elhmmali, Roberts, & Evershed, 2002). Studies of cholesterol and its metabolites in human feces show cholesterol accounts for approximately 20% of the neutral sterol concentration in feces, coprostanol 65%, coprostanone 10%, and cholestanol + cholestanone + epicoprostanol approximately 5% (Jing, Grebenok, & Behmer, 2013).

A meticulous extraction technique followed by sensitive and selective analysis is required to understand the effect of steroids in the water-sediment system (Sadílek et al., 2016). Analysis of steroid hormones and sterols in sediment samples is usu-

ally performed by gas chromatography (GC-MS) tandem mass spectrometry (Biache & Philp, 2013; Sojinu, Sonibare, Ekundayo, & Zeng, 2012; Pisani et al., 2013; Birk, Dippold, Wiesenberg, & Glaser, 2012). However, very few papers are available using the LC-MS method. These studies were also carried out in river sediments (Matić, Grujić, Jauković, & Laušević, 2014, Matić Bujagić, Grujić, Jauković, & Laušević, 2016). There is also a study on the Golden Horn (Sea of Marmara, Turkey) Estuary sediment (Aydoğan & Yurdun, 2021).

Although up to 90% of solids are removed in wastewater treatment plants, some chemical compounds such as nitrogen, phosphorus, lead, EDCs, and steroid hormones, which are hydrophobic and resistant to biodegradation, are also found in effluent because they accumulate on small particles (Gutendorf & Westendorf, 2001; Dartan et al., 2022). The fact that steroid hormones found in wastewater treatment plant effluent waters are also found in drinking water inlet waters at the same rate indicates the necessity of advanced technology treatment systems for these endocrine disruptors (Yarahmadi et al., 2018).

The aim of this study is to analyze 31 selected human, animal and plant sterols and hormones in marine sediment samples using LC-MS/MS with the electrospray ionization technique.

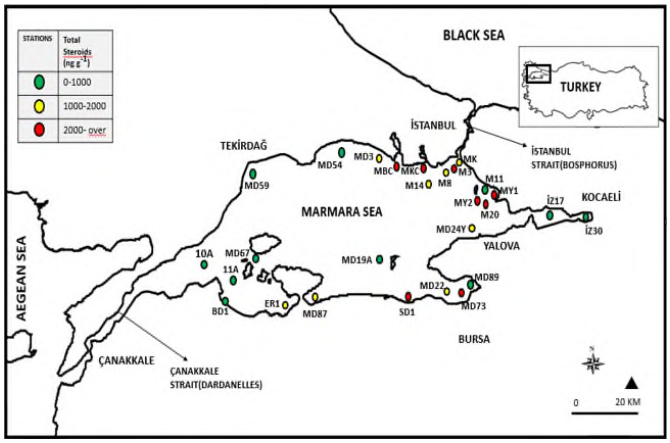

Figure 1. Sampling points in the Marmara Sea and steroid concentrations in sediment samples (ng g^{-1} dw)

MATERIALS AND METHODS

Chemicals and reagents

Depending on the frequency of use and detection in environmental samples, the hormones to be analyzed in the sediments were determined. In this study, a total of 31 steroid hormones and sterols were selected. Human and animal sterols: 17α– ethinylestradiol (Dr. Ehrenstorfer GmbH), estriol (Dr. Ehrenstorfer GmbH), estrone (Dr. Ehrenstorfer GmbH), levonorgestrel (Dr. Ehrenstorfer GmbH), mestranol (Cayman Chemical Company), norethindrone, equilin (Dr. Ehrenstorfer GmbH), 11-deoxycorticosterone, 11-deoxycortisol, 17α-OH-progesterone, 4-androstenedione, 17α-pregnenolone, aldosterone, androsterone, corticosterone, cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAs), dihydrotestosterone, 17β-estradiol, pregnenolone, progesterone, testosterone, cholesterol (Cayman Chemical Company), 5α-cholestan-3-one (cholestanone) (Alfa Aesar), 5β-cholestan-3β-ol (coprostanol) (Sigma-Aldrich), 5β-cholestan-3α-ol (epicoprostanol) (Sigma-Aldrich), 5α-cholestan-3β-ol (β-cholestanol) (Alfa Aesar); Plant sterols: desmosterol (Cayman Chemical Company), campesterol (Cayman Chemical Company), stigmasterol (Supelco). Standard materials, whose company names are not given, were included in a kit (JSM-CL-6500, Sem Laboratory Equipment Marketing Industry and Trade Inc., Turkey). HPLC grade methanol from Riedel-de-Haen and formic acid from Lachema cat.nr. 30587 (Czech Republic) were obtained.

The QuEChERS extract tubes were provided by Agilent Technologies (Massy, France). The extraction kit (QuEChERS extract salt packet 5982-6755 AOAC method, 2007) contained 6 g magnesium sulfate and 1.5 g sodium acetate. The clean-up kit (dispersive SPE 5982-5158 15 mL fatty samples AOAC) contained 1.2 g magnesium sulfate, 400 mg PSA, and 400 mg c18E. The steroid hormone and sterols' standard solutions were prepared at 1 mg mL^{-1} and 100 µg mL^{-1}. Methanol was used as a dilution solvent to prepare working standards and they were diluted to 1 µg mL^{-1} by mixing the appropriate amounts of the standard solutions. All samples were stored at -20 °C. The standard curves of the steroid hormones and sterols were linear in concentration ranges of 50, 100, 250 and 500 ng mL^{-1}.

Sample collection

Figure 1 shows the sampling points in the Sea of Marmara. Marmara Sea sediment samples were obtained from the Istanbul University Institute of Marine Sciences and Management.

Sample extraction

In our study, two different extraction methods were tried. The first is the extraction method with Methanol, the second is the extraction method with QuEChERS. Additionally, in our study, internal standards (IS) with deuterium were used to eliminate the Matrix effect.

Methanol extraction

Sample extraction was performed using 1 g of dry sediment samples. Five mL of methanol with 0.1% formic acid was added to the sediment, vortexed (LMS VTX-3000L 20W Harmony Mixer Uzusio) for 1 min. and sonicated (Elma Ultrasonic LC30) for 10 min. It was then centrifuged (Hettich Zentrifugen D-78532 Tuttlingen) at 2,000 x g for 10 min. The supernatant

was transferred to a conical glass tube. This process was repeated 2 more times and repeated three times in total and the extracts were combined. The clear solution collected in the glass tube was evaporated just to dryness in a 40 °C heater (Stuart SBH130D) under a gentle stream of nitrogen. The dry residues were dissolved using 50 µL acetone and vortexed for 1 min. Then, 950 µL of methanol was added and vortexed for 1 min again. It was centrifuged for 10 min at 2,000 x g. Then, the supernatant was transferred to the vial for injection into the LC-ESI-MS/MS.

QuEChERS extraction

The QuEChERS extraction method was performed according to the Phenomenex Applications note (TN-0096) (Estil et al.2016). In the QuEChERS extraction method, 1 g of dried sediment sample was taken, 10 mL of deionized water was placed on it and vortexed for 1 min. Then, 10 mL of 1% Acetic acid in Acetonitrile was added and vortexed for 1 min. Three point five grams of QuEChERS 5982-0755 salt was weighed and added to the falcon tube and vortexed again for 1 min. Since this extraction method was applied with 2 g of sediment, QuEChERS 5982-0755 salt was taken as 3.5 g instead of 7 g. After vortexing, it was centrifuged at 4,000 rpm for 5 minutes. It was left at -20 °C for 1 night. Approximately 9 mL of supernatant was taken from each sample and added to the ready QuEChERS 5982-5158 tube, vortexed for 1 min. It was then centrifuged at 3000 rpm for 10 minutes. Approximately 5 mL of supernatant was taken and placed in a tube, and the solvent was evaporated until dry under nitrogen flow at 35 °C using a hot heater. Then, 50 µL of acetone was added and vortexed for 1 min. Finally, 950 µL of methanol: water (1:1) was added, and vortexed for 1 min. It was centrifuged at 4,000 rpm for 10 min. After centrifugation, the supernatants were transferred to clean tubes, centrifuged again with Quickspin, transferred to vials and made ready for the LC-MS/MS analysis.

LC-ESI-MS/MS analysis

This method was made according to the work of Aydoğan & Yurdun, 2021. Analyses of sediments were performed on the Agilent Infinity 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer settings of the kit (Sem Laboratory Equipment Marketing Industry and Trade Inc., Istanbul, Turkey) were as follows: drying gas flow 11 L min^{-1}, drying gas temperature 350 °C, sheath gas flow 11 L min^{-1}, sheath gas temperature 400 °C, nebulizer pressure 30 psi, capillary voltages were 5,500 and 3,000 V for positive and negative respectively with 500 V nozzle voltage for both of the polarities. Values of compound steroid mass spectrometer parameters and method performance parameters are the same as Aydoğan & Yurdun, 2021's work. Recovery results are shared in Table 2. Limits of detection (LODs) and quantification (LOQs) are shown in Table 3.

RESULTS

In the study, 27 of the Marmara Sea sediment samples were studied by methanol extraction and 21 of them were studied using QuEChERS Extraction, and both were analyzed by LC-MS/MS. The chromatogram of the steroid mix standard solutions is shown in Figure 2.

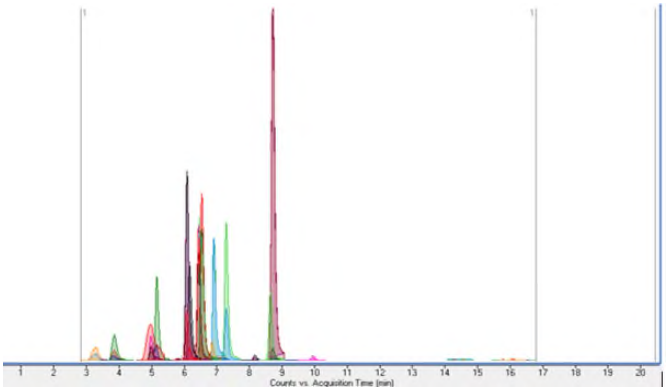

Figure 2. Chromatogram of steroid mix standards (for retention time and details, see Aydoğan & Yurdun, 2021).

In sediment samples taken from 27 points of the Marmara Sea, 31 thirty-one selected steroid hormones and sterols were analyzed with methanol extraction. The following compounds were detected: Androgens: androsterone (24.50-1718.18 ng g^{-1}), testosterone (86.30-1600.32 ng g^{-1}); estrogens: mestranol (33.73-228.32 ng g^{-1}), equilin (53.44-1232.53 ng g^{-1}); progestogens: pregnenolone (37.50-374.76 ng g^{-1}), progesterone (39.96-405.60 ng g^{-1}); levonorgestrel (325.25 and 937.93 ng g^{-1}); fecal sterols: cholestanone (57.57-1726.32 ng g^{-1}), coprostanol + epicoprostanol (51.43-1370.33 ng g^{-1}); plant sterol: campesterol (35.30-1859.90 ng g^{-1}). However, since cholesterol, cholestanol and stigmasterol could not be detected by ESI, analyses could not be made in the sediments. The percentage amounts of sterols in the sediments are shown in Figure 3.

The amounts of deoxycortisol, deoxycorticosterone, aldosterone, androstenedione, corticosterone, cortisol, desmosterol, DHEA, DHEAs, dihydrotestosterone, estradiol, estriol, estrone, norethindrone could not be determined because they were below the LOQ value. In the LC-MS/MS device, DHEAs was studied in the negative mode and all other steroid hormones and sterols were studied in the positive mode. The amounts of steroid hormones and sterols detected in sediment samples are shown in Table 1.

In methanol extraction, the recovery studies were prepared by adding 100 ng g^{-1} (62.5- 101.0) and 500 ng g^{-1} (58.3-

Table 1. Steroid concentrations in Marmara Sea sediment samples (extracting with QuEChERS) (ng g^{-1} dw)

Stations	holestanone	ndrosterone	uilin	Mestranol	regnenolone	rogesterone	estosterone	otal Steroids
MD89	LO	129.04	134.84	62.02	43.02	LO	30.07	398.99
MD87	LO	129.23	78.12	42.26	79.15	LO	LO	328.76
M11	78.32	111.59	LO	LO	LO	LO	502.81	692.72
MD73	106.11	504.81	87.82	LO	LO	78.41	375.99	1153.14
MD22	72.07	569.23	41.83	LO	LO	92.75	55.40	831.28
MD19A	67.13	108.72	LO	LO	LO	25.83	280.52	482.20
M	105.03	134.77	LO	LO	LO	30.56	384.15	654.51
Z-30	LO	147.80	114.77	52.51	45.66	LO	LO	360.74
MD-3	84.75	365.21	LO	LO	LO	59.81	207.43	717.20
SD1-3	LO	134.55	115.98	133.85	42.18	LO	31.13	457.69
M14	LO	89.29	87.45	48.34	66.68	LO	30.28	322.04
MD-8	120.95	113.52	LO	LO	LO	61.55	174.27	470.29
MY1	LO	102.15	414.45	84.15	845.65	LO	32.83	1479.23
MD59	LO	120.10	101.44	45.99	44.32	LO	29.52	341.37
M C-D	115.81	325.99	68.66	LO	LO	358.12	117.49	986.07
ER1	LO	86.82	85.97	48.79	96.20	LO	LO	317.78
MD67	LO	66.71	147.63	41.06	LO	LO	28.83	284.23
M3	LO	147.14	82.33	61.05	LO	LO	32.41	322.93
MY2	LO	99.61	202.07	52.32	LO	LO	29.91	383.91
MD20	86.38	136.96	65.46	LO	LO	51.91	187.43	528.14
MD72	LO	79.02	83.22	45.56	LO	LO	LO	207.80

112.4) of each standard solution to the sediment samples before extraction and they were left to dry at room temperature for one night. Then the extraction procedure was applied. For methanol extraction analyses, method performance parameters are the same as in the study by Aydoğan & Yurdun, 2021.

Recovery with QuEChERS extraction was studied by adding 100 and 500 ng g^{-1} steroids to the sediment with the above method, but the recovery results were low 31.8- 142.7 (100 ng g^{-1}), and 21.6- 155.1 (500 ng g^{-1}). Also, estriol, campesterol, DHEAs, coprostanol+epicoprostanol, desmosterol, and androsterone could not be detected (Table 3).

Results were found by using the parameters (steroids mass spectrometer parameters and method performance parameters) in our previous study on steroids (Aydoğan & Yurdun, 2021). Analyses of both studies were carried out at the same time.

DISCUSSION

In the published research, few studies have been found on the analysis of steroid hormones and steroids in the Golden Horn (Sea of Marmara, Turkey) Estuary sediment (Aydoğan & Yurdun, 2021; Lyons et al., 2015; Readman, Fillmann, Tolosa, Bartocci, & Mee, 2005; De Castro Martins, Montone, Carvalho Gamba, & Pellizari, 2005) and the studies are generally in river sediments (Chou & Liu, 2004; Matić et al., 2014; Matić Bujagić et al., 2016; Frena, Bataglion et al., 2016; Frena, Santos, et al., 2016; Hájková et al., 2007; Froehner, Martins, & Errera, 2009; López de Alda, Gil, Paz, & Barceló, 2002).

Overall, the general distribution of hormones in the Sea of Marmara sediment samples is as follows: Cholestanone> testosterone> androsterone> equilin> campesterol> coprastanol+ epicoprastanol> pregnenolone> progesterone> mestranol> levonorgestrel. The highest concentration of steroids was MY-1 (6479.34 ng g^{-1}), and the lowest concentration was 11A (310.80 ng g^{-1}).

Total steroid concentrations in the Sea of Marmara stations were determined in the range of 310.80-6479.34 ng g^{-1} (Table 1). The highest values were found at sediment sampling points MY-1 (6479.34 ng g^{-1}), M3 (4548.81 ng g^{-1}), SD1 (4273.99 ng g^{-1}), MKC-D (2368.53 ng g^{-1}), MD73 (2305.39 ng g^{-1}), M20 (2228.73 ng g^{-1}), MY2 (2051.00 ng g^{-1}) and MBC (2004.85 ng g^{-1}). Cholestanone, testosterone, androsterone, and equilin amounts were found high in the sediments. This is a strong indication that the pollution sources of Marmara Sea sediments are generally of human origin due to equilin, vegetable origin due to campesterol, and feces origin due to cholestanone (Figure 3).

