İstanbul Journal of Pharmacy

**INDEXING AND ABSTRACTING**

Web of Science - Emerging Sources Citation Index (ESCI)

TÜBİTAK-ULAKBİM TR Dizin

CAS Source Index

SOBİAD

EBSCO Central & Eastern European Academic Source

EBSCO Academic Search Ultimate

Cabells Journalytics

İstanbul Journal of Pharmacy

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**Publication Type:** Periodical

İstanbul Journal of Pharmacy

# EDITORIAL MANAGEMENT BOARD

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## EDITORIAL BOARD

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# INSTRUCTIONS TO AUTHORS

## AIMS AND SCOPE

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

İstanbul Journal of Pharmacy (Istanbul J Pharm) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of pharmaceutical sciences. The journal publishes original articles, short reports, letters to the editor and reviews.

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

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

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

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

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

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

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İstanbul Journal of Pharmacy

# INSTRUCTIONS TO AUTHORS

## 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.

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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%.

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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 Association 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 accepted for publication. Manuscripts submitted for evaluation 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 submitted 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.

### 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.

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

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

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

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

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

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

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

**PEER REVIEW PROCESS**

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

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

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

- Is the language acceptable?

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

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

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

**PUBLICATION ETHICS AND MALPRACTICE STATEMENT**

An approval of research protocols by the Ethics Committee in accordance with international agreements (World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects," amended in October 2013, www.wma.net) is required for experimental, clinical, and drug studies. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning 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.

All submissions are screened by a similarity detection software (iThenticate by CrossCheck) at any point during the peer-review or production process. Even if you are the author 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.

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- Principles of integrity, quality and ransparency should be sustained in designing the research, reviewing the design and conducting the research.
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- 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.
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It is authors' responsibility to ensure that the article is in accordance with scientific and ethical standards and rules. And authors must ensure that submitted work is original. They must certify that the manuscript has not previously been published elsewhere or is not currently being considered for publication elsewhere, in any lan-

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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.

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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, Editing, 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.

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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 provide 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 subunits. 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 × 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

**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 |

main text. The abbreviation should be provided in parentheses following the definition.

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

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

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

## REFERENCES

### Reference Style and Format

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

- American Psychological Association. (2010). Publication manual of the American Psychological Association (6th 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* (8th 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). T*ürkçe 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* (6th ed.). Washington, DC: Author.

**Article**

***a) Turkish Article***

Mutlu, B., & Savaşer, S. (2007). Çocuğu ameliyat sonrası yoğun bakımda olan ebeveynlerde stres nedenleri ve azaltma girişimleri [Source and intervention reduction of stress for parents whose children are in intensive care unit after surgery]. *İstanbul University Florence Nightingale Journal of Nursing, 15*(60), 179–182.

***b) English Article***

de Cillia, R., Reisigl, M., & Wodak, R. (1999). The discursive construction of national identity. *Discourse and Society, 10*(2), 149–173. http://dx.doi.org/10.1177/0957926599010002002

***c) Journal Article with DOI and More Than Seven Authors***

Lal, H., Cunningham, A. L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S.-J. ... Heineman, T. C. (2015). Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. *New England Journal of Medicine, 372,* 2087–2096. http://dx.doi.org/10.1056/NEJMoa1501184

***d) Journal Article from Web, without DOI***

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

***e) Journal Article wih DOI***

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

***f) Advance Online Publication***

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

***g) Article in a Magazine***

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

**Doctoral Dissertation, Master's Thesis, Presentation, Proceeding**

***a) Dissertation/Thesis from a Commercial Database***

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

***b) Dissertation/Thesis from an Institutional Database***

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

***c) Dissertation/Thesis from Web***

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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 submitting 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 ARTICLES

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# CONTENTS

## ORIGINAL ARTICLES



## REVIEW

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**Original Article**

# Development and validation of an RP-HPLC method for simultaneous determination of curcumin and metronidazole in combined dosage form

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**ABSTRACT**

**Background and Aims:** The present study aimed to develop and validate a simple reverse phase-high pressure liquid chromatography (RP-HPLC) method for simultaneous determination of natural compound curcumin and metronidazole in bulk and its combined dosage form.

**Methods:** *In situ* gel formulation containing curcumin and metronidazole was prepared as a model combined system. The chromatographic separation was accomplished isocratically on Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm particle size) column using UV-detection at 254 nm. The optimized mobile phase contained a mixture of Phosphate Buffer pH4.5-Acetonitrile (50:50, v/v), and the flow rate was set to 1.0 mL/min with 10 µL injection volume. The method was validated in compliance with International Council for Harmonisation (ICH) standards, and it was successfully used for quality control assays for their combined drug product

**Results:** The results for retention times were 8.60 and 1.40 min for curcumin and metronidazole, respectively. The method indicated linear responses within the concentration ranges of 3.0-80 and 4.8-128 µg/mL with LOD values of 0.62; 1.03 µg/mL and LOQ values of 1.88; 3.13 µg/mL for curcumin and metronidazole, respectively. Precision results were within acceptable limits (RSD<2%), and the determination of the two active substances was not interfered with by any formulation components.

**Conclusion:** The proposed validated RP-HPLC method was successfully applied to determine the total contents of curcumin and metronidazole *in situ* gel formulation. The validation results showed that the proposed method was simple, specific, and precise, and that it could be used for routine quality control for their combined pharmaceutical application.

**Keywords:** Curcumin, Metronidazole, Simultaneous-quantification, HPLC method development

## INTRODUCTION

Curcumin (CUR) [(E,E)-1,7-bis(4-hydroxy-3-METhoxy-phenyl)-1,6-heptadiene-3,5-ione] (Figure 1 (A)) is the main active ingredient of *Curcuma longa* (turmeric rhizome). Therapeutic use of this herbal drug has been recorded in Asian traditional medicine for over thousand years. Even at very high doses CUR is safe and has not been associated with any toxicities or adverse side effects that have been documented or studied at the population level. (Basnet & Skalko-Basnet, 2011; Berginc, Škalko-Basnet, Basnet, & Kristl, 2012; Wachter et al., 2014). However, due to its low aqueous solubility, photosensitivity, rapid hydrolysis at alkaline pH, and rapid systemic elimination, its clinical use has been limited. Novel drug delivery technologies or combinations with other drugs are commonly used in order to improve the potency of CUR (Yuan et al. 2012).

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Moreover, CUR has emerged as an appealing natural ingredient in combination therapy with potential to enhance the clinical outcomes of many antibiotics (Chanda & Rakholiya, 2011; Ejim et al., 2011; Lakshmi et al., 2016; Sasidharan et al. 2014). Combination treatment is one of the most effective ways to avoid defense mechanisms and dose-related adverse reactions. Synergistic combinations of two or more therapeutically relevant compounds that operate *via* distinct pathways increase therapeutic efficacy by allowing for a multi-target therapy strategy (Jain et al., 2016). CUR's anti-parasitic action has lately been thoroughly researched, revealing that it has a great potential to serve as an effective medication alone or in combination against a variety of parasites (Cheraghipour et al. 2018; Rangel-Castañeda et al. 2018).