Cholesterol, cholestanol, cholestanone, coprostanol, and epicoprostanol are sterols that are indicators of fecal contamina-

Table 2. Limits of detection (LODs) and quantification (LOQs) (Aydoğan& Yurdun, 2021) for QuEChERS extraction: recoveries at two concentration levels, method repeatability (relative standard deviations, RSDs)

Steroid ormones/Sterols	tracting with u h RS Reco er (RSD)		L D ($ngmL^{-1}$)	L ($ng\ mL^{-1}$)
	Spiking le el $ng\ g^{-1}$	Spiking le el ng g		
Estrogens				
Estriol	n.d.	n.d.	10.91	36.35
17- Ethin lestradiol	80.4 4.4	74.3 11.1	12.07	40.23
Estradiol	56.7 10.9	53.0 12.2	7.09	23.63
Estrone	33.1 7.7	47.8 12.4	12.23	40.77
Mestranol S nthetic	47.7 4.9	103.2 2.3	8.11	27.02
E uilin S nthetic	67.5 7.8	77.2 5.9	12.35	41.15
ndrogens				
Androstenedione	54.5 8.2	52.7 2.2	11.91	39.72
Androsterone	n.d.	n.d.	6.35	21.16
Testosterone	53.5 7.0	51.3 5.1	8.61	28.71
DHEA	74.7 14.8	43.7 7.8	9.04	30.13
DHEAs	n.d.	n.d.	4.35	14.51
Dih drotestosterone	142.7 8.5	153.6 6.8	4.67	15.55
rogestogens				
Pregnenolone	66.5 5.3	129.8 7.7	11.05	36.85
Progesterone	41.5 3.6	45.4 9.9	7.70	25.68
17- -OH- pregnenolone	31.8 4.9	41.7 12.1	7.79	25.96
17- -OH- progesterone	53.5 8.9	52.3 7.6	2.54	8.48
Levonorgestrel S nthetic	32.2 1.7	32.7 6.5	12.78	42.60
Norethindrone S nthetic	98.9 4.5	155.1 3.2	5.97	19.91
e tero s				
Coprostanol Epicoprostanol	n.d.	n.d.	9.41	31.37
Cholestanone	76.9 3.3	43.5 1.9	11.22	37.40
nt stero s				
Campesterol	n.d.	n.d.	9.80	32.66
Desmosterol	n.d.	n.d.	9.24	30.80
o ort o ds				
Deo cortisol	56.4 7.0	44.9 15.2	6.40	21.32
Cortisol	39.8 11.3	21.6 16.1	4.80	15.99
M ner o ort o ds				
Corticosterone	71.3 8.3	55.2 8.3	4.27	14.24
Deo corticosterone	50.2 5.1	51.7 2.3	11.94	39.80
Aldosterone	53.2 9.7	36.5 7.2	6.49	21.62

Table 3. Steroid concentrations in Marmara Sea sediment samples (extracting with methanol) (ng g^{-1} dw)

Stations	Cholestanone	Androsterone	Campesterol	Coprostanol Epicoprostanol	E uilin	Levonorgestrel	Mestranol	regnenolone	rogesterone	Testosterone	Total Steroids
11A	57.57	53.04	O	88.42	78.04	O	33.73	O	O	O	310.80
MD-89	308.76	184.42	51.70	132.40	61.15	O	137.11	43.32	O	O	918.85
MD-87	780.11	296.89	104.86	72.40	175.39	O	121.25	151.63	O	O	1702.52
10A	111.14	95.22	36.26	131.53	119.19	O	58.92	53.41	O	O	605.65
MBC	741.30	340.07	228.34	220.11	262.48	O	175.05	37.50	O	O	2004.85
M11	86.28	112.03	81.07	106.82	119.19	O	90.75	59.72	O	O	655.86
MD73	670.84	377.28	126.43	85.71	538.67	O	181.77	324.71	O	O	2305.39
MD22	704.10	345.07	88.48	O	165.60	O	228.32	374.76	O	O	1906.33
MD19A	119.55	85.49	O	156.67	126.01	O	63.06	191.11	O	O	741.89
MK	105.90	75.69	O	74.67	1232.53	O	210.70	197.44	O	O	1896.93
Z-30	127.29	123.80	86.50	303.67	179.76	O	O	77.57	O	O	898.59
MD-3	522.64	263.24	319.90	278.53	82.13	O	191.95	41.84	O	O	1700.23
BD1	67.79	144.89	O	O	184.73	O	85.66	136.87	O	O	619.93
MD-54	186.29	188.89	58.07	88.25	259.94	O	63.25	O	O	O	844.69
SD1	787.82	346.69	1859.90	328.25	594.91	O	109.49	246.93	O	O	4273.99
M14	175.68	162.82	O	75.28	179.19	325.25	O	O	194.06	86.30	1198.57
M8	98.33	412.65	76.16	57.74	O	O	O	O	145.38	217.32	1007.58
MY1	1726.32	1718.18	O	1370.33	990.88	O	O	O	134.09	539.56	6479.34
MD59	71.79	51.14	O	51.43	O	O	O	O	39.96	588.09	802.40
MKC-D	279.17	209.52	128.93	184.94	O	937.93	O	O	405.60	222.44	2368.53
ER-1	258.34	138.94	93.07	133.30	195.20	O	O	O	117.63	280.67	1217.16
MD-24Y	234.12	128.07	151.61	168.49	53.44	O	O	O	131.66	458.43	1325.82
M20	87.36	136.10	45.79	203.23	57.14	O	O	O	98.79	1600.32	2228.73
MD-67	128.64	24.50	O	122.68	115.12	O	O	O	54.15	494.55	939.65
M3	1055.99	252.34	1468.91	142.72	O	O	O	O	195.32	1433.54	4548.81
Z-17	74.78	152.14	O	104.89	156.81	O	O	O	114.23	334.76	937.60
MY2	194.59	56.67	35.30	118.40	O	O	O	O	124.99	1521.05	2050.10

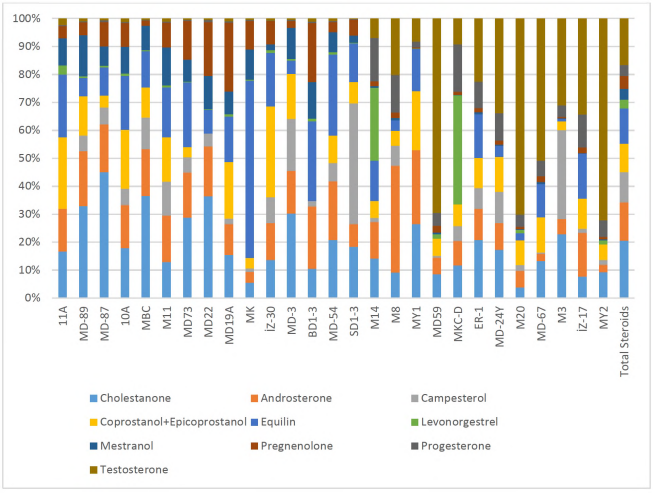

Figure 3. Distribution of steroid hormones in sediments of Sea of Marmara stations

tion. In our study, coprostanol and epicoprostanol results were given together because they could not be differentiated with the LC-ESI-MS/MS detector. Coprostanol + epicoprostanol 51.43-1370.33 ng g^{-1} and cholestanone 57.57-1726.32 ng g^{-1} were detected in sediment samples. Coprostanol + epicoprostanol concentrations in the sediment were found to be considerably higher in other studies (except Aydoğan &Yurdun, 2021's and Sojinu et al., 2012's) compared to our study. Results found by other researchers are as follows: 174- 4170 ng g^{-1} (Matić et al., 2014); 6.5- 1555 ng g^{-1}(Martins et al., 2007); 10- 2350 ng g-1 (Martins et al., 2011); 8.03- 465.54 ng g^{-1} (Sojinu et al., 2012); 34.37- 2603 ng g^{-1} (Lyons et al., 2015); and 42.82- 103.26 ng g^{-1} (Aydoğan &Yurdun,2021). Additionally, the cholestanone concentrations in our study (57.57-1726.32 ng g^{-1}) are higher than all of the other studies: Patos Lagoon sediments 6.9-172.2 ng g^{-1} (Martins et al., 2007), Danube River 79 and 899 ng g^{-1}(Matić et al., 2014), Niger Delta 2.55-771.58 ng g^{-1}(Sojinu et al., 2012) and the Golden Horn Estuary 157.57-1163.07 ng g^{-1} (Aydoğan &Yurdun, 2021). Also, the highest levels of cholesterol (37-16000 ng g^{-1}), coprostanol (12-440 ng g^{-1}) and cholestanol (37-1900 ng g^{-1}) were detected in sediment samples from the Bosphorus (Readman et al., 2005). Some authors emphasized that coprostanol levels between 10-100 ng g^{-1} are an indicator of uncontaminated environments, values greater than 100 ng g^{-1} are an indicator of sewage pollution in determining the pollution levels in the sediment, and they stated that 500 ng g^{-1} indicates meaningful sewage pollution. (Gonzalez-Oreja & Saiz-Salinas, 1998; Lyons et al., 2015; Tolosa, Mesa, & Alonso- Hernandez, 2014). In our study, the amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points (İZ17, 104.89 ng g^{-1}; M11, 106.82 ng g^{-1}; MY2, 118.40 ng g^{-1}; MD67, 122.68 ng g^{-1}; 10A, 131.53 ng g^{-1}; MD89, 132.40 ng g^{-1}; ER1, 133.30 ng g^{-1}; M3, 142.72 ng g^{-1}; MD19A, 156.67 ng g^{-1}; MD-24Y, 168.49 ng g^{-1}; MKC-D, 184.94 ng g-1; M20, 203.23 ng g^{-1}; MBC, 220.11 ng g^{-1}; MD3, 278.53 ng g-1; İZ30, 303.67 ng gCholesterol, cholestanol, cholestanone, coprostanol, and epicoprostanol are sterols that are indicators of fecal contamination. In our study, coprostanol and epicoprostanol results were given together be-

cause they could not be differentiated with the LC-ESI-MS/MS detector. Coprostanol + epicoprostanol 51.43- 1370.33 ng g^{-1} and cholestanone 57.57-1726.32 ng g^{-1} were detected in sediment samples. Coprostanol + epicoprostanol concentrations in the sediment were found to be considerably higher in other studies (except Aydoğan &Yurdun, 2021's and Sojinu et al., 2012's) compared to our study. Results found by other researchers are as follows: 174- 4170 ng g^{-1} (Matić et al., 2014); 6.5- 1555 ng g^{-1}(Martins et al., 2007); 10- 2350 ng g-1 (Martins et al., 2011); 8.03- 465.54 ng g^{-1} (Sojinu et al., 2012); 34.37- 2603 ng g^{-1} (Lyons et al., 2015); and 42.82- 103.26 ng g^{-1} (Aydoğan &Yurdun,2021). Additionally, the cholestanone concentrations in our study (57.57-1726.32 ng g^{-1}) are higher than all of the other studies: Patos Lagoon sediments 6.9-172.2 ng g^{-1} (Martins et al., 2007), Danube River 79 and 899 ng g^{-1}(Matić et al., 2014), Niger Delta 2.55-771.58 ng g^{-1}(Sojinu et al., 2012) and the Golden Horn Estuary 157.57-1163.07 ng g^{-1} (Aydoğan &Yurdun, 2021). Also, the highest levels of cholesterol (37-16000 ng g^{-1}), coprostanol (12-440 ng g^{-1}) and cholestanol (37-1900 ng g^{-1}) were detected in sediment samples from the Bosphorus (Readman et al., 2005). Some authors emphasized that coprostanol levels between 10-100 ng g^{-1} are an indicator of uncontaminated environments, values greater than 100 ng g^{-1} are an indicator of sewage pollution in determining the pollution levels in the sediment, and they stated that 500 ng g^{-1} indicates meaningful sewage pollution. (Gonzalez-Oreja & Saiz-Salinas, 1998; Lyons et al., 2015; Tolosa, Mesa, & Alonso- Hernandez, 2014). In our study, the amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points (İZ17, 104.89 ng g^{-1}; M11, 106.82 ng g^{-1}; MY2, 118.40 ng g^{-1}; MD67, 122.68 ng g^{-1}; 10A, 131.53 ng g^{-1}; MD89, 132.40 ng g^{-1}; ER1, 133.30 ng g^{-1}; M3, 142.72 ng g^{-1}; MD19A, 156.67 ng g^{-1}; MD-24Y, 168.49 ng g^{-1}; MKC-D, 184.94 ng g-1; M20, 203.23 ng g^{-1}; MBC, 220.11 ng g^{-1}; MD3, 278.53 ng g-1; İZ30, 303.67 ng g^{-1}1; SD1-3, 328.25 ng g^{-1}; MY1, 1370.33 ng g^{-1}1) and above 500 ng g^{-1} at only one point (MY1, 1370.33 ng g-1). According to these results, we can think that pollution is starting at the 16 points mentioned. At the highest point (MY1), maybe we can say that there is pollution.; SD1-3, 328.25 ng g^{-1}; MY1, 1370.33 ng g^{-1}1) and above 500 ng g^{-1} at only one point (MY1, 1370.33 ng g^{-1}). According to these results, we can think that pollution is starting at the 16 points mentioned. At the highest point (MY1), maybe we can say that there is pollution.

Another finding was that amounts of pregnenolone (37.50-374.76 ng g^{-1}) were found in all sediments. As far as we have researched, only one study (Aydoğan & Yurdun, 2021) of pregnenolone analysis has been conducted in sediment samples, and the result was 44.19-418.00 ng g^{-1}. For the first time in marine sediment research, sediment analysis was performed with this study and the result was obtained. Pregnenolone is the main steroid from which all other steroid hormones are formed. Pregnenolone is consideredto be a strong indicator of human-induced pollution because it is used as the main metabolite of cholesterol and cholesterol to pregnenolone conversion with cytochrome P-450 side chain cleavage enzyme, and therefore, it is recommended to perform pregnenolone analysis in sediment in similar studies to be carried out from now on. Since an ESI ion source is used in our study but an APCI ion source is required for cholesterol analysis, we think that pregnenolone analysis is meaningful, especially in cases where an ESI ion source is used.

Hormones are the most potent endocrine disruptors even at ng L^{-1} levels. The presence of progesterone in aquatic environments even at low levels (0.1-10 ng L^{-1}) has been linked with different steroidal effects in aquatic species (Díaz-Cruz et al., 2009). This is because they are able to interact with the endocrine system. As such, they interfere with reproductive, growth and development systems in both humans and animals. Some associated changes that have been slowly creeping into the wild fish populations include a reduction in fertility, changes in sex ratio (alteration of sexual development) incidence and inducing feminization. In the study by Mulabagal, Wilson, & Hayworth, 2017, the amount of progesterone found in the sediment was 2.91-22.3 pg g^{-1}. In another study conducted by Omar, Aris, Yusoff, & Mustafa, 2018, it was found to be between 0.7-5.34 ng g^{-1}. Lastly, in another study by Aydoğan & Yurdun, 2021, it was found to be between 1.59-6.03 ng g^{-1}. Considering these results, we can say that the amount of progesterone in our study was significantly higher than in other studies (39.96-405.60 ng g^{-1}). According to the values we found, we predict that some sea creatures in the Marmara Sea and its surroundings may experience negative effects such as feminization, masculinization, and damage to growth and development systems.

The levonorgestrel values in our study (325.25 and 937.93 ng g^{-1}) were found to be significantly higher than the results of previous analyses. The study performed by López de Alda et al., 2002 found it to be 0.05-2.18 ng g^{-1}, and Aydoğan & Yurdun, 2021 found it to be 1.55-7.78 ng g^{-1}. We think that the use of oral contraceptives may be more due to the dense population, a correlation exists between a dense population and the concentration of oral contraceptives released into the environment, and therefore the amount of levonorgestrel may have been found to be high.

To our knowledge, there are only three studies that have analyzed and detected mestranol (Aydoğan & Yurdun, 2021; Matić et al., 2014; Matić Bujagić et al., 2016). In the study by Matić et al., 2014, (Danube River), only one of six sediments (10 ng g^{-1}), and in the study by Matić Bujagić et al., 2016, only 2 of 11 sediments (Danube River and Topčiderka River) contain small amounts (11 ng g^{-1}, 19 ng g^{-1}). Also, mestranol was found in the study by Aydoğan & Yurdun, 2021 (82.34-335.82 ng g^{-1}). In our study, mestranol was found in all 27 marine sediments (33.73-228.32 ng g^{-1}). Mestranol is a synthetic steroid hor-

mone, a prodrug of ethinylestradiol, which enters the body as a result of its use as a contraceptive drug and is then excreted. Therefore, it is considered to be an indicator of estrogenic pollution.

As we researched, there is only one study detecting equilin in river or marine sediment (Aydoğan & Yurdun, 2021). In that study, it was determined as 54.46- 2201.00 ng^{-1}. In our study, 53.44-1232.53 ng g^{-1} of equilin was found in very high amounts in all 27 sediments. Equilin is a substance obtained from mares and produced synthetically and used for contraceptive purposes. It is thought that the amount of oral contraceptive use is high in this region, and therefore the high amount of equilin is a very strong indicator of the presence of human-induced pollution. The fact that the amount of equilin is high at MK, SD1, and MY1 points may make us think that estrogen-induced pollution, that is, human-induced pollution, is high at these points.

Androgenic steroids' excretion from the human body is via the urinary system. For this reason, they mix with the seas through the sewers and cause negative effects on the reproduction-development systems of sea creatures. At the same time, as a result of microbial degradation of paper mill wastes, progesterone and androstenedione are synthesized over converted to phytosterols. The most common phytosterols that undergo this conversion in the paper mill are sitosterol (72%), stigmastanol (11%) and campesterol (8%). According to one study (Jenkins, Wilson, Angus, Howell, & Kirk, 2003), the amount of androstenedione in the Fenholloway River sediment is 0.7±0.2 µg/L. In another study (Aydoğan & Yurdun, 2021), it was found to be 19.91-22.71 ng g^{-1}.

No previous androsterone and testosterone analyses have been found in marine sediments except in one study. In the study conducted by Aydoğan & Yurdun, 2021 in Haliç-Istanbul-Turkey, 72.66-467.56 ng g^{-1} androsterone, and 12.54-16.1 ng g^{-1} testosterone were detected. We also analyzed the amounts of androsterone, which are extremely high in our study (24.50-1718.18 ng g^{-1}). The amount of testosterone we found was extremely high too (86.30-1600.32 ng g^{-1}).

According to the study by Matić et al., 2014, campesterol amounts were 97-733 ng g^{-1}; in Matić Bujagić et al., 2016's study 52-1106 ng g^{-1} campesterol was detected; according to the study by Ali, Humrawali, & Latif, 2009, campesterol amounts were 0.98-14.70 µg g^{-1}; Aydoğan & Yurdun, 2021 found campesterol levels to be 143.90-1423.90 ng g^{-1}. In our study, similar results (35.30-1859,90 ng g^{-1}) were obtained.

In the QuEChERS extraction, sediment samples were studied with fewer samples than the samples studied with methanol extraction. As the recovery results were low using QuEChERS, some steroids could not be detected and the results obtained with methanol extraction are more significant. The results obtained using methanol extraction were taken into account in this study, as can be seen in Tables 1 and 2.

CONCLUSION

The amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points and above 500 ng g^{-1} at only one point. Based on this data, it can be considered that there is fecal pollution in the Marmara Sea. This is an indication that there is sewage pollution in the area. Moreover, we can say that there is no pollution between 0-1000 ng g^{-1}, there is medium pollution between 1000-2000 ng g^{-1}, and above 2000 ng g^{-1} there is pollution according to total steroid amounts. Accordingly, we can say that there is pollution in these sediment sampling points, MY^{-1} (6479.34 ng g^{-1}), M3 (4548.81 ng g^{-1}), SD1 (4273.99 ng g^{-1}), MKC-D (2368.53 ng g^{-1}), MD73 ($^{-1}$), M20 (2228.73 ng g^{-1}), MY2 (2051.00 ng g^{-1}) and MBC (2004.85 ng g^{-1}). In general, it can be said that there is pollution in the Marmara Sea according to the total amount of steroids. High levels of both total coprostanol + epicoprostanol and total steroid levels indicate that there is fecal and steroid pollution in the Marmara Sea. However, we think that studies should continue in order to reach a definitive conclusion.

Methanol and QuEChERS extraction method were used in the extraction of steroids. Significant results were obtained with methanol extraction in the analysis of steroids in the sediment samples. Because the recovery results were low in the QuEChERS extraction method and the data was better using methanol extraction. As seen in Table 1 and Table 2, the data obtained with methanol extraction was taken into account in the results of this study

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Original Article

Taxonomic significance of anatomy and achene micromorphology of selected *Cousinia* Cass. species (Asteraceae)

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ABSTRACT

Background and Aims: The genus *Cousinia* has about 700 taxa all over the world. It is a hard and controversial group to classify in terms of taxonomy. This study aims to determine the achene micromorphological and anatomical characteristics of two selected *Cousinia* species, as well as their taxonomic significance.

Methods: In anatomical studies, the sections were set in paraffin, cut with a microtome, and stained with safranin-fast green. For both *C. eriocephala* Boiss. & Hausskn. and *C. calocephala* Jaub. & Spach species selected, an independent sample T-test analysis was performed using quantitative data to determine the importance of anatomical characters. In addition, PCA analysis and heatmap analyses were performed. SEM images were taken to determine the micromorphological features of the achenes.

Results: In the transver section of stems in *C. eriocephala*, from the epidermis to the center, there are 9–12 rows of cortexs layers composed of parenchymatic cells. In the transver section of stem in *C. calocephala* from the epidermis to the center, there are 5–8 rows of cortical layers composed of parenchymatic cells. In the cross-sections of the leaf in *C. eriocephala*, it was determined that the midrib shape was semi-orbicular, and a total of 9 vascular bundles, 3 large and 6 small, were counted. In the cross-sections of the leaf in *C. calocephala*, it was determined that the midrib shape was semi-orbicular, and a total of 6 vascular bundles, 3 large and 3 small, were counted. The achene surface ornamentation of *C. eriocephala* is striate-scrobiculate, while *C. calocephala* is striate and scrobiculate-faveolate.

Conclusion: According to the findings, it was determined that anatomical characters are important in the differentiation of species, as supported by both PCA and heatmap analysis.

Keywords: Asteraceae, *Cousinia eriocephala, C. calocephala*, Plant anatomy, Principal component analysis, Turkey

INTRODUCTION

In the Asteraceae family, the genus *Cousinia* (Asteraceae, Cardueae) is comprised of approximately 700 taxa, which are distributed in Turkey, Iran, Afghanistan, and Central Asia. The genus *Cousinia* has high species diversity and endemism and is characterized by the Iranian Turan phytogeographic region (Djamali et al., 2012).

The first detailed studies of the genus *Cousinia* were made by Bunge (1865) based on morphological data. Bunge (1865) found 126 species of the *Cousinia* genus in 23 sections, Boissier (1875) found 141 species in 14 sections, based on Bunge's studies, Tscherneva (1962) evaluated 260 species in 50 sections. The genus *Cousinia* has been evaluated with more than 350 species in 58 sections of the Iranian flora, including the Pakistan mountains, Iranian plateaus, Turkmenistan and Afghanistan (Rechinger, 1972). According to Rechinger (1986), *Cousinia* probably has a high proportion of species in a limited range with a unique degree of differentiation.

The *Arctium-Cousinia* complex and the genus *Arctium L.* are both included in the non-monophyletic genus *Cousinia* (Susanna et al., 2003; Lopez-Vinyallonga et al., 2009).

The genus *Cousinia* was first described by Cassini in 1827 as *Carduus orientalis* Adams. It is defined based on its type. The genus *Cousinia* is represented by a total of 38 species, 26 of which are endemic, within 6 sections in the Flora of Turkey (Huber-Morath, 1975). According to the list of plants in Turkey, there are 39 species (Tugay, 2012). With the recently published *Cousinia agridaghensis* Tugay, Ertuğrul & Ulukuş, the total number of species of *Cousinia* in Turkey has reached 40 (Tugay et al., 2019).

Cousinia sect. *Cynaroideae* Bunge contains 89 species from

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all over the world (Rechinger, 1986). There are a total of 8 species, 4 of which are endemic, in the *Cousinia* genus, sect. *Cynaroideae*, in Turkey (Huber-Morath, 1975).

To date, a great deal of taxonomical studies have been conducted on the genus *Cousinia* sect. *Cynaroideae* (Tscherneva, 1962; Huber-Morath, 1975; Rechinger 1972, 1979; Winkler 1892, 1897; Mehregan & Kadereit 2008; Attar & Ghahreman 2000, 2006; Attar & Djavidi, 2010, Attar & Rad, 2019). Recently, palynological and molecular studies have been carried out on sect. *Cynaroideae* (Atazadeh, Sheidai, Attar, Ghahremaninejad & Koohdar, 2020; Atazadeh, Sheidai, Attar & Koohdar, 2021).

There has been only one study of *Cynaroideae* anatomy. In this study, Attar & Ghahreman (2000) studied the leaf, stem, and root anatomy of *C. mobayenii* Ghahr. & Attar. The aim of this research is to reveal the taxonomic importance of the stem, leaf anatomy and achene micromorphology of *C. eriocephala* and *C. calocephala* distributed in Turkey and to contribute to future taxonomic research on the genus *Cousinia*.

MATERIALS AND METHODS

Plant Material

Between the years of 2011 and 2013, while taxonomic revision of the genus *Cousinia* was being carried out in Turkey, plant samples were collected from various places around the country (O.Tugay-8461 & O.Tugay-8471). The KNYA Herbarium at Selcuk University was in charge of storing the specimens. The herbarium specimens were analyzed using the Flora of Turkey and East Aegean Islands with a stereobinocular microscope.

Anatomy

Living material was preserved in a 70% ethanol solution for the purpose of anatomical research. When cutting cross sections of the stems and leaves, the paraffin process was utilized. Following the embedding of the specimens in paraffin wax, a Leica RM2125RTS rotary microtome was used to cut sections with a thickness ranging from 5 to 10 micrometers. After staining with safranin-fast green, each section was mounted with Entellan (Johansen, 1940). The Leica DM1000 binocular light microscope with the Leica DFC280 camera was used to take the measurements as well as the photographs.

Achene Micromorphology

Seed surface ornamentation was identified using scanning electron microscopy images. The surface ornamentation of seeds was evaluated using the terminology proposed by Stearn (1983).

Statistical Analysis

In order to examine the anatomy of the stem, leaf, and midrib based on cell size, at least thirty cell measurements were taken and the minimum, mean, maximum, and standard deviation were calculated (Table 1). For all statistical tests, R 4.1.2 software was utilized. (R core Team, 2021). PCA analysis was conducted using the quantitative characters of anatomical stem, leaf, and midrib characteristics in the species studied. The heat map was created by using the cluster method (R 4.1.2 with library heatmap) of the anatomical features of the species (Figure 7). Independent sample T-tests were used to assess the statistical significance of quantitative stem, midrib, and leaf features (R 4.1.2). P-values <0.05 were regarded as statistically significant (Table 2).

RESULTS

In addition to showing the anatomical and micromorphological features of the species studied, also photographs of the flowers of the species was featured (Figure 1).

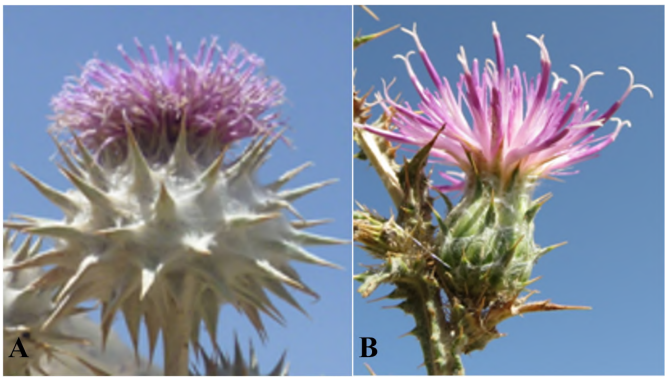

Figure 1. Photo: Prof. Dr. Osman TUGAY
Photographs of studied *Cousinia eriocephala* (A) *and C. calocephala* (B)

Stem Anatomy

Cousinia ericocephala

In the cross-sections of the stem, there is a single layer of protective epidermis tissue on the outermost. Epidermis cells consist of oval and rectangular cells with cell dimensions of 7.11-33.77 × 4.44-20.44 µm. On the epidermis, there is a thin layer of cuticle. From the epidermis to the center, there are 9–12 rows of cortex layers composed of parenchymatic cells of 17.77-65.77 µm in rectangular, pentagonal, and oval shapes. The thickness of sclerenchymatous fibers is between 67.82 and 135.60 above the external phloem and between 34.78 and 113.00 µm above the internal phloem. The vascular bundles are arranged parallel to the stem axis and are well developed. The phloem layer is composed of dense small cells and its dimensions are be-

tween 32.22 and 91.11 µm. The cambium layer is not clearly visible. Xylem ranges in size from 56.52 to 146.3 µm. There are many elliptical vascular bundles. In the center, there is the pith region, which is usually composed of pentagonal-shaped parenchymatic cells (Table 1. Figure 2A-B).

Cousinia calocephala

In the cross-sections of the stem, there is a single layer of protective epidermis tissue on the outside. Epidermis cells consist of oval and rectangular cells with cell dimensions of 4.89-16.3 × 5.43-14.67 µm. On the epidermis, there is a thick layer of cuticle. From the epidermis to the center, there are 5-8 rows of cortical layers composed of parenchymatic cells of 13.91-145.2 µm in rectangular, pentagonal, and oval shapes. The thickness of sclerenchymatous fibers is between 76.52 and 168.6 µm above the external phloem and between 29.56 and 120.00 µm above the internal phloem The vascular bundles are arranged parallel to the stem axis and are well developed. The phloem layer is composed of dense small cells, and its dimensions are between 49.27 and 107.20 µm. The cambium layer is not clearly visible. Xylem ranges in size from 21.73 to 105.70 µm. There are many elliptical vascular bundles. In the center, there is the pith region, which is usually occupied by pentagonal-shaped parenchymatic cells (Table 1. Figure. 2C-D).

Leaf Anatomy

Cousinia ericocephala

In the cross section of the leaf, there are the upper and lower epidermis layers arranged in a single row. The upper epidermis layer is mostly rectangular, and its dimensions are between 10.37-28.14 × 10.37-24.44 µm. The cells of the lower epidermis are slightly smaller than the upper ones. Lower epidermis cell sizes range from 8.00-32.00 × 4.44-21.33 µm. The mesophyll layer (275.50-400.00 µm) between the lower and upper epidermis is parenchymatic palisade, sponge, and palisade. 2-3 rows of palisade parenchyma cells contain abundant chloroplasts, and their dimensions are between 11.11-47.77 × 26.66-112.20 µm. The leaves are equifacial and there is a large collateral vascular bundle consisting of phloem and xylem in the midrib (Table 1, Figure 3A-B).

Cousinia calocephala

In the cross section of the leaf, there are the upper and lower epidermis layers arranged in a single row. The upper epidermis layer is mostly rectangular, and its dimensions are between 15.51-57.75 × 6.03-25.00 µm. The cells of the lower epidermis are slightly smaller than the upper ones. Lower epidermis cell sizes range from 10.75-32.75 × 6.03-17.24 µm. The mesophyll layer (208.60–296.50 µm) between the lower and upper epidermis is parenchymatic palisade, sponge, and palisade. 2-3 rows of palisade parenchyma cells contain abundant chloroplasts, and their dimensions are between 5.17-14.65 × 43.96-66.37 µm. The leaves are equifacial, and there is a large collateral vascular bundle consisting of phloem and xylem in the midrib (Table 1, Figure 3C-D).

Midrib

Cousinia ericocephala

In the cross-sections of the leaf, it was determined that the midrib shape was semi-orbicular, and a total of 9 vascular bundles, 3 large and 6 small, were counted. Phloem and xylem tissues are surrounded by dense sclerenchyma cells. There are collenchyma and parenchymatic cells up to the epidermis in both the upper and lower parts of the conducting bundles. Parenchymatic cells are pentagonal and hexagonal in shape. The phloem layer is composed of very small cells, the size of the layer is between 69.56-108.6 µm. The xylem tissue is well developed, and sizes range from 134.7-260.8 µm (Table 1, Figure 4A-B).

Cousinia calocephala

In the cross-sections of the leaf, it was determined that the midrib shape was semi-orbicular, and a total of 6 vascular bundles, 3 large and 3 small, were counted. Phloem and xylem tissues are surrounded by dense sclerenchyma cells. There are collenchyma and parenchymatic cells up to the epidermis in both the upper and lower parts of the conducting bundles. Parenchymatic cells are pentagonal and hexagonal in shape. The phloem layer is composed of very small cells, the size of the layer is between 65.21-102.1 µm. The xylem tissue is well developed, and sizes range from 63.04-193.4 µm (Table 1, Figure 4C-D).

Achene micromorphology

Cousinia eriocephala

Achenes are broadly obovate prominent margins at the wrinkled end and are not clearly toothed. Their achene surface pattern is striate and scrobiculate. The surface of cells are hollow and anticlinal walls are flat. Periclinal walls are concave or flat (Figure 5A-B).

Cousinia calocephala

Achenes are oblong-obovate with prominent margins at the wrinkled end and are clearly toothed. Their achene surface pattern is striate and scrobiculate-faveolate. The surface of cells are hollow and anticlinal walls are flat. Periclinal walls are concave or flat (Figure 5C-D).

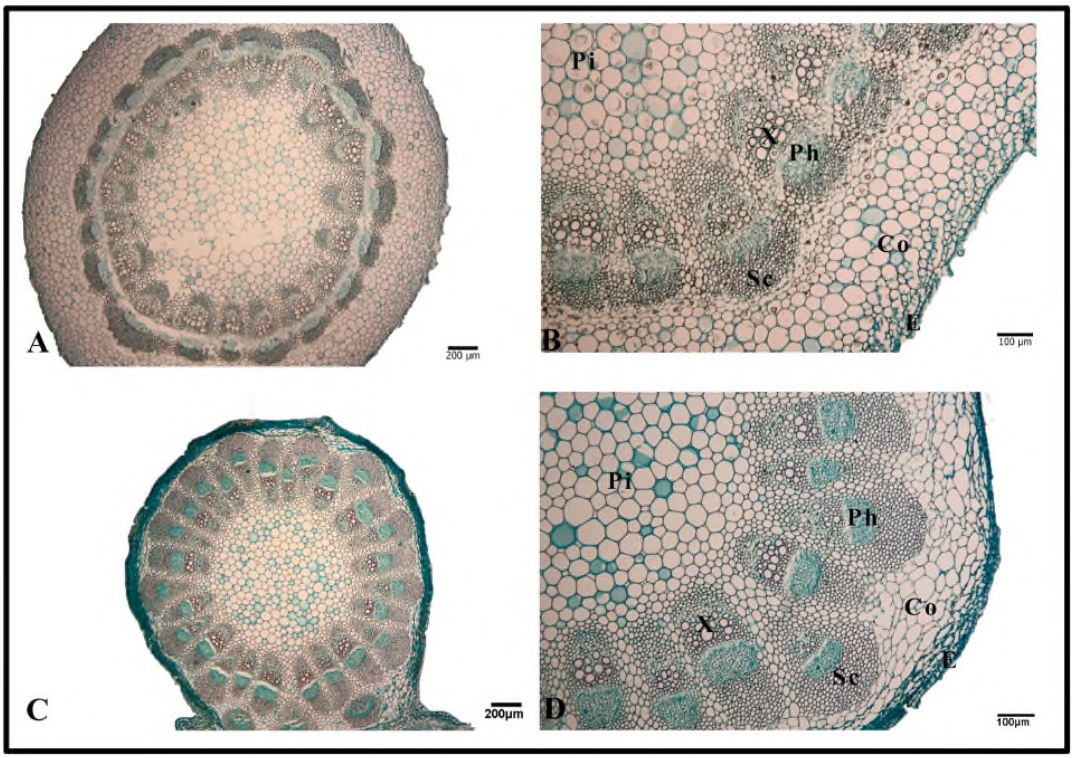

Figure 2. Transverse section of the stem; (A, B) *Cousinia eriocephala*, (C, D) C. *calocephala*. (E: epidermis, Co: cortex, Sc: sclerenchyma, Ph: phloem, X: xylem, Pi: pith region).