Metronidazole (MET; Figure 1-B) is a highly effective broad-spectrum antibiotic used to treat gastrointestinal infections as well as sexually transmitted diseases (STDs) such as *trichomoniasis*, *giardiasis,* parasitic infections, and bacterial *vaginosis*. Topical delivery of MET has been favored due to the circumvention of its numerous drawbacks caused by the systemic administration of drug (Held, 1987; Ibrahim et al., 2012; Topal et al., 2015). Therefore, finding novel treatments that are more effective and have fewer adverse effects is critical. One of these innovative therapies is combination therapy, which employs antibiotic resistance inhibitors. Some edible natural and dietary ingredients have recently been found to improve the antiparasitic action of certain drugs such as metronidazole and artemisinin (Isacchi et al. 2012; Rangel-Castañeda et al. 2018). Moreover, CUR has been shown to protect DNA against damage and oxidative stress produced by some drugs and environmental mutagens, including MET (Singh & Giri 2013). As previously mentioned, several studies on the biological activities of CUR have been conducted, but the effects of this natural substance in combination with various antibiotics have yet to be thoroughly investigated (Teow & Ali 2015; Mun et al. 2013; Sasidharan et al. 2014).

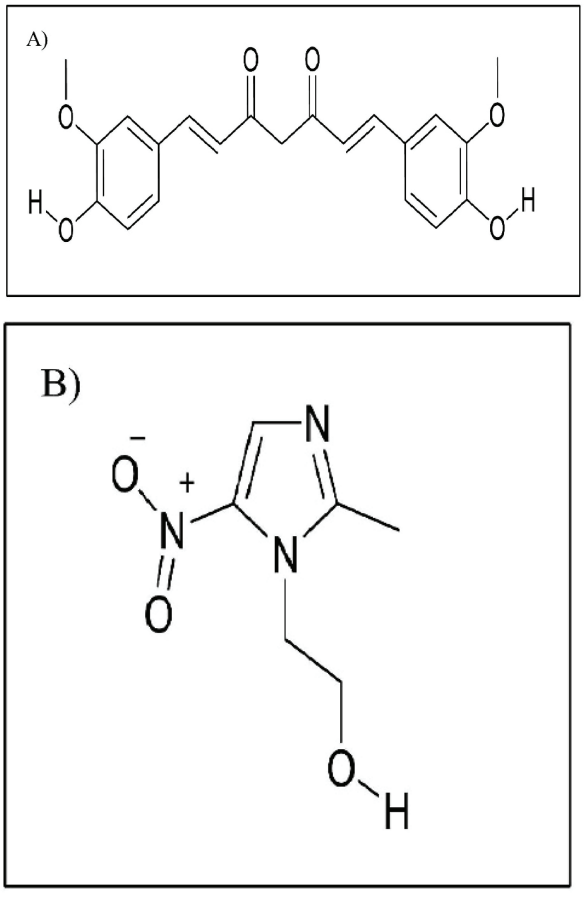


**Figure 1.** Chemical structures of CUR (A) and MET (B).

A variety of different HPLC determination methods have been proposed individually for CUR and MET in the literature (Chaudhary et al. 2012; Ji et al. 2009; USP 2015; Venkateshwaran and Stewart 1995). Since there is not any product on the market for this combination, there is yet to be a validated analytical method for the simultaneous evaluation of these drugs. The current study aimed to develop and validate a simple sensitive, precise, and reproducible RP-HPLC method for determining both active substances in bulk and in its dosage form. In this analysis, single *in situ* gel system containing CUR and MET was prepared and used as a representative formulation for aforementioned combined model system.

## MATERIAL AND METHODS

CUR was purchased from Merck (Darmstadt, Germany). MET was obtained from Ibrahim Etem Ulagay Menarini (Istanbul, Turkey). Poloxamer 188 (PLX 188), Poloxamer 407 (PLX407) and potassium dihydrogen phosphate were provided from Sigma-Aldrich (Saint-Quentin Fallavier, France). Pharmasolve® (PHR) was obtained from Ashland (Oregon, USA). Phosphate buffer pH 4.5 (PBS pH 4.5) and Acetonitrile (ACN) were HPLC grade and purchased from Merck (Darmstadt, Germany). Cellulose sterile acetate syringe filters (Pore size: 0,45µm) were obtained from ISOLAB (Eshau, Germany). Double distilled water was used for all experiments.

### Instrumentation and chromatographic conditions

This research was conducted using the Agilent 1260 Infinity HPLC system (Wilmington, DE, USA) fitted with a solvent degasser, a quaternary pump, an auto sampler, a column oven, and a diode array detector. The Agilent ChemStation software was used for the management and data processing of instrument operations. The column Eclipse XDB-C18 (5 µm, 150 mm x 4.6 mm) was used for separation. The optimized mobile phase contained mixture of PBS pH 4.5:ACN (50:50, v/v), and flow rate was set to 1.0 mL/min with 10 µL injection volume. The column oven was conditioned at 37 °C, and analysis run time took 15 minutes. 254 nm wavelength (λ max) was determined by standard scanning for CUR and MET with DAD detector between 200-400 nm. Before each injection, column was equilibrated until the UV signal, and back pressure were stabilized with the mobile phase flowing through the system.

### Standard solutions and preparation of the samples

A standard stock solution was prepared by dissolving 0.2 $mg.mL^{-1}$ CUR and 0.32 $mg.mL^{-1}$ MET in ACN. The solution was placed in an ultrasonic bath (Selecta Ultrason HD, Spain) for 30 min, aiming complete dissolution of the combination.

**Preparation and analysis of *in situ* gels with CUR and MET**

*In situ* gel system was prepared with a cold method technique (Baloglu et al., 2011b; Garala et al., 2013); PLX 407 (20%); PLX 188 (5%), PHR (15%) and, 0.7% CUR and 0.7% MET (w/w) combination were gradually added to cooled and distilled water placed in an ice bath (4 °C) with constant stirring. The final gel system (CUR-MET-Gel) was left at 4 °C for 24 h in order to ensure a full wetting and elimination of the air bubbles. The same protocol was also adopted in order to prepare a drug free *in situ* gel sample.

Following organoleptic and physical examination of the CUR-MET-Gel, critical parameters such as *sol-gel* transition temperature, gelation time, and viscosity characterized with *in situ* gel systems were also analyzed using the HR-1 Discovery Hybrid Rheometer (TA Instruments, England). This evaluation was conducted using a steel probe with a diameter of 40 mm and a set interval of 500 µm with a fixed frequency of 0.01 Hz. The change in the viscosity (Pa.s) of the samples was monitored by heating the samples at a rate of 2 °C/minute within the range of 15-50 °C. The region where viscosity changes significantly was taken as basis for determination of the *sol-gel* transition temperature and gelation time (Baloglu, Karavana, Senyigit, & Guneri, 2011a; Edsman, Carlfors, & Petersson, 1998). The pH of the CUR-MET-Gel was examined by pH–meter (Ohaus Starter 3100, USA).

**Analytical method validation**

The method was validated in scope of system suitability, linearity, limits of detection (LOD) and quantitation (LOQ), precision, accuracy, specificity, selectivity and stability in compliance with ICH guidelines (ICH 2005).

*Linearity*

Linear calibration curves of the proposed method were obtained by diluting stock solutions with mobile phase (PBS pH 4.5:ACN) (50:50) (v/v) for both of the drugs with concentrations values of 3, 5, 10, 20, 40, 60 and 80 µg/mL for CUR and 4.8, 8, 16, 32, 64, 96 and 128 µg/mL for MET. Linearity was evaluated by fitting least-squares regression analysis.

*Specificity*

The specificity was determined by assessing chromatograms of the interference of excipient(s) with CUR and MET determination. To accomplish this, chromatograms of drug free *in situ* gel solution, bulk solution with concentration of 10 µg/mL from CUR and 20 µg/mL of MET and mobile phase were injected into the chromatographic system.

*Accuracy*

The accuracy of the analytical method was confirmed by comparing the experimental results to the theoretical findings. For this objective, three sets of CUR (4 µg/mL, 12 µg/mL, and 30 µg/mL) and MET (6.4 µg/mL, 19.2 µg/mL, and 48 µg/mL) concentrations were added to the matrix samples of pH 4.5 phosphate buffer and acetonitrile (50:50) medium. The results were reported as percent recovery.

*Precision*

Precision of the system was validated in terms of repeatability, intermediate accuracy, and reproducibility. For repeatability, six individual samples at a concentration of 30 µg/mL for CUR and 48 µg/mL for MET were prepared and injected to HPLC system. Furthermore, intermediate precision was tested by preparing six solutions of the same concentration (CUR: 30 µg/mL and MET: 48 µg/mL) and checking them on two consecutive days by two different analysts. All results were evaluated in terms of standard deviation (SD) and relative standard deviation (RSD).

*Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The limits of detection and quantification value were determined based on the standard deviation (SD) of the responses and the slope (S). The equations (Eq.1 and Eq.2) were used to calculate LOD and LOQ values:

$$LOD = 3.3\ SD/S \qquad \text{(Eq.1)}$$

$$LOQ = 10\ SD/S \qquad \text{(Eq.2)}$$

*Short-term Stability of Curcumin and Metronidazole Solution*

A solution containing 70 µg/mL CUR and MET was prepared and tested for short-term solution stability. For 48 hours, the prepared solution was maintained at 37 °C. Samples were collected and analyzed after 0, 24, and 48 hours (n=3).

*Assay procedure for in situ gel formulations*

Drug contents of the CUR and MET in gel formulations were determined by dissolving an accurately weighed quantity of gel (about 500 mg) in ACN. This solution was transferred to 50 mL volumetric flask, and appropriate dilutions were made with the mobile phase (PBS pH 4.5:ACN (50:50, v/v). The solution was sonicated for 20 minutes to achieve complete dissolution of the active pharmaceutical ingredients (API) with yield concentrations 70 µg/mL for CUR and 70 µg/mL for MET. The resulting solution was then filtered through 0.45 µm syringe membrane filters (ISOLAB, Eshau, Germany) and proceed for HPLC analysis.

## RESULTS AND DISCUSSION

**Preparation and analysis of *in situ* gels with CUR and MET**

*In situ gel* system was successfully prepared with cold method. Obtained gel had a light orange color, elegant appearance, homogeneous texture, and it was free of gritty particles. Findings for *sol-gel* transition temperature and gelation time, viscosity and pH are given in Figure 2. The *sol-gel* transition temperature of stimulus sensitive gels is the temperature at which the rheological properties of the system shift its rheological behavior from *Newtonian/elasto-viscous* to *viscoelastic*. As can be seen from Figure 2 (A), the viscosity profile of the formulation decreased as the sliding speed increased at 37 °C. The gelation temperature for mucosal formulations preferred to be in range of 30-36 °C. Gelation temperatures below 30°C facilitate the forming of gel at room temperature, creating difficulties in manufacturing, handling and administration, whereas gelation temperatures above 37 °C cause gel to remain in liquid state, resulting in rapid elimination after administration (Giuliano et al., 2018). Rheological performance of topical gel formulations plays an important role in achieving maximum clinical efficacy by affecting both ease of application and re-

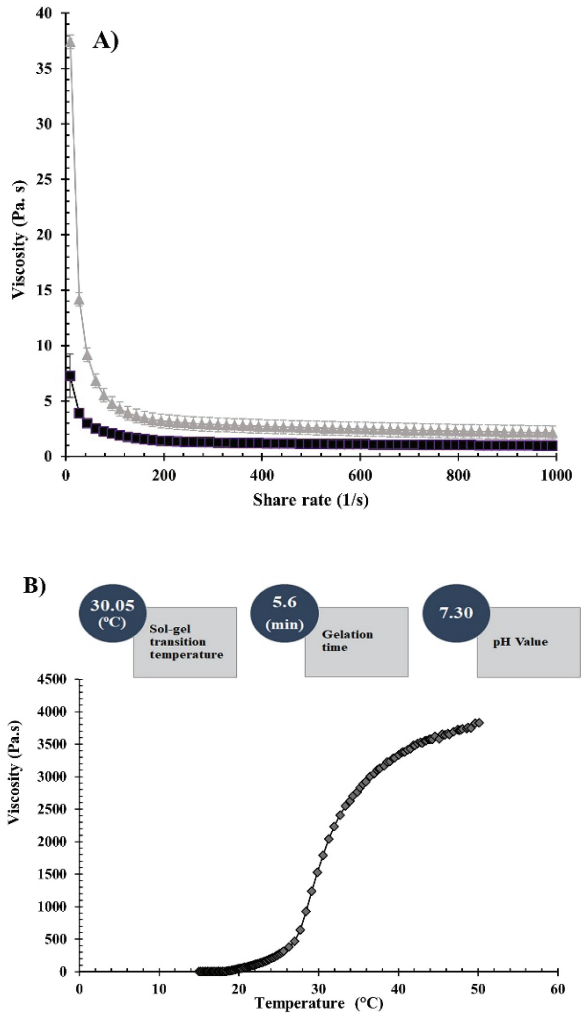


**Figure 2.** Flow rheograms measured at 37 °C (-▲-) and 25 °C (-■-) (A); Viscosity versus temperature graph, *Sol-gel* transition temperature, gelation time and pH value for CUR-MET-GEL formulation (B).

tention on vaginal surface. Prepared gel sample demonstrated suitable features for mucosal administration in terms of flow property, optimum *sol-gel* transition temperature and gelation time (30.05 °C; 5.6 min) and, pH (7.30 ± 0.08) in scope of physiological limitations (Baloglu, et al., 2011b; Yu et al., 2011).

## HPLC method development and optimization

There are various HPLC methods available in the literature for both active substances separately; based on these studies, numerous trials were conducted to develop an optimal chromatographic method for the simultaneous estimation of CUR and MET in combination. For this aim, combinations of different solvents (tetrahydrofuran: water; 0.1% ortho phosphoric acid: ACN; 0.01 M monobasic potassium phosphate buffer pH 4.5: methanol) as a mobile phase were varied to optimize the separation conditions. Knowing that for isocratic elution, a mixture of buffered solution and water-miscible organic solvents approach is frequently utilized for drug combinations. When compared to tetrahydrofuran or methanol, ACN was chosen as an organic phase since it has the lowest viscosity, strongest eluting power, and the highest selectivity for the separation of curcuminoids (Chaudhary et al., 2012; Galmier et al., 1998; Jangle and Thorat, 2013; Jayaprakasha et al., 2002; Ji et al., 2009; Venkateshwaran & Stewart, 1995). From all trailed combinations PBS pH 4.5 and ACN (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70 v/v) showed the optimum separation. Due to the form and symmetry of the peaks, the best result was achieved with PBS pH 4.5 and ACN with a ratio of 50:50. Under these conditions, MET and CUR were eluted at 1.40 min and 8.60 min, respectively, and the peaks for both APIs were specifically defined. The current approach has offered the advantage of having a relatively short run time, which allows increased production (Chaudhary et al. 2012; Jayaprakasha et al. 2002). Additionally, the lowered acid content in the mobile phase, the use of only two solvents, and the lower flow rate (1.0 mL/min) insured that the column and system would survive longer (Jangle and Thorat, 2013). Furthermore, the use of isocratic elution rather than gradient elution for this research provided low cost, simplicity, and consistency over the entire testing timeframe. The summary of the HPLC conditions, retention time and symmetry factor are presented in Table 1.