Table 1. Comparative anatomy of the, stem, leaves and midrip *C. eriocephala* and *C. calocephala* Abbreviations: Mean: Average, SD: Standart deviation, Min: Minimum, Max: Maximum, µm: Micrometer

		C. eriocephala				*C. calocephala*			
		Width m		Length m		Width m		Length m	
		min-ma	mean SD	min-ma	mean SD	min-ma	mean SD	min-ma	mean SD
tem	Epidermis cell	7.11 33.77	16.05 6.48	4.44 20.44	11.13 3.78	4.89 16.3	9.14 2.32	5.43 14.67	9.58 2.32
	Corte cell	17.77 65.77	39.52 12.95			13.91 145.2	29.67 23.54		
	Outer sclerench ma la er	67.82 135.60	83.40 17.23			76.52 168.60	122.22 19.87		
	Inner sclerench ma la er	34.78 113.00	71.73 21.79			29.56 120.00	61.38 23.30		
	Phloem la er	32.22 91.11	55.17 14.88			49.27 107.20	79.99 13.05		
	lem la er	56.52 146.30	105.99 21.26			21.73 105.70	55.88 18.49		
	Pith	23.33 93.33	63.40 17.23			19.13 69.56	45.32 12.24		
Leaf	Upper epidermis	10.37 28.14	19.50 4.08	10.37 24.44	17.22 2.65	15.51 57.75	26.00 10.64	6.03 25.00	15.62 4.84
	Lo er epidermis	8.00 32.00	14.12 4.85	4.44 21.33	10.63 3.33	10,75 - 32,75	16.17 5.45	6.03 17.24	12.15 2.82
	Mesoph ll	275.50 400.00	329.87 32.24			208.60 – 296.50	249.83 27.98		
	Palisade parench ma	11.11 47.77	21.47 8.74	26.66 112.20	68.73 20.00	5.17 14.65	11.05 2.55	43.96 66.37	54.33 6.29
Midri	Lo er collench ma	343.4 943.4	662.27 235.84			143.40 – 760.80	445.02 196.43		
	Upper sclerench ma	101.80 308.60	162.83 89.74			132.60 – 132.60	132.60 5.78		
	Lo er sclerench ma	113.00 423,10	272.10 111.75			45.65 93.47	72.46 20.28		
	Phloem la er	69.56 108.60	89.39 16.06			65.21 102.10	78.67 13.77		
	lem la er	134.7 260.8	209.20 47.93			63.04 193.40	124.74 49.34		

Statistical analysis

According to PCA analyses based on stem, leaf, and midrib characters of *C. calocephala* and *C. eriocephala*, the two species were distinguished from each other (Figure 6).

The independent sample T-test show that stem epidermal cell width, cortex cell width, outer schylerenchyma width, xylem layer width, pith width, and phloem layer width are all substantially different between *C. calocephala* and *C. eriocephala* (Table 2, P<0.05). Leaf mesophyll, palisade length and width characteristics seem to be important in the differentiation of *C. calocephala* and *C. eriocephala* (Table 2, P<0.05). Except for the upper schylerenchyma width, the remaining midrib fea-

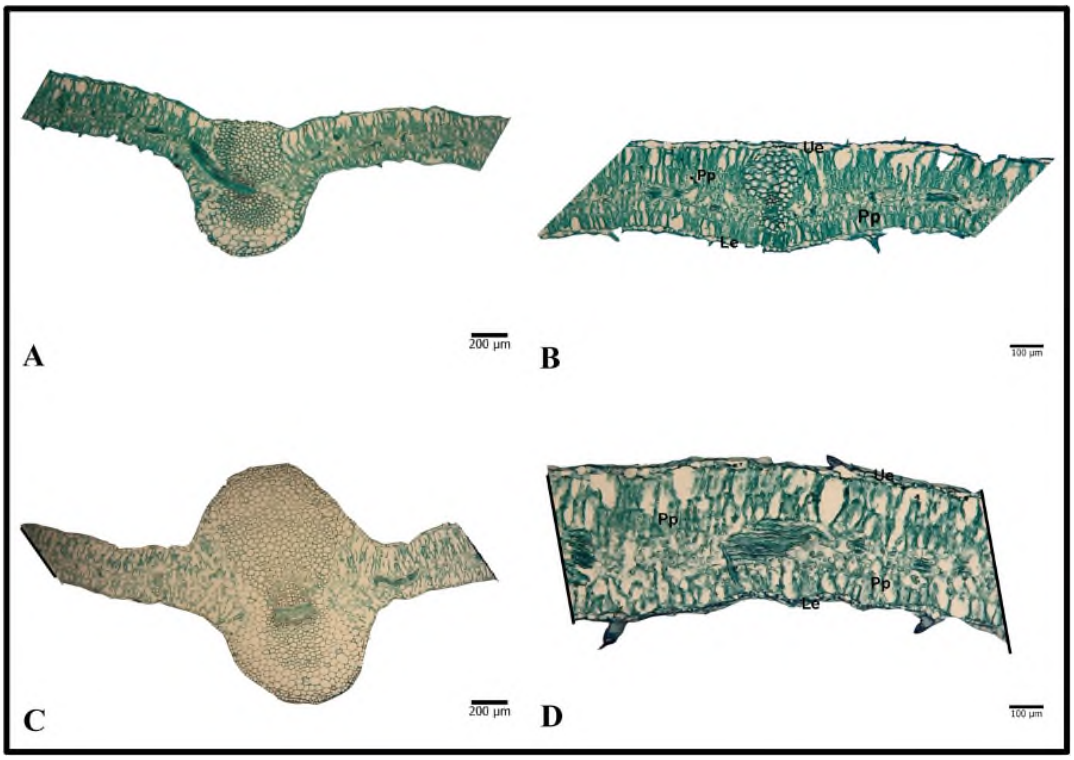

Figure 3. Transverse section of the lamina; (A, B) *Cousinia eriocephala*, (C, D) C. *calocephala*. (Le: lower epidermis, Pp: palisade parenchyma, Ue: upper epidermis).

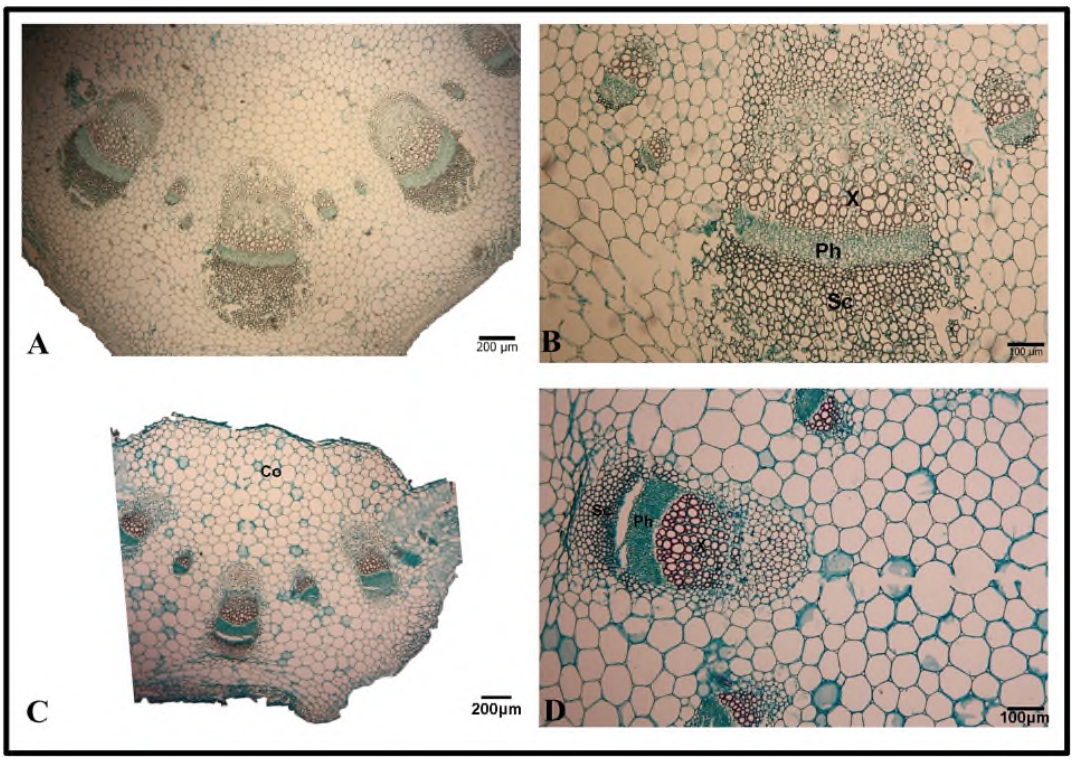

Figure 4. Transverse section of the midrib; (A, B) *Cousinia eriocephala*, (C, D) C. *calocephala*. (Co: collenchyma, Ph: phloem, Sc: sclerenchyma, X: xylem).

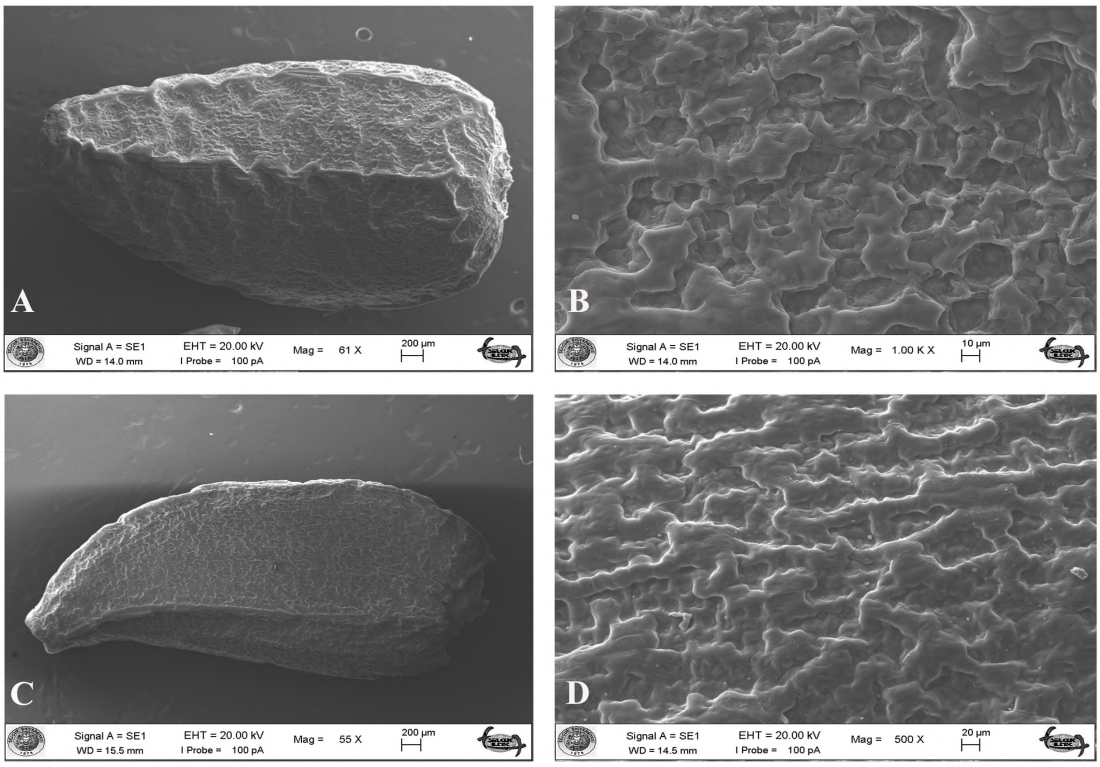

Figure 5. SEM micrographs of achenes of *Cousinia eriocephala* (A, B) and C. *calocephala* (C, D).

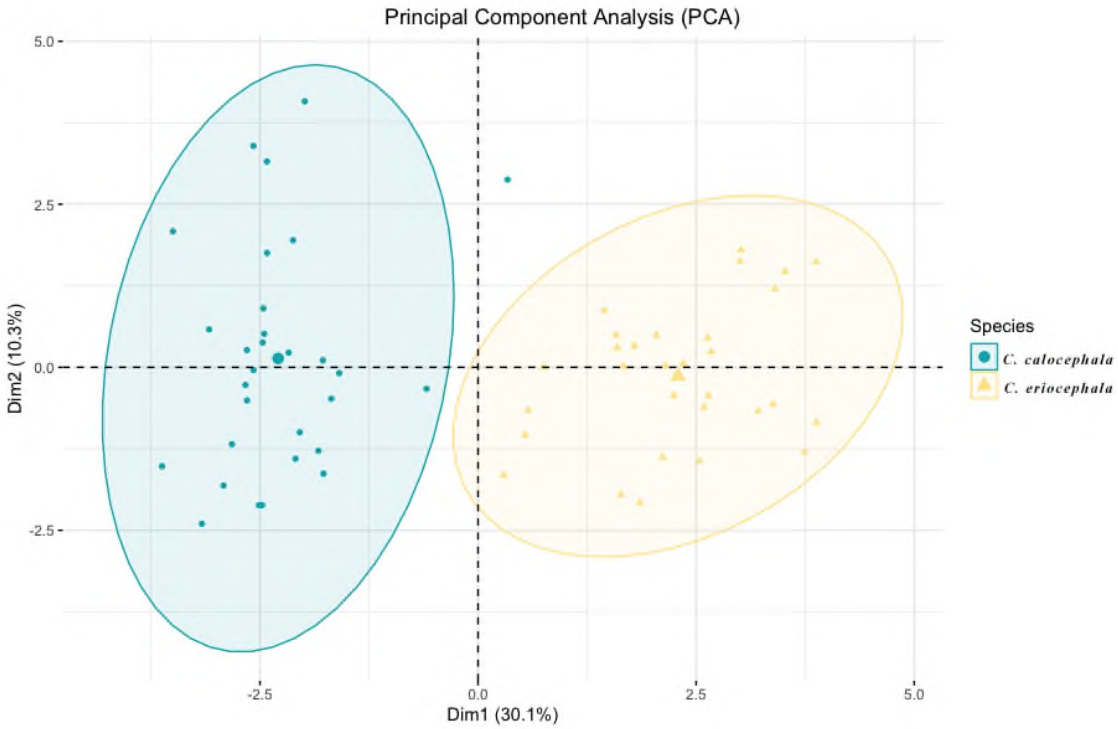

Figure 6. PCA for examined *Cousinia* species

tures were found to be important in the differentiation of *C. calocephala* and *C. eriocephala* (Table 2, P<0.05).

Table 2. Independent sample T-test based on the anatomical characters of the studied species

	Characteristics	*C. calocephala-C. eriocephala*
tem	Sep	P 0.05
	Sepl	P 0.05 NS
	Scor	P 0.05 NS
	Soutsc	P 0.05
	Sinsc	P 0.05 NS
	Sphl	P 0.05
	S l	P 0.05
	Pi	P 0.05
Leaf	Lue	P 0.05
	Luel	P 0.05 NS
	Lle	P 0.05 NS
	Llel	P 0.05 NS
	Lmeso	P 0.05
	Lpp	P 0.05
	Lppl	P 0.05
Midri	Mdlocol	P 0.05
	Mdupsc	P 0.05 NS
	Mdlosc	P 0.05
	Mdphl	P 0.05
	Md l	P 0.05

NS non-signi icant.
Signi icant at the level o 0.05.

Sepw: epidermis cell width of stem, Sepl: epidermis cell length of stem, Scorw: cortex cell width of stem, Soutscw: outer schylerenchyma width of stem, Sphlw: phloem width of stem, Sxylw: xylem width of stem, Piw: pith cell width of stem, Luew: upper epidermis width of leaf, Luel: upper epidermis length of leaf, Llew: lower epidermis width of leaf, Llel: lower epidermis length of leaf, Lmesow: mesophyll width, Lppw: palisade parenchyma cells width, Lppl: palisade parenchyma cells length, Mdlocolw: lower collenchyma width of midrib, Mdupscw: upper schylerenchyma width of midrib, Mdloscw: lower schylerenchyma width of midrib, Mdphlw: phloem width of midrib, Mdxylw: xylem width of midrib.

The results of the heat map analysis, which was based on anatomical characteristics, demonstrated that the two species that were analyzed could be distinguished from one another (Figure 7).

DISCUSSION

The data provided from stem, leaf, and midrib anatomical findings in this research indicated significant results that will contribute to the identification of the studied species within the *Cousinia* sect. *Cynaroideae* (Table 2). According to the stem anatomy findings, the size of the epidermis cells, cortex layers, outer schylerenchyma, phloem, xylem, and pith cells have taxonomically significant characters (Table 2). These stem anatomical characteristics can be integrated with morphological characteristics to identify species. Depending on the studied stem anatomical features, *C. eriocephala* differs from *C. calocephala* by its 9–12 layered parenchyma cells in cortex (Figure 2). According to the leaf anatomy results, the size of the epidermis cells, the size of the mesophyll layer width, and the size of the palisade parenchyma cells are taxonomically significant characters (Table 2, Figure 3). According to midrib anatomical characters, except for the upper schylerenchyma, other midrib characters are taxonomically important (Table 2). Recently, some anatomical studies have been carried out related to *Cousinia*. In these studies, Ulukuş & Tugay (2019b) investigated the stem, leaf, and midrib anatomy of *C. iconica* Hub.-Mor. Our anatomy findings partially concur with their findings. Ulukuş & Tugay (2019b) stated that mesophyll type is bifacial; in our study, we observed that the species examined are equifacial. Ulukuş & Tugay (2019b) reported that the number of vascular bundles of *C. iconica* in the midrib is 10. According to our study, while there are nine vascular bundles in *C. eriocephala*, respectively, there are six vascular bundles in *C. calocephala* (Figure 4). Ulukuş & Tugay (2019a) studied the anatomy of *C. halysensis*. Our findings partially accord with theirs concerning anatomy. Ulukuş (2019) stated that number of midrib vascular bundles have 10. However, we observed that the number of vascular bundles has nine and six studied species, respectively (Figure 4A-D). According to Atasagun, Ulukuş & Tugay (2021), *C. aucheri* DC. has 7-8 layered parenchyma cells in the cortex, but in our study, we found that *C. eriocephala* has 9–12 layered parenchyma cells in the cortex (Figure 2A–B). Ulukuş, Atasagun & Tugay (2021) stated that *C. decolarans* have 3 vascular bundles, However, in this study, we observed that the number of vascular bundles was nine and six in *C. eriocephala* and *C. calocephala*, respectively (Figure 4A–D).