**Table 1. Data for optimized HPLC method.**

| Parameters | |
|---|---|
| Mobile phase: | Isocratic mixture: Phosphate buffer pH 4.5: acetonitrile (50:50, v/v) |
| Flow rate: | 1.0 mL/s |
| Injection volume: | 10 µL |
| Wavelengths: | 254 nm |
| Dilution solvent: | Mobile phase |
| Retention time for MET: | 1.40 min |
| Retention time for CUR: | 8.60 min |
| Symmetry factor for MET: | 0.75 |
| Symmetry factor CUR: | 0.80 |

## Analytical method validation

The Method was validated with respect to system suitability, linearity, limits of detection (LOD) and quantitation (LOQ), precision, accuracy, specificity and selectivity in accordance with ICH guidelines (ICH 2005) with additionally short-term solution stability analysis.

### *Specificity*

Methodology for specificity was found to be specific as there was no interference between the chromatograms of active substances within the gel formulation excipients or mobile phase constituents (Figure 3).

### *Linearity*

Standard lines were plotted within 3-80 and 4.8-128 µg/mL concentration ranges, with the linear regression equation y=21.946x+0.738 ($R^2$=0.999) and y=12.975x+9.434 ($R^2$=0.999) for

CUR and MET, respectively. The calculated coefficients of determination of both CUR and MET were close to 1, and standard deviation was low, which indicates that the equipment response is in proportional relationship to the drugs concentrations in the analyze. The analyses of calibration are shown in Figure 4.

*Accuracy and recovery*

The percentage of recovery results for CUR and MET are shown in Table 2. Obtained 95% confidence interval, and RSD values illustrated good precision and accuracy of the method for both APIs.

*Precision*

Neither of the peak areas changed by more than 2% for CUR and MET, suggesting that the method was highly repeatable. Intermediate precision checked by two analysts, and during the two consecutive days was evaluated by six analyses. All RSD results were lower than 2% in all assays, which meets the criteria for precision (Çelebier et al. 2010; Chaudhary et al. 2012). Tables 3 and 4 summarize the key results.

*Limit of Detection (LOD) and Limit of Quantification (LOQ)*

In order to assess the sensitivity of the method, the LOD and LOQ values were determined using Eq. 1 and 2. The obtained findings are shown in Table 5 indicating that the method is sensible enough to evaluate both CUR and MET in combination.

*Short-term stability of curcumin and metronidazole solution*

The CUR and MET solution short-term stability test findings demonstrated no change in retention time or deterioration in peak characteristics for the detected HPLC peaks. Both drugs were stable at 37 °C for 48 hours with an RSD of less than 2%.

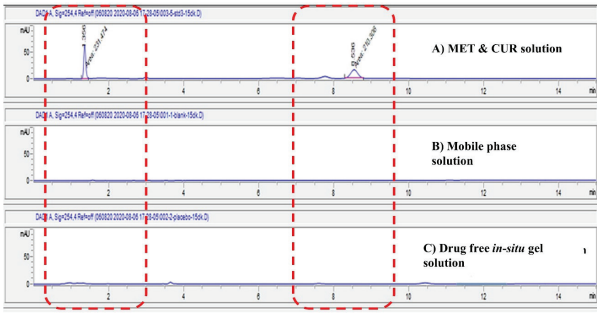


**Figure 3.** Chromatogram of (A) CUR and MET injection, (B) mobile phase solution-placebo and C) drug free *in situ* gel solution.

**Application of the Method for Assay of APIs Within the *in situ* Gel Formulation**

The proposed validated method was successfully applied to determine the total drug content of CUR and MET for prepared *in situ* gel formulation (CUR-MET-Gel). The obtained results were similar with matching labelled amounts (Table 6).

## CONCLUSION

It is well recognized that the validation process is a crucial part of the development of the analytical method. The developed

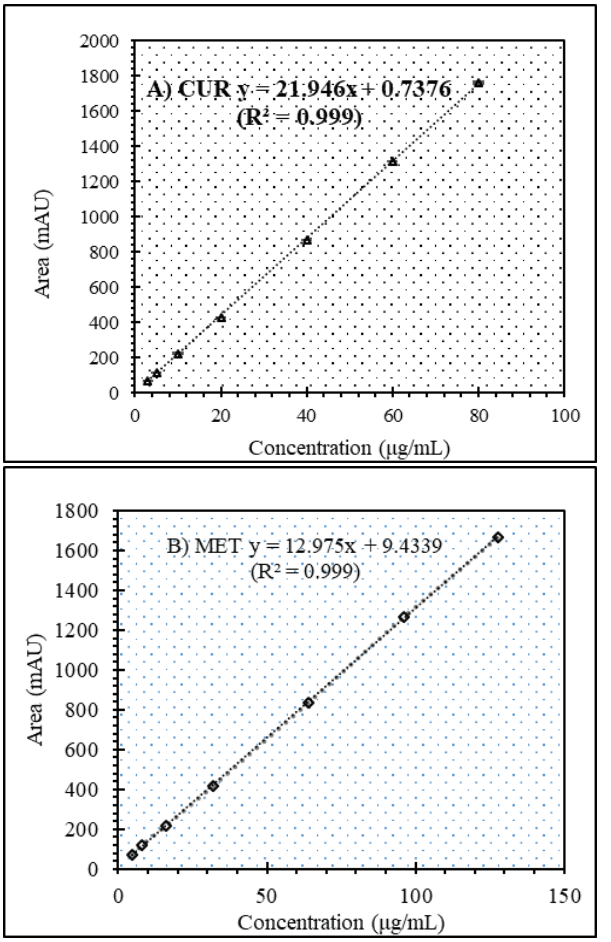


**Figure 4.** Calibration curves of CUR (A) and MET (B).

**Table 2. Recovery results for CUR and MET.**

| API | n | Theoretical Concentration (µg/mL) | Practical Concentration (µg/mL) | Recovery (%) | SD | RSD (%) | CI (95%) |
|---|---|---|---|---|---|---|---|
| **CUR** | 6 | 4.00 | 3.95 | 98.70 | 0.42 | 0.17 | 98.22-99.18 |
| | 6 | 12.00 | 12.09 | 100.78 | 2.58 | 1.05 | 98.37-104.29 |
| | 6 | 30.00 | 29.71 | 99.04 | 0.95 | 0.39 | 98.27-100.27 |
| **MET** | 6 | 6.40 | 6.81 | 106.42 | 1.10 | 0.45 | 105.15-107.68 |
| | 6 | 19.20 | 19.28 | 100.46 | 1.68 | 0.68 | 98.53-102.39 |
| | 6 | 48.00 | 49.21 | 102.52 | 1.07 | 0.44 | 101.29-103.75 |

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

**Table 3. Precision test results of CUR and MET in pH 4.5 phosphate buffer and acetonitrile (50:50) medium.**

| Sample number | AUC for CUR (30 µg /mL) | AUC for MET (48 µg /mL) |
|---|---|---|
| 1 | 649.00 | 654.20 |
| 2 | 650.40 | 650.00 |
| 3 | 648.90 | 642.20 |
| 4 | 652.80 | 640.30 |
| 5 | 645.90 | 646.40 |
| 6 | 660.70 | 650.00 |
| AVR | 651.28 | 647.35 |
| SD | 4.68 | 5.05 |
| RSD (%) | 1.91 | 1.96 |
| CI (95%) | 645.89-656.67 | 641.54-653.15 |

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

**Table 4. Intermediate precision checked by two analysts and on two different days.**

| API | | 1. Analyst | 2. Analyst | 1. Day | 2. Day |
|---|---|---|---|---|---|
| CUR (%) | Concentration: 30 µg/mL (n=6) | 99.31 | 98.90 | 99.86 | 100.01 |
| | SD | 0.62 | 0.77 | 0.59 | 0.45 |
| | RSD (%) | 0.25 | 0.31 | 0.24 | 0.18 |
| | CI (95%) | 98.65-99.96 | 98.09-99.70 | 99.24-100.48 | 99.55-100.48 |
| MET (%) | Concentration: 48 µg/mL (n=6) | 104.74 | 103.27 | 102.84 | 101.88 |
| | SD | 1.98 | 0.51 | 0.51 | 1.34 |
| | RSD (%) | 0.81 | 0.21 | 0.21 | 0.55 |
| | CI (95%) | 103.48-106.57 | 102.79-103.75 | 102.25-103.44 | 100.64-103.12 |