In achene micromorphology studies related to *Cousinia*, Ulukuş & Tugay (2019) found that theachene structure pattern is reticulate in *C. iconica*, According to our study, we concluded that the achene structure patterns of *C. eriocephala* and *C. calocephala* are scrobiculate and scrobiculate-faveolate, respectively (Figures 5B–D). Ulukuş & Tugay (2019) stated that *C. halysensis* has a retipilate achene structure pattern. Both Atasagun et al. (2021) and Ulukuş et al. (2021) reported that studied species have a retipilate of achene surface ornamentation. However, we found that achene surface ornamentation is scrobiculate and scrobiculate-faveolate in the studied species (Figure 5B-D).

CONCLUSION

In this study, it was seen in both heatmap and PCA analyzes that the anatomical features used could be an important taxonomic character in the differentiation of species with the support of the statistical analysis results.

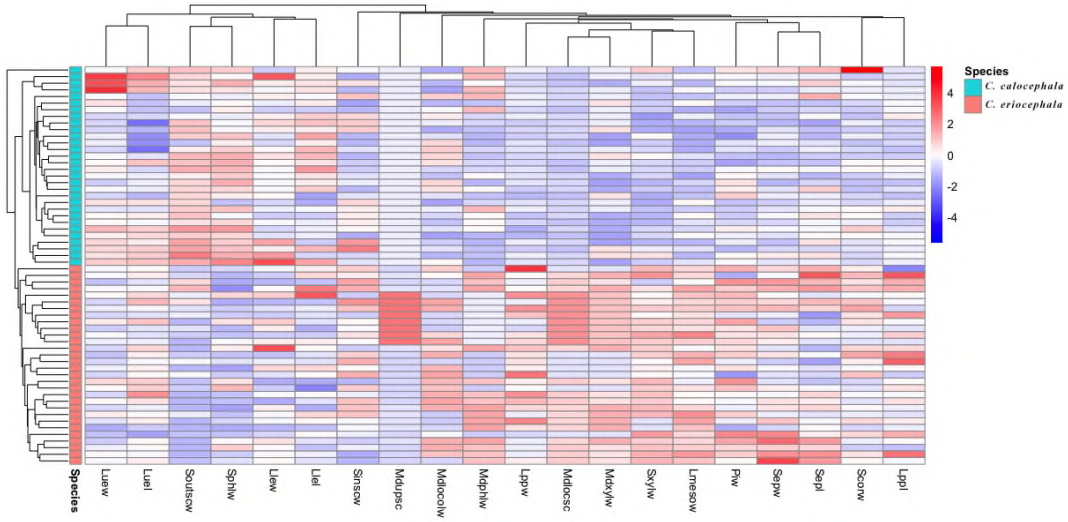

Figure 7. Heatmap for *C. calocephala* and *C. eriocephala* examined

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Original Article

Attitudes and perceptions of pharmacy students toward pharmacognosy and related competencies of the national core education program in Türkiye

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ABSTRACT

Background and Aims: Competency-based education (CBE) and the use of natural health products have been increasingly discussed in pharmacy. The national core education program of Türkiye has 108 mandatory competencies. This study investigated pharmacy students' thoughts about pharmacognosy and their preparedness to provide related competencies.

Methods: A descriptive online survey consisting of a 35-item questionnaire was administered to pharmacy students in Türkiye between June and July 2023. A 3-point Likert scale was used to assess students' opinions. Data were analyzed using SPSS 23.0 ($P < 0.05$).

Results: A total of 404 students in the third, fourth, and fifth years from 19 different faculties participated in the study. The interest in pharmacognosy was high, and students attributed significant value and importance to the field. Most of the students believed that their pharmacognosy education (67.1%) is sufficient, particularly on herbal medicinal plants (62.4%) and traditional and complementary medicine (59.2%). However, their satisfaction rates with education on marine pharmacognosy (10.4%) and drugs sourced from animals (30.2%) and microorganisms and minerals (32.2%) were low. Students rated their preparedness toward related competencies at concerning levels. The lowest value was observed in homeopathy (21.0%). Students felt more confident in academic and industrial practices (52.4%) than in community and hospital pharmacy requirements (35.3%). The impacts of national accreditation status and the education model of the faculties were found to be limited.

Conclusion: An overall review may be needed to adapt the field to outcome-based education or CBE.

Keywords: competency, integrated curriculum, pharmacognosy outcomes, pharmacy

INTRODUCTION

Pharmacy education is fundamental in healthcare systems for supplying contemporary, qualified pharmaceutical professionals meeting several societal needs and expectations. Similar to medical and other healthcare education systems, it has been undergoing major paradigm changes to align with the priorities of the 21st century. These global changes include a shift from time-based education (TBE) to competency-based education (CBE). Briefly, traditional TBE defines the systems mostly relying on fulfilling the admission and curriculum criteria at a predetermined time interval. TBE mainly focuses on the processes, whereas the graduates (end products) are almost the only thing that matters in CBE. CBE systems aim to provide graduates equipped to deal with all demands of the stakeholders. Too much emphasis on the outcomes while ignoring the time spent on learning and becoming a professional has been the main criticism of the CBE models (Anderson & Arakawa, 2021; Hodges, 2010; McMullen, Arakawa, Anderson, Pattison, & McGrath, 2023; Park, Hodges, & Tekian, 2016). However, a worldwide consensus has emerged regarding the planning, adaptation, and development of CBE systems in pharmacy education. One of the main topics of the Pharmacy Education Action Plan prepared by the World Health Organization, United Nations Educational, Scientific and Cultural Organization, and International Pharmaceutical Federation was developing a competency framework for pharmaceutical services (Anderson et al., 2008; Bruno, Bates, Brock, & Anderson, 2010). The "National Qualifications Framework for Higher Education" in Türkiye was announced in 2001 as a part of the Bologna Process according to the Lisbon Strategy published by the European Union. This general process was carried out by all programs of all universities in Türkiye. A more detailed, comprehensive, and field-specific study named the "National Phar-

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macy Core Education Program (NPCEP)" was conducted by the Council of Deans of Faculties of Pharmacy of Türkiye in 2015. The implementation of this guide by all pharmacy faculties in the country became mandatory in the same year. Furthermore, the NPCEP has become a fundamental part of the first and yet only national accreditation program of Türkiye for pharmacy faculties (National Society of Assessment and Accreditation of Pharmacy Education [ECZAKDER]). The study was revised in 2019, and 108 competencies are available in the current version. However, this document was prepared as a guide and did not propose any obligatory curriculum or education methods. Thus, although a few faculties developed novel hybrid systems (e.g., integrated and modular) to ensure that their graduates have the essential competencies, most of them reached a compromise by mapping their course contents with the competencies and some changes in their curricula. These hybrid systems are still time/content/discipline restricted but are enriched with new CBE applications and measurement/assessment techniques.

The use of natural health products is increasing and breaking new records every year. The growing popularity of traditional/complementary/alternative/integrative medicine applications contributes greatly to this increase. The definition and terminology of natural health products and these fields of medicine are tremendously variable from country to country. Nevertheless, there is a common opinion that these products should be included in the pharmacists' scope of practice as a public health requirement (Geldenhuys, Cudnik, Krinsky, & Darvesh, 2015; Homberg et al., 2021; Lee et al., 2018). However, as the variety and use of these products increase, the expectations and needs of patients, users, and other healthcare professionals have undergone a significant evolution at the same time. Questions arise about the education of natural health products in pharmacy, which is mainly the responsibility of the field of pharmacognosy. Pharmacognosy may be the oldest modern science and the very first core of the pharmacy profession. It is generally defined as dealing with natural crude drugs (e.g., sourced from plants, animals, microorganisms, algae, and minerals) and their metabolites applicable to any pharmaceutical field (Cahlíková et al., 2020; Sarker, 2012). Pharmacognosy is a multidisciplinary field. However, since the 19th century, chemical disciplines such as isolation and analytical phytochemistry, preparative organic chemistry, and structure elucidation methods stand out in traditional approaches after biological disciplines including macroscopic and microscopic analyses, ethnobotany, biochemistry, pharmacology, and toxicology. Along with these disciplines, modern approaches impose new responsibilities on pharmacognosy education about pharmaceutical care in areas such as clinical pharmacognosy, traditional/complementary/alternative/integrative medicine, rational use of natural health products, drug interactions, and wellness because most healthcare professionals do not have adequate education or time to focus on the source of drugs they use (Cahlíková et al., 2020; Geldenhuys et al., 2015; Kinghorn, 2002; Tiralongo & Wallis, 2008; Zhang, Phipps, & McDaniel, 2017). This metamorphism can actually be described as a return to the spirit, as the original roots of the field lay on "materia medica," knowledge of drugs and pharmacology.

This descriptive questionnaire study aimed to investigate students' perceptions of pharmacognosy, its education, and their preparedness to provide related competencies of the NPCEP in Türkiye.

MATERIALS AND METHODS

Research population

This study was conducted among pharmacy students between June and July 2023. The study population included the third, fourth, and fifth year students from all faculties in Türkiye. The total number of students was determined as approximately 12,900 according to the quotas of 49 pharmacy faculties in Türkiye (YÖK, 2023). However, the minimum sample size was 374, using OpenEpi Version 3.01 under the following parameters: design effect 1 (unknown prevalence), 5% error level, 95% confidence interval, and 80% power, without sample selection (OpenEpi, 2023). Students who agreed to participate in the study were included. Questionnaires with incomplete, contradictory, and/or inappropriate answers in the data collection form were excluded.

Survey form and data collection

Data were collected using an online survey developed by the authors. The survey comprised an informed consent form and 13 questions asked in four sections. The questions in the first section were about the sociodemographic and personal aspects of the participants. In the second and third sections, students' thoughts about pharmacognosy and its education were probed. The last section was about preparedness of the participants toward pharmacognosy-related national competencies provided by the NPCEP of Türkiye in 2019. These questions were designed with respect to the required learning levels given by the same program. The final version of the questionnaire was determined after applying the survey to 10 individuals to check for possible deficiencies. A 3-point Likert scale was used to assess students' opinions. "This is a security question, if you are reading this please check 'Not sure'" was added to the Likert scale sections of the questionnaire. The data of the students who provided a different answer to this proposition were excluded from the analysis. In this way, the validity and reliability of the Likert scale questions were tested.

Data analysis

Data analysis of the survey was conducted using IBM SPSS (Statistical Package for Social Sciences Version 23.0; SPSS,

Inc., Chicago, Illinois, USA). Descriptive statistics were presented as numbers and percentages for categorical variables and means, standard deviations, and minimum, and maximum values for numerical variables. The chi-square test families (Pearson chi-square test and Fisher exact test) were used to compare categorical variables in independent groups. The level of statistical significance was set as $P < 0.05$.

Ethics approval

This study was approved by the Ethics Committee of Dicle University Faculty of Medicine (17.05.2023/160). Official invitations consisting of information about the study, ethical approval, and link to the online survey were sent to all faculties. Participants' informed consent was obtained before the survey questions.

RESULTS AND DISCUSSION

Of the 459 pharmacy students who participated in the survey, 404 were eligible for the study. Table 1 summarizes the key characteristics of the respondents. The number of female students surveyed was more than three times higher than that of males. The distribution of the students in the third and fourth years was homogeneous. However, the rate of the fifth year students was 10% less than those of the others. Of the students, 95% and 67% had grade point averages of >2.5 and >3, respectively. Different factors, particularly parents, affect and sometimes even determine students' profession choices in the first place. This may significantly influence how students define the profession and its education. However, 82.4% of the participants stated that the choice was their own. These characteristics imply a willing and academically successful population. The students were from 19 different faculties. Of these faculties, 21.5% provided a hybrid (e.g., integrated/modular) course model, and 72.8% had accreditation certificates (including full and conditional) given by the National Society of Assessment and Accreditation of Pharmacy Education (ECZAKDER).

Table 1. Key characteristics of the participants (n = 404)

Characteristics	n	
Sex		
Female	309	76.5
Male	95	23.5
Age (min, 20; max, 37)	(Year, mean	SD) 22.6 1.6
ear in school		
Third	158	39.1
Fourth	156	38.6
Fifth	86	21.3
Above fifth	4	1.0
rade point average		
0–2.00 (0–53.33)	4	1.0
2.01–2.50 (53.56–65)	16	4.0
2.51–3.00 (65.23–76.66)	113	28.0
3.01–3.50 (76.90–88.33)	209	51.7
3.51–4.00 (88.56–100)	62	15.3
Course model		
Time discipline based	317	78.5
Hybrid (integrated modular)	87	21.5
National accreditation status of the faculty		
Yes	294	72.8
No	110	27.2
O n choice of profession		
Yes	333	82.4
No	71	17.6

Pharmacognosy and its education

Pharmacognosy is a highly multidisciplinary field and may be considered the very first core of the pharmacy profession. Its ancient history causes extraordinary fractionation and the emergence of new disciplines over centuries. This fractionating has reached such great proportions upon the specialization trends of modern science that it has become questionable whether there is a certain discipline left from pharmacognosy. However, crucial needs in natural health products, natural crude drugs, discovery of new molecules, and intersections of biology and chemistry with the increasing incidence of traditional/complementary/alternative/integrative medicine practices make pharmacognosy-educated pharmacists essential (Cahlíková et al., 2020; Sarker, 2012; Steinhoff & Committee, 2013; Zhang et al., 2017). This claim is consistent with the current results revealing that 91.3% of the students believed that "pharmacognosy is indispensable for pharmacy education." Furthermore, 85.4% of the students believed that "pharmacognosy courses make them feel they are in the faculty of pharmacy," and 71.8% believed that they will use what they learned in pharmacognosy courses in their professional life. Opinions of the students from different years (third, fourth, and fifth) and subjected to different education models on all three propositions did not significantly differ (Table 2). However, the number of students who agreed with the first two opinions was significantly lower in accredited schools. Seventy percent of the students believed that the courses given by the Pharmacognosy Department were interesting. However, 30% of them stated that they would choose pharmacognosy elective courses as their first choice. In addition, 22.4% stated that they would choose pharmacognosy topics for graduating projects (asked to third and fourth year students). Nevertheless, 24.5% of the students planning master's or PhD degree in education (n = 151) stated that pharmacognosy would be their first choice. This rate may be high considering all fields of pharmacy. It should be noted that the percentages of students who would not choose pharmacognosy elective courses or project topics or choose these as their last choice were only 13.1% and 17.4%, respectively.

Pharmacognosy education in Türkiye is provided in two main sections: theoretical and practical courses (laboratory). Of the

Table 2. Thoughts of the surveyed pharmacy students about pharmacognosy and its education (n = 404)

		ear in school				ccreditation			ducation model (h rid)		
uestions	otal	hird	ourth	i th	a	es	o	a	es	o	a
Pharmacognos courses are indispensable or pharmac education.	n ( )	n ( )	n ( )	n ( )		n ( )	n ( )		n ( )	n ( )	
Agree	369 91.3	144 91.1	144 92.3	81 90.0		262 89.1	107 97.3		78 89.7	291 91.8	
Not sure	20 5.0	9 5.7	8 5.1	3 3.3	0.522	19 6.5	1 0.9		5 5.7	15 4.7	0.717
Disagree	15 3.7	5 3.2	4 2.6	6 6.7		13 4.4	2 1.8		4 4.6	11 3.5	
Pharmacognos courses ma e me eel li e I am in the acult o pharmac .											
Agree	345 85.4	135 85.4	135 86.5	75 83.3		244 83.0	101 91.8		69 79.3	276 87.1	
Not sure	41 10.1	17 10.8	13 8.3	11 12.2	0.844[b]	36 12.2	5 4.5		13 14.9	28 8.8	0.159
Disagree	18 4.5	6 3.8	8 5.1	4 4.4		14 4.8	4 3.6		5 5.7	13 4.1	
I ill use m pharmacognos no ledge in m pro essional li e.											
Agree	290 71.8	114 72.1	113 72.4	63 70.0		207 70.4	83 75.5		61 70.1	229 72.2	
Not sure	91 22.5	35 22.2	34 21.8	22 24.4	0.993	72 24.5	19 17.3	0.247	20 23.0	71 22.4	0.806
Disagree	23 5.7	9 5.7	9 5.8	5 5.6		15 5.1	8 7.3		6 6.9	17 5.4	
M education on herbal medicinal plants is su icient.											
Agree	252 62.4	92 58.2	110 70.5	50 55.6		193 65.6	59 53.6		45 51.7	207 65.3	
Not sure	119 29.5	51 32.3	37 23.7	31 34.4	0.112	81 27.6	38 34.5		34 39.1	85 26.8	0.059
Disagree	33 8.2	15 9.5	9 5.8	9 10.0		20 6.8	13 11.8		8 9.2	25 7.9	
M education on marine pharmacognos is su icient.											
Agree	42 10.4	21 13.3	16 10.3	5 5.6		33 11.2	9 8.2		8 9.2	34 10.7	
Not sure	152 37.6	71 44.9	52 33.3	29 32.2		105 35.7	47 42.7	0.368	30 34.5	122 38.5	0.655
Disagree	210 52.0	66 41.8	88 56.4	56 62.2		156 53.1	54 49.1		49 56.3	161 50.8	
M education on animal crude drugs is su icient.											
Agree	122 30.2	54 34.2	46 29.5	22 24.4		90 30.6	32 29.1		24 27.6	98 30.9	
Not sure	152 37.6	63 39.9	55 35.3	34 37.8	0.237	107 36.4	45 40.9	0.698	31 35.6	121 38.2	0.578
Disagree	130 32.2	41 25.9	55 35.3	34 37.8		97 33.0	33 30.0		32 36.8	98 30.9	
M education on crude drugs o microorganisms and minerals is su icient.											
Agree	130 32.2	51 32.3	54 34.6	25 27.8		104 35.4	26 23.6		33 37.9	97 30.6	
Not sure	144 35.6	68 43.0	48 30.8	28 31.1		94 32.0	50 45.5		18 20.7	126 39.7	
Disagree	130 32.2	39 34.7	54 34.6	37 41.1		96 32.7	34 30.9		36 41.4	97 29.7	
M education on traditional and complementar medicine is su icient.											
Agree	239 59.2	87 55.1	100 64.1	52 57.8		172 58.5	67 60.9		48 55.2	191 60.3	
Not sure	112 27.7	52 32.9	35 22.4	25 27.8	0.342	84 28.6	28 25.5	0.823	23 26.4	89 28.1	0.258
Disagree	53 13.1	19 12.0	21 13.5	13 14.4		38 12.9	15 13.6		16 18.4	37 11.7	
Pharmacognos course materials are su icient.											
Agree	242 59.9	98 62.0	102 65.4	42 46.7		179 60.9	63 57.3		49 56.3	193 60.9	
Not sure	107 26.5	36 22.8	37 23.7	34 37.8		76 25.9	31 28.2	0.805	23 26.4	84 26.5	0.520
Disagree	55 13.6	24 15.2	17 10.9	14 15.6		39 13.3	16 14.5		15 17.2	40 12.6	
In general, m education about pharmacognos is su icient.											
Agree	271 67.1	107 67.7	111 71.2	53 58.9		202 68.7	69 62.7		53 60.9	218 68.8	
Not sure	101 25.0	39 24.7	36 23.1	26 28.9	0.276	69 23.5	32 29.1	0.484	24 27.6	77 24.3	0.258
Disagree	32 7.9	12 7.6	9 5.8	11 12.2		23 7.8	9 8.2		10 11.5	22 6.9	