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

**Table 5. Limits of detection (LOD) and quantitation (LOQ) for CUR and MET.**

| | CUR (µg/mL) | MET (µg/mL) |
|---|---|---|
| **Limits of detection - LOD** | 0.62 | 1.03 |
| **Limits of quantitation - LOQ** | 1.88 | 3.13 |

**Table 6. *CUR and MET*assay for prepared *in situ* gel dosage form.**

| Prepared *in-situ* gel formulation | n | Recovery for CUR (%) ± RSD (%) | Recovery for MET (%) ± RSD (%) |
|---|---|---|---|
| **CUR-MET-GEL** | 6 | 94.00 ±0.16 | 98.00±1.25 |

RSD: Relative standard deviation

method was tested in accordance with the ICH guidelines. Regarding the validation results, the proposed method was found to be simple, specific, accurate, and precise and could be applied to the quantitative analysis of CUR along with a MET combination in a bulk solution and as well in a formulation as *in situ* gel. Furthermore, the method is suitable for regular analysis and quantitative testing of CUR and MET combinations in pharmaceutical dosage forms.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- L.B.P., E.B.; Data Acquisition- L.B.P., E.V.B.; Data Analysis/Interpretation- L.B.P., E.V.B., E.B.; Drafting Manuscript- L.B.P., E.V.B.; Critical Revision of Manuscript- L.B.P., E.B.; Final Approval and Accountability- L.B.P., E.V.B., E.B

**Conflict of Interest:** The authors have no conflict of interest to declare.

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**Original Article**

# Effects of thymoquinone and etoposide combination on cell viability and genotoxicity in human cervical cancer hela cells

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**ABSTRACT**

**Background and Aims:** It is thought that thymoquinone might have a crucial role in preventing DNA damage, regulating DNA repair mechanisms, and inhibiting the formation of a cancer. Studies on the cytotoxic and genotoxic effects of thymoquinone together with etoposide in cervical carcinoma cells (HeLa) are not adequate. The objective of this study is to evaluate the effect of combinations with thymoquinone on etoposide cytotoxicity and genotoxicity in HeLa cells.
**Methods:** Cytotoxicity was evaluated by MTT assay and genotoxicity was determined by Comet assay.
**Results:** The $IC_{50}$ values of thymoquinone were 233.6 µM and 145.5 µM, and the $IC_{50}$ values of etoposide were 167.3 µM and 52.7 µM for 24 and 48 h, respectively. Thymoquinone significantly decreased the approximate $IC_{50}$ value of etoposide in doses of 15.63 µM and above for 24 h and 31.5 µM and above for 48 h in a dose-dependent manner. 0.1-5 µM thymoquinone and 1 µM etoposide alone did not cause DNA damage, but at higher doses increased DNA damage significantly in a dose-dependent manner. Thymoquinone significantly reduced DNA damage induced by 10 µM etoposide at the doses of 0.1-10 µM.
**Conclusion:** Our results show that thymoquinone might increase the cytotoxic and genotoxic effects of etoposide in HeLa cells at high doses and reduce DNA damage at low doses that are not cytotoxic, which suggests that etoposide may increase its anticancer effect at high doses, but comprehensive studies are needed on this subject. This study is a preliminary study and will contribute to the development of new treatment strategies.
**Keywords:** Thymoquinone, etoposide, cytotoxicity, genotoxicity, comet assay, HeLa cells

## INTRODUCTION

Cancer is a leading cause of death, and it is among the global problems affecting public health and the economy. Cervical cancer ranks fourth in cancer-related deaths in women, according to the Global Cancer Observatory (GLOBOCAN) database (Sung et al., 2021). Radiotherapy and chemotherapy, capable of improving patients' survival considerably, are used in the treatment of cervical cancer (Green, Kirwan, & Tierney, 2001). Multiple drug regimens are preferred in chemotherapy due to drug resistance and drug-induced toxicity limit treatment. Nowadays, the combination of cisplatin and etoposide is one of the common chemotherapy regimens used (Salvo, Gonzalez Martin, Gonzales, & Frumovitz, 2019; Kluska & Wozniak, 2021).

Studies on this topic started to increase due to the positive effects of phytochemicals in cancer treatment. Current studies suggest that combinational chemotherapy of phytochemicals having different anticancer mechanisms may be successful (Xiaofei

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et al., 2021). The predominant macromolecular effect of etoposide, a semi-synthetic derivative of podophyllotoxin, is the inhibition of DNA-topoisomerase II enzyme or the induction of DNA strand breaks by the formation of free radicals (PubChem, 2022). Its mechanism of action is primarily in the late S and G2 phases of the cell cycle. It inhibits cell cycle progression at a premitotic phase (late S and G2), probably via inhibition of DNA synthesis. Myelosuppression is the dose-limiting toxicity of etoposide. It can cause many side effects including nausea, vomiting, diarrhea or constipation, abdominal pain, weakness, alopecia, and vision problems (Sinkule, 1984). Cisplatin, one of the most commonly used drugs for cancer chemotherapy, has a very high potential for drug toxicity. Some of the well-known adverse reactions to this drug include nausea, vomiting, renal toxicity, ototoxicity, peripheral neuropathy, hypersensitivity reactions and electrolyte disturbances. Some of the rarer reactions include hypocalcemia, headache, salivation, and dizziness (Surendiran et al., 2010).

It was suggested that thymoquinone, isolated from *Nigella sativa L.* (Ranunculaceae), may show anticancer effects by regulating different molecular targets in various cancer cells (Hafiza & Latifah, 2014). The suggested action mechanisms of thymoquinone in the anticancer treatment include increasing the production of reactive oxygen species, regulation of apoptosis, genotoxicity and inhibition of tumor angiogenesis (Shoieb, El-gayyar, Dudrick, Bell, & Tithof, 2003; El-Mahdy, Zhu, Wang, Wani, & Wani, 2005; Woo, Kumar, Sethi, & Tan, 2012; Racoma, Meisen, Wang, Kaur, & Wani, 2013). Thymoquinone was shown to inhibit proliferation, induce apoptosis and have a chemosensitizing effect by suppressing signal transducer and activator of transcription-3 activation in human multiple myeloma cells (Li, Rajendran, & Sethi, 2010). Some of the tumor suppressor genes and proteins (p53, PTEN, p21, p27, BRCA1) were found to be overexpressed or activated by thymoquinone. Moreover, thymoquinone was determined to inhibit some oncogenic signaling molecules and pathways, phosphoinositide 3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/ERK (Gali-Muhtasib, Abou Kheir, Kheir, Darwiche, & Crooks, 2004; Rahmani, Alzohairy, Khan, & Aly, 2014; Diricanet al., 2015). Recent studies revealed that thymoquinone can modulate epigenetic mechanisms, such as changing histone acetylation and deacetylation. Thymoquinone can also change genetic expression of various non-coding RNAs such as miRNA and lncRNA, which are considered key parts of cellular epigenetics (Khan, Tania, & Fu 2019).

A number of well-characterized chemotherapeutic drugs as well as several natural products with anticancer or chemopreventive properties are topoisomerase II poisons. Thymoquinone was reported to have the activity of human topoisomerase IIα due to the similarities to known topoisomerase II poisons. Results indicate that purified thymoquinone, black seed extract, and black seed oil all increase levels of enzyme-mediated DNA cleavage. This is thought to be responsible for its anticancer properties. These enzymes modulate levels of torsional stress in the genetic material and remove knots and tangles from the genome. They function by creating a transient double-strand break in one double helix and passing a separate intact DNA segment through the opening. To maintain genomic integrity while the DNA is cleaved, type II topoisomerases covalently attach to the newly generated 5′ termini of the cleaved helix. This covalent enzyme-cleaved DNA complex is known as the cleavage complex (Ashley & Osheroff, 2014).

It seems that research studies should focus on the discovery of innovative drug strategies to improve treatment outcomes in chemotherapy (Pucci, Martinelli, & Ciofani, 2019). It is claimed that thymoquinone might have an important role in preventing DNA damage, regulating DNA repair mechanisms, and inhibiting carcinogenesis. There are limited studies on the cytotoxic and genotoxic effects of thymoquinone in case of using in combination with etoposide in cervical cancer. The objective of this study is to determine the effects of thymoquinone combinations on etoposide cytotoxicity and genotoxicity in cervical cancer cell lines (HeLa cells) by MTT and alkaline Comet assay, respectively.

## MATERIAL AND METHODS

### Chemicals

The chemicals used in the experiments were purchased from the following suppliers: etoposide from Koçak Farma (Turkey); dimethyl sulfoxide (DMSO), ethanol, ethidium bromide (EtBr), L-glutamine, fetal bovine serum (FBS), low melting point (LMPA) agarose, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), normal melting point (NMPA) agarose, sodium bicarbonate, thymoquinone, trypan blue, trypsin–EDTA, and Dulbecco's phosphate-buffered saline (PBS) from Sigma (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin from Biowest (France); millipore filters from Millipore (Billerica, MA, USA); all other plastic materials from Corning (Corning Inc., NY, USA). The purity of thymoquinone is ≥ 98.5%.

### Cell culture

HeLa cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in DMEM containing low glucose (1000 mg/L) and sodium bicarbonate. The media were supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 1% penicillin-streptomycin solution (10000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C. The cells were sub-cultured in 75 cm cell culture flasks. The culture medium was changed every 3 days. The passage numbers used in our study for the cell lines were between passage 18 and passage 20.

### Determination of cytotoxicity

The effects of thymoquinone and etoposide and their combination on cell viability were determined by MTT assay (Mosmann, 1983; Hansen, Nielsen, & Berg, 1989). According to the cell viability data, $IC_{50}$was estimated. Cells were plated in 96-well plates containing 200 µL medium at a density of $1 \times 10^4$ cells/well and incubated to adhere to the plate for 24 h. The number of cells was calculated by trypan blue dye exclusion. The stock solution of thymoquinone was freshly prepared in PBS with DMSO and filtered with millipore filters (0.20 µm). DMSO concentration did not exceed 0.5% (v/v) in medium.

The cells were treated with etoposide (25-400 µM), thymoquinone (3.91-1000 µM), or the combination at the related culture medium for 24 h and 48 h. Negative control experiments were carried out with the culture medium containing DMSO (0.5%) or PBS (1%), for thymoquinone and etoposide, respectively. At the end of the incubation, 5 mg/mL MTT solution was added to each well and incubated for another 4 h at 37°C in the dark. Then the medium was discarded. The formazan crystals were dissolved in 100 µL of DMSO and absorbance of each sample was detected at 570 nm using the microplate reader (SpectraMax M2, Molecular Devices Limited, Berkshire, UK). The percentage of cell viability was calculated using the formula: "Percentage of cell viability = (The absorbance of sample/ control) x 100". The cytotoxic concentration that killed cells by 50% ($IC_{50}$) was determined from absorbance versus concentration curve.

### Determination of genotoxicity

The genotoxicity of thymoquinone and etoposide were measured in HeLa cells using Comet assay. The basic alkaline technique described by Singh, McCoy, Tice, & Schneider (1988) which is a fast and easy technique widely used in the detection of single cell DNA damage, was used for the detection of DNA damage in the cells (Collins, Dobson, Dusinka, Kennedy, & Stetina, 1997; Becit & Aydın Dilsiz, 2020). Cells were plated in 96-well plates containing 200 µL medium at a density of $1 \times 10^4$ cells/well using trypan blue dye exclusion and incubated to adhere to the plate for 24 h. HeLa cells were incubated with thymoquinone (0,1-100 µM) and etoposide (1-50 µM) at non-cytotoxic doses for 1 h (preincubation). Moreover, 0.5% DMSO was applied as a negative control. According to the Comet results obtained; the combination of 0.1-50 µM thymoquinone with 5 µM etoposide was also studied. After treatment, the cells were trypsinized and washed. The cell pellets (~$1 \times 10^4$ cells) were then suspended in 50 µL PBS to reach $1 \times 10^4$ cells/50 µL. The cell suspension mixed with 1% LMPA were then embedded on slides precoated with a layer of 1% NMPA. The slides were allowed to solidify on ice for 5 min. The cover slips were then removed. All slides were immersed in cold lysing solution (pH 10) for a minimum of 1 h at 4°C. The slides containing the cells were removed from the lysing solution, drained, and then placed in a horizontal gel electrophoresis tank filled with freshly prepared alkaline electrophoresis solution (300 mmol/L NaOH, 1 mmol/ EDTA-2Na, pH 13.0) for 20 min at 4°C to allow unwinding of the DNA and expression of DNA damage. Electrophoresis was then conducted at 4°C for 20 min at 25 V/300 mA. The slides were neutralized at room temperature by washing 3 times in neutralization buffer (0.4 mol/L Tris-HCl, pH 7.5) for 15 min. After neutralization, the slides were then incubated in 50%, 75%, and 99% of ethanol for 5 min successively. Before reading, the slides were left to dry for at least 1 day. All these steps were performed in the dark to avoid additional DNA damage. The dried microscope slides were stained with EtBr (20 µg/mL in distilled water, 30 µL/slide) and covered with a cover glass prior to analysis with a fluorescence microscope (Leica DM1000, Wetzlar, Germany) equipped with an excitation filter of 515- 560 nm. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, Version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize the DNA damage, the slides were examined at 400X. For each condition, 100 randomly selected comets from each of two replicate slides were scored (without knowledge of the group codes). DNA damage parameters were expressed as DNA tail intensity %.

### Statistical analysis

The statistical analysis was performed with SPSS 10.5 (SPSS, Chicago, IL, USA). The means of data were compared by One-way variance analysis test (ANOVA) and post hoc analysis of group differences was performed by least significant difference (LSD) test. All experiments were carried out four times at different times. The results were presented as the mean ± standard deviation (SD). A p value of less than 0.05 was considered statistically significant.

## RESULTS

### Cytotoxic effects of thymoquinone and etoposide in HeLa cells

Thymoquinone did not show significant cytotoxic effect at the doses of 3.91-125 µM and at the doses of 3.91-62.5 µM when compared to the negative control (0.5% DMSO) after 24 h and 48 h of treatments, respectively; however, the cell viabilities were significantly decreased above 250 µM and 125 µM doses of thymoquinone ($p<0.05$) after 24 h and 48 h of treatments, respectively, in a dose-dependent manner. The $IC_{50}$ values of thymoquinone were 233.6 µM and 145.5 µM after 24 h and 48 h of treatments, respectively (Figure 1).