0.05 a, Pearson test b, Fisher e act test.

participants, 35.6%, 59.4%, and 5.0% defined the content of the theoretical courses as more than enough, sufficient, and insufficient, respectively. For the practical courses, the rates were 9.6%, 71.8%, and 18.6%, respectively. The results showed that pharmacy students may have a demand for changing the theoretical/practical content ratio in favor of laboratory practices. Furthermore, the results did not show any significant differences regarding accreditation status and education model.

Herbal medicinal plants, crude marine drugs, and drugs from other biological sources (e.g., animals and microorganisms) with traditional and complementary medicine are almost the main actors of all pharmacognosy definitions. Thus, the opinions of the students about their education on these topics were questioned (Table 2). Despite the relatively high percentage of the students who agreed that their pharmacognosy education is generally sufficient (67.1%), the mean percentage of the agreeing students on each aforementioned topic was 38.9%. This difference may indicate that the students did not have a common idea about the scope of pharmacognosy. The satisfaction rates of the students with the education of herbal medicinal plants and traditional and complementary medicine were relatively high (62.4% and 59.2%, respectively). This may be a result of the emphasis on phytochemistry and phytotherapy in the curricula. However, these results cannot provide any ignorance about the dramatic results of marine drugs. Of the students, 89.6% either defined the education of marine pharmacognosy as insufficient (52.0%) or not sure about it (37.6%). The responses did not show any significant difference regarding either the accreditation status of the faculties or the education model. All drugs of natural origin are within the scope of pharmacognosy, and this includes marine sources as much as terrestrial sources. Marine pharmacognosy is a challenge to the field because of its brutal environment with little known living organisms and their taxonomy. More than 70% of the earth is covered by water, and the biodiversity of life in oceans is glamorous. In addition, tough external factors in the environment can cause unique secondary metabolites. Thus, its education as a part of pharmacognosy has become crucial (Bisaria, Sinha, Srivastava, & Singh, 2020; Cahlíková et al., 2020; Kinghorn, 2002).

The current results suggest an emphasis on this topic in the national curricula of Türkiye, which is a peninsula surrounded by sea on three sides. Significant improvement in the third and fourth year students' opinions may indicate a precession; however, there is still a long way to reach sufficient levels. The results for animal, microorganism, and mineral sources are also concerning. In particular, recent developments in biotechnology regarding isolation or production of new pharmaceutical compounds using microorganisms are remarkable (Verpoorte, 2000). Education and research in this field deserve more attention in pharmacognosy.

Students' opinions on the education of herbal medicinal plants significantly differed in favor of accreditation but not in the year in school and education model. However, no significant differences were observed in any of the three parameters for the "In general, my education about pharmacognosy is sufficient" proposition. In addition, the least agreement rate with the proposition was determined in the fifth year students. Pharmacognosy course materials were found to be sufficient by 59.9% of students, with a significant decrease in the fifth year students (46.7%). These two low rates of the fifth year students may be caused by the fifth year curricula structured mostly with elective courses and mandatory internships, which may convert their expectations from theoretical knowledge to community practice. Thus, more elective courses regarding students' possible internship needs should be considered.

National competencies related to pharmacognosy

The NPCEP of Türkiye has 108 competencies. It cannot be denied that each course, more or less, contributes to all competencies. Otherwise, it should be reconsidered and revised according to CBE. However, most courses are meant to be responsible or one of the major contributors for a particular competency. Ten competencies were selected regarding this fact. Some of the selected competencies may involve pharmacognosy, pharmaceutical botany, and pharmacology or other fields as major actors together. It should be noted that all related courses are not given under the same department in all faculties. For instance, courses dealing with medicinal teas, identification of plants, drug/natural product interactions, or supplements can be found under one of the aforementioned departments in different faculties. Such competencies and education, measurement, and assessment of the courses aimed at these competencies should be considered opportunities not to be missed for collaboration. All competencies were defined with different learning levels ranging from 1 (lowest) to 4 (highest) in the NPCEP. Level 1 requires knowledge about the topic and to provide guidance, whereas level 2 stipulates conducting the practice with the help of a source/guide/instruction or with assistance. Graduates should be able to provide competency without assistance in general practices at level 3. In contrast, level 4 includes handling complicated cases without any help. All selected competencies require learning levels between 1 and 3. They were divided into two main parts. Competencies more related to community and hospital pharmacy (CH), and others more related to academic and industrial pharmacy (AI). CH competencies include preparing medicinal teas, providing consultancy on medicinal teas, homeopathy, traditional herbal medicinal products, rational and safe use of supplements, and detecting and evaluating drug/natural product interactions. AI competencies include obtaining active substances/excipients from natural sources, identifying medicinal, poisonous, or narcotic plants, performing quality-control operations on natural sources, preparing traditional herbal medicinal products, and developing active compounds/excipients from natural sources. The mean rate of students who believed that they have competencies related to CH was 35.3%, whereas that for AI was 52.4% (Table 3). Although both rates were disturbingly low, these results indicated that students felt more prepared for academic or industrial issues than for patient-oriented duties such as consultancy and guidance on pharmaceutical care. A pharmacist is usually defined as a healthcare professional dealing with all aspects of the supply and use of medicines (World Health Organization, 2019). This process involves research, development, and production of all kinds of medicines, and pharmacists are crucial actors in every step. However, the number of needed workforces in the pharmacies is much higher. Furthermore, it is obvious that most pharmacy graduates are employed in community and hospital pharmacies in Türkiye. Thus, a review and improvement of the curricula and/or educational techniques may be considered for all these competencies, particularly for CH-related ones. Consultancy and guidance duties require communication skills and a multidisciplinary perspective. These may be some of the missing pieces that prevent students from feeling more confident about these competencies. Furthermore, consulting and guiding patients/users/healthcare professionals are core aspects of pharmacology and clinical pharmacy. Thus, further collaborations in these fields should be considered.

The highest self-confidence was found in identifying plants (67.6%), performing quality-control operations (63.1%), and obtaining active substances/excipients (62.6%). The lowest preparedness was observed in identifying homeopathic products and providing guidance on this subject (21.0%). Homeopathy practices are part of the "Traditional and Complementary Medicine Practices Regulation (27.10.2014, 29158)" in Türkiye, and the supply of homeopathic products is restricted to pharmacies only. Despite the recently reported high awareness of pharmacy students in Türkiye (Renda, Gökkaya, Kandemir, Özyiğit, & Kurt, 2023), the current results revealed extremely low confidence in the related competency.

As the competencies are expected to be the results of the entire education, the perceptions of the fifth year students may be more significant. The mean rates of self-confidence of the fifth year students were 39.32% and 51.12% for CH- and AI-

Table 3. Pharmacy students' thoughts on their preparedness for related national competencies (n = 404)

		ear in school				Accreditation			Education model (hybrid)		
Competencies	**Total**	**Third**	**ourth**	**ifth**	[a]	**es**	**No**	[a]	**es**	**No**	[a]
I can prepare medicinal teas and provide consultancy without assistance in general practice (CH).	**n ( )**	**n ( )**	**n ( )**	**n ( )**		**n ( )**	**n ( )**		**n ( )**	**n ( )**	
Agree	171 (42.3)	49 (31.0)	78 (50.0)	44 (48.9)		128 (43.5)	43 (39.1)		45 (51.7)	126 (39.7)	
Not sure	152 (37.6)	64 (40.5)	57 (36.5)	31 (34.4)	**0.001**	105 (35.7)	47 (42.7)	0.431	22 (25.3)	130 (41.0)	**0.02**
Disagree	81 (20.1)	45 (28.5)	21 (13.5)	15 (16.7)		61 (20.7)	20 (18.2)		20 (23.0)	61 (19.2)	
I am knowledgeable about how homeopathy is practiced, and I can identify homeopathic products and provide guidance on this subject (CH).											
Agree	85 (21.0)	24 (15.2)	41 (26.3)	20 (22.2)		64 (21.8)	21 (19.1)		23 (26.4)	62 (19.6)	
Not sure	186 (46.0)	69 (43.7)	72 (46.2)	45 (50.0)	**0.033**	132 (44.9)	54 (49.1)	0.728	34 (39.1)	152 (47.9)	0.249
Disagree	133 (32.9)	65 (41.1)	43 (27.6)	25 (27.8)		98 (33.3)	35 (31.8)		30 (34.5)	103 (32.5)	
I can provide consultancy on traditional herbal medicinal products without assistance in general practice (CH).											
Agree	138 (34.2)	40 (25.3)	61 (39.1)	37 (41.1)		104 (35.4)	34 (30.9)		35 (40.2)	103 (32.5)	
Not sure	185 (45.8)	70 (44.3)	73 (46.8)	42 (46.7)	**0.000**	130 (44.2)	55 (50.0)	0.571	34 (39.1)	151 (47.6)	0.315
Disagree	81 (20)	48 (59.3)	22 (14.1)	11 (12.2)		60 (20.4)	21 (19.1)		18 (20.7)	63 (19.9)	
I can provide consultancy on the rational and safe use of supplements without assistance in general practice (CH).											
Agree	184 (45.5)	58 (36.7)	77 (49.4)	49 (54.4)		137 (46.6)	47 (42.7)		38 (43.7)	146 (46.1)	
Not sure	166 (41.1)	63 (39.9)	69 (44.2)	34 (37.8)	**0.000**	115 (39.1)	51 (46.4)	0.373	36 (41.4)	130 (41.0)	0.864
Disagree	54 (13.4)	37 (23.4)	10 (6.4)	7 (7.8)		42 (14.3)	12 (10.9)		13 (14.9)	41 (12.9)	
I can detect and evaluate drug natural product interactions without assistance in general practice (CH).											
Agree	135 (33.4)	47 (29.7)	61 (39.1)	27 (30.0)		106 (36.1)	29 (26.4)		29 (33.3)	106 (33.4)	
Not sure	185 (45.8)	60 (38.0)	76 (48.7)	49 (54.4)	**0.000**	128 (43.5)	57 (51.8)	0.172	40 (46.0)	145 (45.7)	0.999
Disagree	84 (20.8)	51 (32.3)	19 (12.2)	14 (15.6)		60 (20.4)	24 (21.8)		18 (20.7)	66 (20.8)	
I can obtain active substances excipients from natural sources with the help of a source guide instruction or with assistance (AI).											
Agree	253 (62.6)	97 (61.4)	102 (65.4)	54 (60.0)		183 (62.2)	70 (63.6)		52 (59.8)	201 (63.4)	
Not sure	103 (25.5)	39 (24.7)	36 (23.1)	28 (31.1)	0.543	73 (24.8)	30 (27.3)	0.548	20 (23.0)	83 (26.2)	0.212
Disagree	48 (11.9)	22 (13.9)	18 (11.5)	8 (8.9)		38 (12.9)	10 (91.)		15 (17.2)	33 (10.4)	
I can identify medicinal, poisonous, or narcotic plants with the help of a source guide instruction or with assistance (AI).											
Agree	273 (67.6)	102 (64.6)	111 (71.2)	60 (66.7)	0.673	199 (67.7)	74 (67.3)	0.657	61 (70.1)	212 (66.9)	0.206
Not sure	105 (26.0)	43 (27.2)	37 (23.7)	25 (27.8)		78 (26.5)	27 (24.5)		24 (27.6)	81 (25.6)	
Disagree	26 (6.4)	13 (8.2)	8 (5.1)	5 (5.6)		17 (5.8)	9 (8.2)		2	24 (7.6)	
I can perform uality-control operations on natural resources with the help of a source guide instruction or with assistance (AI).											
Agree	255 (63.1)	97 (61.4)	105 (67.3)	53 (58.9)		183 (62.2)	72 (65.5)		50 (57.5)	205 (64.7)	
Not sure	118 (29.2)	45 (28.5)	40 (25.6)	33 (36.7)	0.221	90 (30.6)	28 (25.5)	0.534	32 (36.8)	86 (27.1)	0.195
Disagree	31 (7.7)	16 (10.1)	11 (7.1)	4 (4.4)		21 (7.1)	10 (9.1)		5 (5.7)	26 (8.2)	
I can prepare traditional herbal medicinal products without assistance in general practice (AI).											
Agree	117 (29.0)	41 (25.9)	47 (30.1)	29 (32.2)		89 (30.3)	28 (25.5)		30 (34.5)	87 (27.4)	
Not sure	190 (47.0)	66 (34.7)	83 (53.2)	41 (45.6)	**0.024**	132 (44.9)	58 (52.7)	0.370	34 (39.1)	156 (49.2)	0.231
Disagree	97 (24.0)	51 (32.3)	26 (16.7)	20 (22.2)		73 (24.8)	24 (21.8)		23 (26.4)	74 (23.3)	
I am knowledgeable about developing active compounds excipients from natural sources and can provide guidance (AI).											
Agree	160 (39.6)	48 (30.4)	78 (50.0)	34 (37.8)		123 (41.8)	37 (33.6)		38 (43.7)	122 (38.5)	
Not sure	170 (42.1)	72 (45.6)	59 (37.8)	39 (43.3)	**0.005**	117 (39.8)	53 (48.2)	0.259	33 (37.9)	137 (43.2)	0.632
Disagree	74 (18.3)	38 (24.1)	19 (12.2)	17 (18.9)		54 (18.4)	20 (18.2)		16 (18.4)	58 (18.3)	

0.05; a, Pearson test; CH, community and hospital pharmacy; AI, academic and industrial pharmacy.

related competencies, respectively. Statistically significant differences were observed in seven competencies regarding the year in school (Table 3). Among them, the fifth year students stated more confidence than the third and fourth year students in only three competencies: providing consultancy on traditional herbal medicinal products (41.1%), providing consultancy on the rational and safe use of supplements (54.4%), and preparing traditional herbal medicinal products (32.2%) with still concerning levels. No significant difference was determined in the preparedness of students for the competencies regarding the accreditation status of the faculties. However, a slightly increased confidence was observed in all CH-related and three AI-related competencies in favor of accreditation. Only a significant difference regarding the education model emerged in preparing medicinal teas and providing consultancy with a higher confidence in students who are subject to hybrid models.

Limitations and strengths

This descriptive questionnaire study reflects the participants' opinions. No sample selection was performed. Therefore, the results may not be valid for the entire population. There are 49 faculties in Türkiye and Turkish Republic of Northern Cyprus. However, the study included participants from 19 of them. All students considered knowing all the mentioned terms about the field. This study was conducted via an online form. Possible environmental influences were not known. Participants were asked to perform a self-evaluation of the competencies without a tangible condition. To the best of the authors' knowledge, this is the first study to evaluate the thoughts and attitudes of pharmacy students studying in Türkiye toward pharmacognosy education and national competencies. Thus, it may lead to more studies on the adaptation of the field to CBE in Türkiye. Fur-

thermore, the study provides premise findings on the impact of national accreditation and education models on the topic.

CONCLUSION

This descriptive questionnaire study investigated the attitudes and perceptions of pharmacy students regarding pharmacognosy and related competencies of the NPCEP in Türkiye. The interest in pharmacognosy was high, and students attributed significant value and importance to the field. However, a revision may be needed regarding the elective courses and the rate of practical (laboratory) courses of pharmacognosy. Most of the students believed that their pharmacognosy education (67.1%) was sufficient, particularly on herbal medicinal plants (62.4%) and traditional and complementary medicine (59.2%). However, most of them responded to the questions about their education on marine pharmacognosy and drugs sourced from animals, microorganisms, and minerals either as insufficient or not sure. Students felt more prepared toward academic and industrial practices (52.4%) such as isolation of natural compounds and performing quality-control operations than community and hospital pharmacy requirements (35.3%) including consultancy and guidance about natural health products. The lowest self-confidence was observed in identifying homeopathic products and providing guidance on the subject (21.0%). The impacts of national accreditation status of the faculties and hybrid (e.g., integrated/modular) education models were limited. An overall review may be needed to adapt the field to outcome-based education or CBE. However, further and periodic studies scoping with a higher number of students are needed.