Etoposide did not cause significant cytotoxic effect at the doses of 25 µM and 50 µM and at the doses of 25 µM when com-

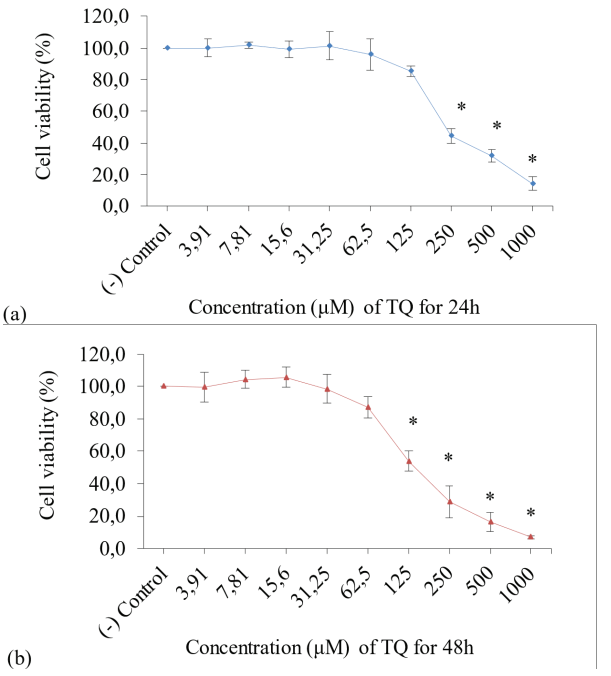


**Figure 1.** Effect of thymoquinone on HeLa cell viability for 24 h (a) and 48 h (b).*$p<0.05$, compared to negative control (0.5% DMSO). TQ: thymoquinone.

pared to the negative control after 24 h and 48 h of treatments, respectively; however, the cell viabilities were significantly decreased above 50 µM and 100 µM of etoposide ($p<0.05$) in a dose-dependent manner after 24 h and 48 h of treatments, respectively. The $IC_{50}$ value of etoposide were 167.3 µM and 52.7 µM after 24 h and 48 h of treatments, respectively (Figure 2).

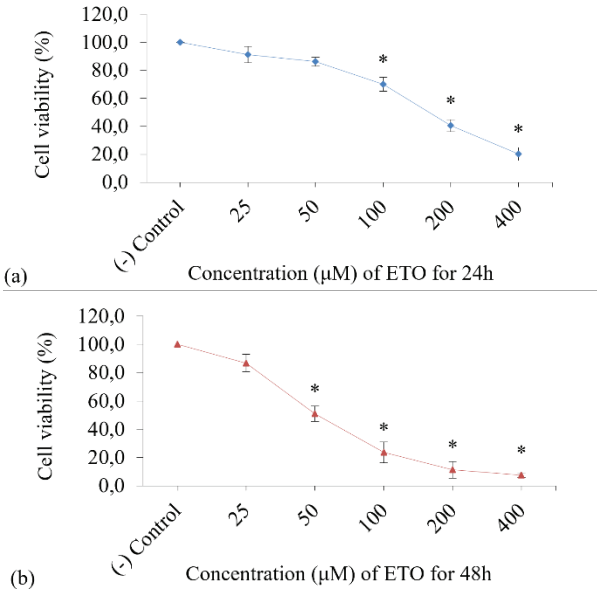


**Figure 2.** Effect of etoposide on HeLa cell viability for 24 h (a) and 48 h (b).*p<0.05, compared to negative control (0.5% DMSO). ETO: etoposide.

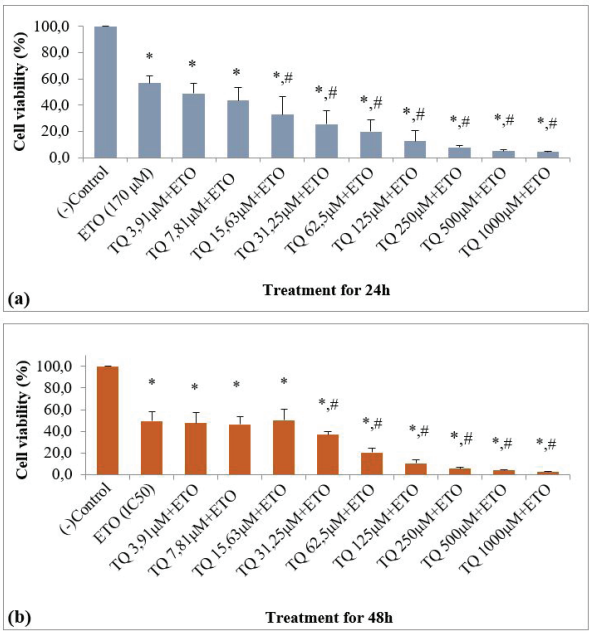


**Figure 3.** Effect of thymoquinone on etoposide cytotoxicity at 24 h (a) and 48 h (b)on HeLa cells. *p<0.05, compared to negative control (0.5% DMSO); #p<0.05, compared to etoposide (IC50: 170 µM for 24h and 50 µM for 48 h) as positive control. ETO: etoposide; TQ: thymoquinone.

### Effects of thymoquinone on etoposide cytotoxicity in HeLa cells

As shown in Figure 3 (a), thymoquinone did not change the $IC_{50}$ value of etoposide (170 µM, approximately) at the concentration ranges of 3.91-7.81 µM after 24 h of treatment; however, the $IC_{50}$ value of etoposide was significantly reduced at the concentration of 15.63 µM and above of thymoquinone (1.73, 2.22, 2.88, 4.50, 7.31, 11.69, 12.72 fold for 15.63 µM, 31,3 µM, 62,5 µM, 125 µM, 250 µM, 500 µM and 1000 µM, respectively) when compared to the negative control after 24 h of treatment ($p<0.05$). As shown in Figure 3 (b), thymoquinone did not change the $IC_{50}$ value of etoposide (50 µM, approximately) at the concentration ranges of 3.91-15.63 µM after 48 h of treatment; however, the $IC_{50}$ value of etoposide was significantly reduced at the concentration of 31.25 µM and above of thymoquinone (1.34, 2.44, 4.80, 9.32, 12.84, 12.37 fold for 31,3 µM, 62,5 µM, 125 µM, 250 µM, 500 µM and 1000 µM, respectively) when compared to the negative control after 48 h of treatment ($p<0.05$). There was no significant difference in cell viability at 48 hours of exposure compared to 24 hours of exposure. It was determined that cell viability did not change in a time dependent manner (Figure 3 a and b).

### Effects of thymoquinone on etoposide genotoxicity in HeLa Cells

The genotoxicity of etoposide at non-cytotoxic (1-50 µM) doses in HeLa cells are given in Figure 4. It was found that etoposide did not significantly change DNA damage at 1 µM concentration ($p>0.05$), but increased DNA damage significantly at 5-50 µM doses ($p<0.05$) when compared to the negative control.

The results of the evaluation of genotoxicity of thymoquinone at non-cytotoxic (0.1-100 µM) doses in HeLa cells using the alkaline Comet assay are given in Figure 4. DNA damage was evaluated in terms of DNA tail intensity. It was found that thymoquinone did not significantly change DNA damage at 1-5 µM doses ($p>0.05$), but increased DNA damage significantly at 10-100 µM doses ($p<0.05$) when compared to the negative control.

Thymoquinone significantly reduced etoposide (10 µM)-induced DNA damage at the doses of 0.1-10 µM ($p<0.05$); however, it induced DNA damage at 50 µM concentration ($p>0.05$) (Figure 5).

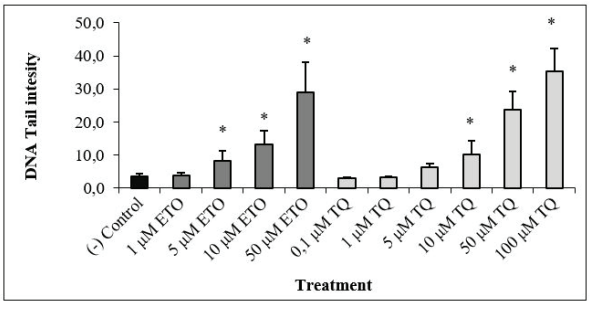


**Figure 4.** Genotoxicity of thymoquinone and etoposide in HeLa cells. *p<0.05, compared to negative control (0.5% DMSO). ETO: Etoposide, TQ: thymoquinone.