Informed Consent: Written consent was obtained from the participants.

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REVIEW ARTICLE

Antioxidant supplements: Positive or negative actors in orthodontic treatment

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ABSTRACT

Antioxidant supplements are popular and commonly considered healthy benefits such as reducing the risk of disease. It should be noted that their advantages/disadvantages are still unclear. Some research on antioxidants shows that they may reduce the risk of cancer, heart disease, neurodegenerative diseases, and some chronic diseases, and have various health benefits such as a positive effect on bone metabolism by supporting bone regeneration. Some of them show that the benefits of antioxidant supplements are not clear and indicate to increase the risk. The effects of antioxidants on orthodontic treatment are now being studied extensively due to their widespread use. Antioxidants that regulate bone modulation can be used to reduce orthodontic treatment time, accelerate tooth movement, or in some cases prevent unwanted tooth movement, but their unconscious use can adversely affect the orthodontic treatment. Understanding the mechanisms of action of antioxidants and their effects on orthodontic treatment can increase the success of treatment and prevent adverse situations that may occur due to the use of antioxidants.

Many inflammatory mediators play a role in the response to mechanical forces in orthodontic treatment. Increased expression of pro-inflammatory cytokines is associated with oxidative stress. Antioxidants can affect remodeling processes in which osteoblast and osteoclast cells play a role, such as relapse, anchorage, and bone formation after maxillary expansion in orthodontic treatment. The use of antioxidants in orthodontic treatment may increase tooth movement and shorten retention time by increasing osteoblastic activity after maxillary expansion, or on the contrary, slow tooth movement and prolong treatment time by reducing oxidative stress and inflammation. Accordingly, factors such as the desired effect in orthodontic treatment and the phase of treatment should be considered when using antioxidants. We aimed to provide information and suggestions for evaluating the effectiveness of antioxidant use in orthodontic treatment with basic information about antioxidants.

Keywords: Antioxidant supplements, orthodontic treatment, oxidative stress

INTRODUCTION

The process of oxidation in the human body damages cell membranes and other structures (cellular proteins, lipids, DNA, etc.) by creating unstable molecules called free radicals (Buczko, Knas, Grycz, Szarmach, & Zalewska, 2017). Over time the damage caused by an overload of free radicals may become irreversible and lead to cellular dysfunctions and certain diseases or cancers. Oxidation can be also accelerated by stress, physical conditions, chemicals, sunlight, and other factors. Nevertheless, as it is well known, antioxidants are man-made or natural substances, which are produced from several sources including minerals, vitamins or food and herbal supplements, that scavenge free radicals from the body cells and may prevent or reduce the health issues caused by oxidation. Antioxidants are classified into basic groups: synthetic and natural on their origin, or endogenous and exogenous depending on their source, or enzymatic or non-enzymatic depending on their action, or water- and lipid-soluble depending on their solubility (Pizzino et al., 2017).

Unconscious use and high doses of exogenous antioxidants can cause oxidative damage by showing a pro-oxidant effect (Sotler et al., 2019). Even if use of antioxidant supplements has been found to be healthy; their protective potentials have become scientifically interesting compounds. High doses of antioxidant supplements can be harmful in some cases. For example, taking high-dose beta-carotene supplements increases the risk of lung cancer in smokers, and using high-dose vitamin E supplements increases the risk of hemorrhagic stroke and prostate cancer (Virtamo et al., 2003). In the dental field, the effect of antioxidant use is controversial.

Orthodontic tooth movement (OTM) occurs as a result of the remodeling of teeth and surrounding tissues by the appli-

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cation of mechanical force. During orthodontic treatment, the periodontal ligament (PDL) compresses on the compressive side and stretches on the tension side when force is applied to the tooth. Compression of the PDL is associated with osteoclast activity and stretching of the PDL is associated with osteoblast formation, resulting in bone formation (Masella & Meister, 2006; Verna, Zaffe, & Siciliani, 1999). Osteoclastic activity and bone resorption are considered rate-limiting factors in OTM (Seong et al., 2022). Osteoclasts remove the hyalinized necrotic tissue in the PDL, allowing the tooth to move within the alveolar bone (Salomao et al., 2014). Although the main factor affecting OTM is force, there are studies reporting that pharmacological active substances, chemical agents, antioxidants, and food supplement applications affect tooth movement by affecting the remodeling mechanisms of the PDL in tension and pressure regions. In addition to pharmacological interventions such as vitamin D, thyroxine, dihydroxycholecalciferol and prostaglandin E2 (PGE2) administration, invasive and non-invasive surgical techniques have been tried to accelerate OTM and modify the limiting step of bone resorption (Chen et al., 2016; Kacprzak & Strzecki, 2018; Kale, Kocadereli, Atilla, & Asan, 2004)

Oxidative stress is one of the biological responses to orthodontic treatment (Buczko, et al., 2017). Orthodontic force is considered a type of physical stress placed on the PDL. Hypoxia and compression occurring in PDL stimulate the production of free radicals (Arai et al., 2010). Oxidative stress, caused by excessive free radical production and/or impaired antioxidant defenses, can adversely affect bone formation by impairing the formation of osteoclasts, osteoblasts, and osteocytes (Kara et al., 2012). Many inflammatory mediators are involved in the response to mechanical forces during orthodontic treatment (Buczko et al., 2017). Oxidative stress and inflammation can be easily induced by each other (Fan et al., 2017). Antioxidants play a role in the anti-inflammatory response by preventing oxidant formation and reducing inflammation and may be effective on OTM (Chae et al., 2011).

In some cases, in orthodontic treatment, besides accelerating tooth movement, tooth movement should be suppressed/optimized for anchor applications that prevent unwanted tooth movement. Although various cytokines and compounds, including PGE2, vitamin D3, calcitonin and bisphosphonates, have been considered as drug candidates to optimize tooth movement by modulating osteoclasts, they have been reported to be unsuitable for clinical use due to their potential adverse effects (Shoji-Matsunaga et al., 2017). Antioxidants may be effective without the risk of oral tissue damage due to mechanical application and side effects of pharmacological agents. Besides, inhibition of osteoclast differentiation and promotion of osteoblast differentiation by regulating bone modulation could support the effectiveness of treatment in clinical applications such as preventing relapse in orthodontic treatment, accelerating the bone formation process after maxillary expansion, and anchoring methods that suppress/optimize unwanted tooth movement (Gad & Soliman, 2023). At the same time, considering that OTM may slow down with the use of antioxidants, issues such as orthodontic treatment duration, orthodontic force activations and appointment frequency should be considered in treatment planning (Bilici Geçer, 2023).

Several authors have investigated the effects of topical and systemic medications, including antioxidant supplements, on OTM. A significant number of patients seeking orthodontic treatment may benefit from medication for common health problems. Furthermore, given the general trend towards increased use of dietary supplements in the populations, it would be helpful to understand the effects of different agents on OTM in order to plan treatments and predict the timing of these treatments.

The duration of fixed orthodontic treatment can be approximately 1 to 3 years, depending on the severity of the malocclusion and the treatment applied. Lifestyle changes in patients undergoing orthodontic treatment have a significant impact on the patient's dietary intake and nutritional requirements (Al-Dlaigan, Shaw, & Smith, 2001). During orthodontic treatment, eating habits are affected by pain and functional limitations, especially in the first 3 to 5 days after treatment, patients may avoid raw vegetables, fruit and hard foods to prevent adequate chewing due to tooth sensitivity and bracket breakage during treatment (Ozdemir, Ilhan, Gorucu-Coskuner, Taner, & Bilgic, 2021). This condition significantly reduces the intake of protein, calcium, fiber and some vitamins, and blood levels of antioxidants such as vitamins C and E may be lower in orthodontic patients (Miresmaeili, Mollaei, Azar, Farhadian, & Mani Kashani, 2015; Ozdemir et al., 2021).

It is well known that the use of antioxidants is common in adults today. Although there are some studies investigating the relationship between orthodontic tooth movement and osteoclastic activity and age, the results are controversial. It has been suggested that alveolar bone and PDL remodeling is slower in adults than in adolescents, due to factors such as reduced cellular activity, vascularity and changes in bone composition. While it has been reported that there was no significant difference in the number, size or activity of osteoclasts in alveolar bone during orthodontic tooth movement in rats (Kabasawa, Ejiri, Hanada, & Ozawa, 1996; Jager & Radlanski, 1991), another study reported that osteoclast formation was slower in adult rats than in young rats (Ren, Kuijpers, & Maltha, 2005). It has also been suggested that orthodontic retention may take longer in adults to prevent relapse (Li et al., 2016). Considering the lower bone dynamics and osteoclastogenic activity during orthodontic treatment in adults, it should be considered that the use of antioxidants may prolong the duration of orthodontic treatment in adult patients, but may contribute to the post-treatment retention process.

As people become more concerned about their oral health,

dental care is becoming more detailed. This article focuses on the use of antioxidants in orthodontic treatment. Different types of antioxidants, different mechanisms of action and amounts of antioxidants used in OTM were reviewed. Challenges and safety assessment of these materials in the current field were also discussed. This review provides background information on antioxidants, summarizes the scientific evidence on antioxidants and health, and suggests additional sources of information on orthodontic treatment.

Exogenous antioxidants are dietary antioxidants found in significant amounts in widely consumed fruits, vegetables, nuts and cereals. Examples include vitamin C (ascorbic acid), vitamin E (alpha-tocopherol), carotenoids, polyphenols (phenolic acid, flavonoids, resveratrol etc.) and some minerals (Zn, Mn, Cu, Se, etc.). Endogenous antioxidants include glutathione, melatonin, uric acid, bilirubin, albumin, coenzyme Q10 (CoQ10), alpha-lipoic acid, ceruloplasmin and transferrin produced by the body (Mironczuk-Chodakowska, Witkowska, & Zujko, 2018; Pizzino et al., 2017).

Vitamin C

Vitamin C is an important water-soluble antioxidant that has been shown to neutralize the effects of free radicals on body fluids and reverse free radical damage at the cellular level (Bolat et al., 2020; Ishikawa, Iwasaki, Komaki, & Ishikawa, 2004). A major source of vitamin C, naturally occurring in citrus fruits, tomatoes, potatoes, broccoli, red and green peppers, kiwisand strawberries (Miresmaeili et al., 2015; Yalcin Bahat, Ayhan, Ureyen Ozdemir, Inceboz, & Oral, 2022; Gregory, 1993; Mangels et al., 1993). The recommended daily intake is 45 mg for 9 to 13 year olds, 65-70 mg for 14 to 18 year olds and 75-90 mg for 19 year olds (Monsen, 2000). For vitamin C, the most common effects are diarrhoea, nausea and abdominal cramps (Jacob & Sotoudeh, 2002; Monsen, 2000). Vitamin C may cause chromosomal and/or DNA damage by acting as a pro-oxidant and should be used with caution (Kazmierczak-Baranska, Boguszewska, Adamus-Grabicka, & Karwowski, 2020).

Vitamin C levels in the blood of orthodontic patients have been found to be 17 to 75% lower than desired (Miresmaeili et al., 2015). Vitamin C is known to be an important factor in bone remodelling and collagen synthesis in the PDL. Its deficiency can lead to a complete halt in osteogenesis, disturbance in the organisation of the PDL and an increase in bone resorption (Fujita, Hirano, Itoh, Nakanishi, & Tanaka, 2001; Van den Berg, Yu, Lemmens, & Beynen, 1994). Vitamin C has been shown to play a critical role in osteoclast stimulation, which occurs during tooth movement (Ozdemir et al., 2021). It also enables stem cells to transform into osteoblasts through collagen type I synthesis, interaction with integrins, activation of protein kinase signalling and phosphorylation of osteoblast-specific transcription factors (Miresmaeili et al., 2015). It has been reported to increase collagen production and induce tooth movement, and its deficiency may reduce OTM due to inhibition of collagen remodelling (Fujita et al., 2001; Motoji, To, Hidaka, & Matsuo, 2020; Van den Berg et al., 1994).

Miresmaeili *et al*., (2015) evaluated the effect on OTM in rats given dietary vitamin C. To achieve the desired blood level, vitamin C (1% water) added to the daily drinking water 7 days before the start of the experiment and applied for 17 days. It has been reported that the amount and rate of OTM and the number of osteoclasts increase. Bolat *et al*., (2020) evaluated the systemic and local effects of vitamins C and E on OTM. Systemically, 150 mg/kg (i.p.) of vitamins C and E were administered once a day. Locally, vitamins C and E (20 µL) were injected into the PDL every three days. It was reported that the most tooth movement was in the local vitamin C group, the least tooth movement was in the local vitamin E group, there was no significant difference in the number of osteoclasts, and the number of osteoblasts increased with the application of the vitamin. Özdemir *et al*., (2021) stated that vitamin C deficiency during orthodontic treatment reduces tooth movement by reducing tissue healing and regeneration in the PDL. Consuming less than the daily requirement of vitamin C may prevent collagen degradation and reformation necessary for tooth movement (Litton, 1974).

A long retention period after maxillary expansion is required to prevent early relapse. Therefore, it is important to promote osteogenesis of the expanding midpalatal suture to prevent relapse. Farhadian *et al.*, (2015) evaluated the effect of dietary vitamin C on osteogenesis of the midpalatal suture in rats during maxillary expansion, and they planned application periods of 3, 9 and 17 days. To achieve the desired blood level, 10 kg/m3 of vitamin C was added to the daily drinking water 7 days before the start of the experiment. It was observed that vitamin C had no significant effect on osteoclasts during maxillary suture expansion in rats, it had a stimulating effect on osteoblast differentiation at the beginning (day 3), but later (day 17), a negative effect on osteoblasts was observed.

Uysal *et al*., (2011) evaluated the effects of vitamin C administration on bone formation in the expansion of the maxillary suture in rats by histomorphometry. A single dose vitamin C (0.5 mg/kg) was administered locally and systemically to rats as an intramuscular and subcutaneous injection. The experimental period consisted of a 5-day expansion period and a 15-day retention period. Systemic administration of vitamin C with rapid maxillary expansion may shorten the duration of the procedure and improve the quality of the regenerated bone, whereas local injection of antioxidants has been reported to be detrimental to bone formation.

Dehis *et al.*, (2018) evaluated the efficacy and safety of local vitamin C injection on impacted canine traction speed and preservation of periodontal integrity in patients with unilateral impacted canines. The use of vitamin C was reported to increase the speed of movement during traction of the impacted

tooth. Healing was evaluated for 12 months after canine maintenance surgery and it was found that the alveolar bone level was preserved, the gingival biotype and the width of keratinized gingival tissues increased. Vitamin C was found to accelerate the eruption of canines by maintaining the integrity of the periodontium.

Vitamin E

Vitamin E is a powerful lipid-soluble antioxidant (Seong et al., 2022). It is found abundantly in olive and sunflower oils, as well as nuts, soybeans, avocados, wheat, and green leafy vegetables (Colombo, 2010). There are eight different forms of vitamin E known to occur in nature: four tocopherols (α, β, γ and δ isomers) and four tocotrienols (α, β, γ and δ isomers) (Clarke, Burnett, & Croft, 2008). Alpha-tocopherol has the highest antioxidant activity and is the most bioavailable in human tissues (Borhanuddin, Mohd Fozi, & Naina Mohamed, 2012; Huang, Chang, Huang, & Chen, 2003). Protects cell membranes from oxidation by lipid radicals generated during the lipid peroxidation chain reaction (Herrera & Barbas, 2001). In addition to being a potent biological antioxidant, it suppresses the production of pro-inflammatory mediators that have been reported to increase bone resorption, such as interleukin (IL)-1, IL-6, PGE2 and tumor necrosis factor-α (TNF-α) (van Tits, Demacker, de Graaf, Hak-Lemmers, & Stalenhoef, 2000). Vitamin E also has anti-inflammatory effects, inhibits platelet aggregation and improves immunity (Seong et al., 2022). The Food and Drug Administration (FDA) has classified α-tocopherol as "Generally Recognized as Safe (GRAS)" (Bolat, 2014). The recommended daily intake is 11 mg for children aged 9 to 13 years and 15 mg for children aged 14 years and over (NIH, 2021).

Many inflammatory mediators are involved in the response to mechanical forces during orthodontic treatment (Buczko et al., 2017). Vitamin E alters cytokine production (Esenlik, Naziroglu, Acikalin, & Ovey, 2012; Xu, Watkins, & Seifert, 1995), suppresses the harmful effects of free oxygen radicals in cells during bone formation (Xu et al., 1995), stimulates bone formation and has beneficial effects on new bone formation (Kurklu et al., 2011) during orthodontic treatment.

In rats, the supplementation with tocotrienol and alpha-tocopherol maintained corticosterone levels at a value appropriate for cellular homeostasis (Nur Azlina & Nafeeza, 2008) and reduced the stress state and inflammation caused by orthodontic force (Sufarnap et al., 2021). In rats fed a vitamin E diet (600 IU/kg), OTM increased on days 4 and 14, the number of osteoclasts increased, and bone volume decreased on day 14 (Seong et al., 2022). They suggested that high levels of vitamin E may help stimulate bone formation by increasing osteoblastic activity and preventing relapse during the retention phase. Although no systemic effects on bone turnover were found, it has been reported that long-term administration of high levels of vitamin E during orthodontic treatment can cause unwanted side effects. Indeed, the several studies reported that it may increase tooth movement by increasing osteoclastic activity (Seong et al., 2022; Sufarnap, Siregar, & Lindawati, 2020), and may reduce OTM by reducing inflammation (Esenlik et al., 2012; Q. Jiang, 2014; Sufarnap et al., 2021).

Wistar rats were given vitamin E (60 mg/kg) by oral gavage 14 days before appliance placement and during the 14-day experiment. It has been reported that vitamin E can reduce cortisol and IL-1β levels, and accordingly reduce the stress caused by orthodontic force (Sufarnap et al., 2021). Another similarly designed study reported that vitamin E increased tooth movement and the number of osteoblasts, but did not affect the number of osteoclasts (Sufarnap et al., 2020).

The results of studies of vitamin E on bone mineral density, markers of bone formation and bone health in humans are inconsistent. While some studies have found a negative association between serum vitamin E levels and markers of bone turnover and bone mineral density (Hamidi, Corey, & Cheung, 2012; Zhang, Hu, & Zhang, 2017) others have found that, on the contrary, higher dietary vitamin E intake is associated with higher bone mineral density (Mata-Granados, Cuenca-Acebedo, Luque de Castro, & Quesada Gomez, 2013; Shi et al., 2016). Esenlik et al., 2012 evaluated the changes in oxidant and antioxidant levels in gingival crevicular fluid in patients receiving orthodontic treatment with vitamin E (300 mg/day) for one month. Vitamin E has been reported to reduce lipid peroxidation levels in the anterior region.

Omega-3

Omega-3 fatty acids, which are polyunsaturated fatty acids, consist of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids have anti-inflammatory effects by reducing levels of inflammatory mediators such as pro-inflammatory cytokines and arachidonic acid-derived eicosanoids (PGE2 etc.) (Calder, 2006; Gad & Soliman, 2023). Omega-3 fatty acids have beneficial effects on bone and cardiovascular diseases due to their immunomodulating and anti-inflammatory effects (Gad & Soliman, 2023). The use of EPA and DHA, separately or together, reduces oxidative stress. This effect is thought to be related to the immunomodulatory effect of polyunsaturated fatty acids and the reduction of leukocyte activation (Mori & Beilin, 2004). The icosapent ethyl and omega-3 acid ethyl esters, which are omega-3 fatty acid products, have been approved by the FDA, and the recommended daily intake are 1-1.2 g for 9 to 13 year olds, 1.1-1.6 g for 14 to 18 year olds and over 19 year olds. Although there are harmless side effects such as fishy smell, indigestion, diarrhea, bloating and nausea, it is generally safe and it has been reported that EPA and DHA intake should not exceed 3 g per day (Krupa, Fritzy, & Parmar, 2023; NIH, 2023).

Omega-3 has been shown to inhibit osteoclast activity and bone resorption, while stimulating osteoblast activity and new

bone formation (Sun et al., 2003). Omega-3 has a beneficial effect on bone health by increasing Ca absorption in the intestine, increasing osteoblast differentiation and activity, and supporting mineral deposition in bone (Gad & Soliman, 2023). Diet rich in omega-3 fatty acids suppresses the inflammatory response, like non-steroidal anti-inflammatory drugs, and the inhibition of prostaglandin synthesis and suppression of the inflammatory response results in osteoclast activation and a decrease in the rate of OTM (Iwami-Morimoto, Yamaguchi, & Tanne, 1999). Gad & Soliman, (2023) evaluated the effect of oral administration of omega-3 fatty acids (200 mg/kg/day) for 21 days on OTM in rabbits and a decrease in active resorption areas was observed due to the strong osteoclastic inhibitory effect of omega-3 fatty acids. It was reported that while an increase in osteoblastic activity was observed, the use of omega-3 reduced the amount of OTM. Similarly; Ogrenim *et al*., (2019) administered oral omega-3 fatty acids (400 mg/kg/day) for 14 days. It has been reported that while omega-3 increases total antioxidant status, it decreases total oxidant status, receptor activator of NF-κβ ligand (RANKL), proinflammatory cytokine levels such as IL-6 and IL-1β, and thus slows the rate of OTM.

Coenzyme Q10 (CoQ10)

CoQ10 is a lipid-soluble, vitamin-like benzoquinone compound that is endogenously synthesized in the human body from tyrosine and functions as a coenzyme in key enzymatic reactions during cellular energy production (Bilici Gecer, 2023). CoQ10, a non-enzymatic antioxidant, is the only lipophilic antioxidant that can be synthesized de novo by cells and has enzymatic mechanisms to regenerate its reduced form (Arenas-Jal, Sune-Negre, & Garcia-Montoya, 2020). CoQ10 is the most widely used dietary supplement after fish oil and multivitamins (Yang et al., 2022). CoQ10 is not FDA-approved for the treatment of any medical condition, but is widely used as a dietary supplement over the counter. Oral formulations are available in doses from 30 to 600 mg. It has been found that CoQ10 supplements are generally well tolerated, with rare side effects such as stomach upset, nausea, vomiting and diarrhea. No toxic effects have been reported even at doses of 1200 mg/day (Sood & Keenagham, 2022)

Beneficial effects of CoQ10 have been reported in many conditions including cardiovascular disease, inflammatory disease, diabetes and cancer (Arenas-Jal et al., 2020). CoQ10 is used as a preventive and supplement in neurodegenerative diseases such as Alzheimer's and Parkinson's, which are associated with ageing and increased oxidative damage (Lopez-Lluch, Rodriguez-Aguilera, Santos-Ocana, & Navas, 2010). Recently, the use of CoQ10 in dermocosmetic products has become widespread due to its skin repair and anti-aging properties. The effects of CoQ10 and selenium on oxidative stress and inflammation in viral infections have been investigated in COVID-19 infection and it was found that they could be used as a supportive approach in the prevention and treatment of diseases (Hargreaves & Mantle, 2021).

The high antioxidant activity of CoQ10 is explained by its intramembranous localization and redox properties (Varela-Lopez, Giampieri, Battino, & Quiles, 2016). Thanks to its highly hydrophobic isoprene side chain, CoQ10 prevents the initiation of lipid, protein and DNA peroxidation by interacting with oxygen-derived free radicals and protects cells from oxidative damage by preventing damage to biomolecules (Crane, 2001). Unstable free radicals become stable with an electron from ubiquinone. The ubiquinol and semi-quinone forms of CoQ10 provide regeneration of the reduced forms of antioxidant compounds such as vitamins E and C (Kawamukai, 2002). CoQ10 has been reported to have an anti-inflammatory effect by inhibiting the expression of RANKL-dependent genes. It has also been shown to increase the peroxisome proliferator-activating receptor-dependent anti-inflammatory response and inhibit the release of cytokines such as TNF-α and IL-6 (Fan et al., 2017; Varela-Lopez et al., 2016).

Bilici Gecer, (2023) evaluated the effects of CoQ10 (100 mg/kg/day) on OTM in rats. It has been reported that CoQ10 reduces orthodontic tooth movement, reduces the number of osteoclasts due to the inhibition of ROS formation, and the morphology of osteoblasts changes to a cubic/cylindrical form, showing osteoblastic alignments, new ossification areas are prominent and wide, and bone matrix formation is more advanced. In immunohistochemical evaluation, it was stated that RANKL and vascular endothelial growth factor (VEGF) levels increased with orthodontic force application and decreased with CoQ10. At the same time, it was stated that while total oxidant status levels decreased in CoQ10 groups, total antioxidant status levels increased. Another study evaluating the effects of CoQ10 (25 mg/kg/day) for 21 days on relapse after orthodontic treatment in rabbits reported that there was no significant difference in the amount of tooth movement due to relapse after removal of the orthodontic appliance, but the number of osteoclasts decreased significantly (Madian et al., 2020).

Resveratrol

Resveratrol, an exogenous antioxidant in the group of polyphenols found naturally in a variety of foods such as grapes, grape seeds, blueberries, peanuts and red wine, is known to have anti-inflammatory, anti-carcinogenic, antioxidant, anti-aging and protective effects on the cardiovascular system and bone tissue (Liu et al., 2020; Okubo, Ishikawa, Sano, Shimazu, & Takeda, 2020). Clinical studies have shown that taking resveratrol in amounts up to 5 g per day is technically safe, but taking more than 2.5 g per day can cause abdominal side effects such as cramping, bloating, and nausea (Ramirez-Garza et al., 2018). Resveratrol increases cellular resistance to oxidative stress, supports osteogenesis by increasing the differentiation of bone mesenchymal cells, and stimulates the prolifera-

tion and differentiation of osteoblasts by inducing the production of alkaline phosphatase and bone morphogenetic protein-2 (BMP-2) (Y. Jiang et al., 2020; Xia, Daiber, Forstermann, & Li, 2017). It also inhibits receptors involved in osteoclast differentiation through RANKL and induces apoptosis of differentiated osteoclasts (Boissy et al., 2005). Resveratrol reduces the production of inflammatory mediators through inhibition of the cyclooxygenase-2 (Cox-2) cascade (Okubo et al., 2020). Resveratrol has no known significant toxic side effects (Russo, 2007) and is being promoted as a complementary alternative medicine candidate for pain management (Okubo et al., 2020).

Several studies have been conducted in the field of dentistry, reporting on the antioxidant capacity of phenolic compounds in the structure of grape seed extract and its beneficial effects on bone tissue. The effects of resveratrol on OTM and orthodontic root resorption at doses of 5 and 10 mg/kg/day for 14 days were investigated and it was observed that resveratrol significantly reduced OTM and orthodontic induced root resorption, decreased RANKL expression and increased the expression of osteoblast-related mediators such as OPG (Liu et al., 2020). Demir, (2020) reported that during rapid maxillary expansion in rats, 150-300 mg/kg/day of grape seed extract administered by oral gavage resulted in increased bone formation in the midpalatal suture, thus shortening the fixation period and preventing relapse. Okubo *et al*., (2020) reported that when resveratrol was administered to rats at a dose of 2 mg/kg (i.p.), it suppressed peripheral and/or central sensitization and reduced mechanical ectopic hyperalgesia induced by experimental tooth movement, making it a potential therapeutic agent for this purpose.

Curcumin

Curcumin is a substance derived from the root of the turmeric plant, which has the characteristic of being a yellow or orange pigment (Unlu, Nayir, Dogukan Kalenderoglu, Kirca, & Ozdogan, 2016). It is increasingly being investigated for its various therapeutic properties, including analgesic, antioxidant, anti-inflammatory and antimicrobial activities. Curcumin inhibits inflammatory cytokine production by regulating lipoxygenase activities. Inhibits RANKL activation for osteoclastogenesis (Cesur et al., 2018). According to EFSA (European Food Safety Authority) reports, up to 3 mg/kg body weight per day is allowed (EFSA, 2014). In another study, some subjects receiving 0.45 to 3.6 g/day curcumin for one to four months reported side effects such as nausea and diarrhea (Hewlings & Kalman, 2017).

Asefi, Seifi, Fard, & Lotfi, (2018) investigated the effect of curcumin (0.03 mL local injection) for 21 days on the OTM rate in rats. Curcumin had no significant effect on OTM, but significantly inhibited root and bone resorption, osteoclastic activity and angiogenesis. In another study investigating the potential effect of topical curcumin (1%) on periodontal tissues and myeloperoxidase activity in the gingival crevicular fluid (GCF) during the first phase of orthodontic movement in patients undergoing orthodontic treatment, it was reported that the curcumin gel formulation reduced myeloperoxidase activity in the GCF 14 days after arch wire placement (Samita, Verma, Sharma, Moinuddin, & Ahad, 2022).

Melatonin

Melatonin is the major pineal hormone synthesized from tryptophan. It stimulates osteoblastic cell proliferation and type I collagen synthesis. Due to its antioxidant properties, it inhibits bone resorption by influencing osteoclast differentiation and protecting bone from oxidative damage (Cesur et al., 2018). Acute toxicity of melatonin is very low in both animal and human studies. Melatonin can cause mild side effects such as headaches, insomnia, skin rashes, upset stomach, and nightmares (Malhotra, Sawhney, & Pandhi, 2004). After rapid maxillary expansion in rats, curcumin (150 mg/day/kg, i.p.) and melatonin (75 mg/day/kg, i.p.) induce new bone formation and may shorten the retention phase (Cesur et al., 2018). However, the clinical trials should be conducted before the agents can be used prophylactically in humans. In a study investigating the effect of simulated orthodontic pressure and tension forces on periodontal ligament fibroblasts, melatonin increased collagen synthesis and expression of inflammatory mediators without effects on genes involved in bone remodeling (Schroder et al., 2022).

CONCLUSION AND CLINICAL RECOMMENDATIONS

Unconscious use and high doses of antioxidants used as dietary supplements can cause oxidative damage by having a pro-oxidant effect. The use of antioxidant supplements has sometimes been found to be healthy, but studies have also shown that they are not effective in treating or preventing disease. Therefore, their protective potential has made them scientifically interesting compounds. High doses of antioxidant supplements can be harmful in some cases, as well as beneficial in orthodontic treatment, and adverse effects may be observed. It is thought that antioxidants, which are widely used today, may be effective in OTM due to their effects on oxidative stress and pro-inflammatory cytokines. In some cases, in addition to accelerating tooth movement, tooth movement should be suppressed/optimized for anchor applications that prevent unwanted tooth movement in orthodontic treatment. In addition, shortening the retention period and increasing bone formation in patients undergoing maxillary expansion are important in reducing the duration of orthodontic treatment. The effective use of antioxidants without the risk of side effects of pharmacological agents may have positive effects in clinical use as well as negative effects such as prolongation of treatment and slowing of tooth movement in unconscious use.

Antioxidants can reduce inflammation during OTM through their effects on oxidative stress and proinflammatory cytokines. Reduced expression of proinflammatory cytokines such as RANKL, IL, TNF-a may result in decreased osteoclast formation and thus tooth movement. Considering that OTM may be reduced, issues such as orthodontic treatment duration, orthodontic force activations and frequency of appointments may need to be considered in treatment planning.

Antioxidants can be effective without the risk of oral tissue damage from mechanical application and the side effects of pharmacological agents. In addition, it can support treatment efficacy in clinical applications such as inhibiting osteoclast differentiation and promoting osteoblast differentiation by regulating bone modulation, preventing relapse in orthodontic treatment, accelerating the bone formation process after maxillary expansion, and anchoring methods that suppress/optimize unwanted tooth movement. It can support the retention process in preventing relapse due to tooth movement after orthodontic treatment and accelerate the bone formation process after maxillary expansion. Local applications in anchorage methods, such as slowing/suppressing tooth movement, can help increase the effectiveness of treatment. Considering the different mechanisms of action of antioxidants and their effects on remodeling during the orthodontic treatment process, clinicians can determine at which stage of treatment they should be used and play an important role in increasing the effectiveness of treatment. In addition, considering the nutritional value of antioxidants, the patient's diet may be as important as the pharmacological drugs in the medical history. Given that OTM may be slowed with the use of antioxidants, issues such as the duration of orthodontic treatment, orthodontic force activations, and the use of antioxidants should be considered.

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Obituary

A Life Dedicated to Pharmacy Education and Science

Prof. Aysel GÜRSOY (05.08.1940-19.07.2023)

Professor Aysel Gürsoy was born in Beşikdüzü, Trabzon, in 1940. She completed her primary and secondary education in Zonguldak, and graduated from the School of Pharmacy, Faculty of Medicine at Istanbul University in 1963. In the same year, she was appointed as an assistant to the Department of Pharmaceutical Chemistry at the Faculty of Pharmacy, Istanbul University. She received her Ph.D. in Pharmacy in 1967, and became an Associate Professor in 1971 after passing the science and trial lecture exams. She was awarded the title of Professor with the joint degree numbered 20521, dated 29.5.1978. She conducted research and investigations at the School of Pharmacy, University of London, in 1967-1968. She was assigned to teach Pharmaceutical Chemistry courses at Ege University School of Pharmacy for the 1971-1973 academic years, and at Istanbul Academy of Economics and Commercial Sciences School of Pharmacy for the 1973-1974 academic year. She served as the Vice Dean of the Faculty of Pharmacy at Istanbul University for three terms (1985-1994), Head of the Department of Pharmaceutical Chemistry for one term (1998-2001), Member of the Senate of the Faculty of Pharmacy at Istanbul University for one term (1998-2000), and Dean of the Faculty of Pharmacy at Istanbul University for two terms (2000-2006). A highly respected mentor to many professors, Prof. Aysel Gürsoy made significant contributions to pharmacy education and science, particularly in pharmaceutical chemistry. Known for her unwavering commitment to fairness and objectivity, she excelled in her administrative roles, effectively resolving conflicts and building consensus through her strong leadership qualities. She showed great determination and effort in the relocation of our faculty, which was severely damaged as a result of the 1999 Marmara Earthquake Disaster, to the Faculty of Science under those difficult conditions and in the continuation of educational and training activities. Her efforts to repair the Faculty's Historic Block A and the difficulties she experienced will always be remembered. Professor Gürsoy participated in numerous domestic and international congresses throughout her distinguished career. She is a prolific author with 72 original scientific publications, most of which are in prestigious SCI-indexed journals. Additionally, she co-authored three books with Professor Nedime Ergenç and Professor Öznur Ateş. She retired from Istanbul University, Faculty of Pharmacy in 2007. She was appointed as a founding faculty member to the Department of Pharmaceutical Chemistry at Istanbul Health and Technology University, Faculty of Pharmacy in 2020. These accomplishments as a valuable scientist, faculty member, and administrator are the consequence of Prof. Gürsoy's relentless commitment to work, her dependability in human connections, and her reputation as a valued scientist. For her selfless contributions to pharmacy education and science, her efforts, the thousands of pupils she has nurtured, and the good actions she has done, our dear professor will be remembered with love, respect, and longing.

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