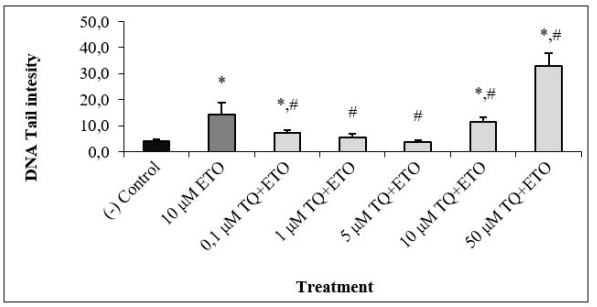


**Figure 5.** Effect of thymoquinone against etoposide-induced DNA damage in HeLa cells. [a]p<0.05, compared to negative control (0.5% DMSO); [b]p<0.05, compared to etoposide (10 µM) as positive control. ETO: Etoposide, TQ: thymoquinone.

## DISCUSSION

Cervical cancer remains a significant cause of morbidity and mortality (Sung et al., 2021). Currently, the combination of cisplatin and etoposide is a commonly used chemotherapy regimen (Salvo et al., 2019). Despite their effectiveness, however, concerns about their adverse effects and drug resistance issues continue. In addition, it is very important to avoid or minimize redundant costs in treatment. Research studies on the combination therapy with phytochemicals are increasing, with a view to increase the effectiveness of cancer treatment and reduce toxicity (Negrette-Guzman, 2019). It is suggested that thymoquinone may be promising in cancer treatment because of its anticancer and chemosensitizing properties (Mahmoud &Abdelrazek, 2019). Studies on the efficiency of thymoquinone along with etoposide in cervical cancer are not sufficient. The aim of this study was to evaluate the cytotoxic and genotoxic effects of the combination of thymoquinone and etoposide on HeLa cells viability.

In our study, the $IC_{50}$ values of thymoquinone in HeLa cells were 233.6 µM and 145.5 µM at 24 and 48 h, respectively, and the $IC_{50}$ values of etoposide were 167.3 µM and 52.7 µM at 24 and 48 h, respectively, by MTT assay. The results show that the cell viability reducing effect of thymoquinone and etoposide in HeLa cells were both in a dose- and time-dependent manner. Thymoquinone significantly decreased the approximate $IC_{50}$ value of etoposide at 15.63 µM and above doses for 24 h and at 31.25 µM and above doses for 48 h, in a dose-dependent manner. Our study shows that thymoquinone can increase the cytotoxic effect of etoposide in HeLa cells. The *in vitro* effect of TQ lowers the $IC_{50}$ of etoposide and it can be interpreted that the necessary etoposide dose for therapy can be decreased, resulting in fewer adverse effects.

In several studies, the $IC_{50}$ value of etoposide was observed to be different. The $IC_{50}$ values of etoposide for 48 h incubation of human gastric cancer (BGC-823), HeLa, and lung cancer (A549) cells were reported as 43.74 ± 5.13 µM, 209.90 ± 13.42 µM, and 139.54 ± 7.05 µM, respectively (Xiao et al., 2014). In another study, however, the viability of HeLa cells treated with 50 µM etoposide were found to be 81.6% for 48 h incubation and 37.5% for 72 h incubation (Rello-Varona et al., 2006).

Consistent with our findings, many studies demonstrated that thymoquinone might have a cytotoxic effect on various cancer cells. However, it is apparent that cytotoxic profiles may be different, depending on different methods applied and cell lines used. The $IC_{50}$ values of thymoquinone were determined by the sulforhodamine B assay as 44.8 µM and 35.1 µM, for 24 h of HeLa and hepatocellular carcinoma (HepG2) respectively (ElKhoely et al., 2015). The $IC_{50}$ values of thymoquinone in different cell lines (HeLa, SiHa, 3T3, Vero cells) were determined by MTT assay to be119.2 µM, 87.8 µM, 70.6 µM, and 21.8 µM at 24 h exposure, 72.1 µM, 52.3 µM, 69.3 µM, and 17.7 µM at 48 h exposure, and 29.6 µM, 23.4 µM, 61.7 µM, and 17.4 µM at 72 h exposure, respectively (Hafiza & Latifah, 2014). In a study conducted by MTT assay, $IC_{50}$ values for thymoquinone were determined to be 2.4 µM, 10.3 µM, and 8.3 µM, respectively, for 24 h incubation of different glioblastoma cell lines (T98G, U87MG ve Gli36DEGFR). Thymoquinone was determined to cause enzyme release from lysosomes as well as apoptotic cell death in a p53-independent and caspase-dependent manner (Racoma et al., 2013). In another study using MTT assay, the $IC_{50}$ value of thymoquinone was determined to be 25 µM for 48 h incubation of breast cancer (MCF-7) cells. It was also shown in that study that thymoquinone regulated the expression of apoptosis-related genes, (BAD, bax, and p53) (Yıldırım, Azzawri, & Duran, 2019). It was reported that $IC_{50}$ concentration of thymoquinone in lung (LNM35), liver (HepG2), colon (HT29), melanoma (MDA-MB-435), and breast (MDA-MB-231 and MCF-7) cells for 24 h was 34 µM for HepG2 and between 50 and 78 µMfor other cells, and decreased cell viability dose-dependently. In that study, high doses (100 µM) of thymoquinone were noted to have toxic effects by causing DNA damage and activating mitochondrial-proapoptotic signaling pathways (Attoub et al., 2013). The $IC_{50}$ values of thymoquinone determined by MTT assay and trypan blue dye test after 72 h in human cervical squamous carcinoma (SiHa) cells, were determined as 64 µM and 55.9 µM, respectively (Ng, Yazan, & Ismail, 2011).

We have not seen any *in vitro* studies on thymoquinone combined with etoposide in cervical cancer. Thymoquinone can exhibit a synergistic effect in reducing cell viability with anticancer drugs, such as cisplatin, that can cause DNA-damage (El Nabi et al., 2019; Pucci et al., 2019). The potentially synergistic effect of thymoquinone with topotecan was shown on human colon cancer cell lines. After determining the best combination (40 µM thymoquinone and 0.6 µM topotecan) in the study, thymoquinone was reported to increase the efficiency of topotecan by inhibiting proliferation through mechanisms independent of p53 and Bax/Bcl2 and reducing toxicity (Khalife, Hodroj, Fakhoury, & Rizk, 2016). In another study, the $IC_{50}$ values of gemcitabine, in MCF-7 cells, were shown to be significantly decreased following thymoquinone combination. It was concluded that thymoquinone showed promising chemomodulatory effects on gemcitabine in breast cancer cells by inducing apoptosis, necrosis, and autophagy (Bashmail et al., 2018).

In our study, genotoxicity profiles were evaluated by calculating the DNA tail intensity using the alkaline Comet assay. It was found that thymoquinone (at non-cytotoxic doses) did not sig-

nificantly change DNA damage at doses of 1-5 µM in HeLa cells, but significantly increased DNA damage at doses of 10-100 µM. It was found that etoposide did not significantly change DNA damage at 1 µM concentration, but increased DNA damage significantly at 5-50 µM doses. DNA damage induced by etoposide (5 µM) in HeLa cells significantly diminished at 0.1-10 µM doses of thymoquinone; however, it increased at 50 µM concentration of thymoquinone.

There are many studies on the reducing or preventing effects of thymoquinone concerning DNA damage induced by chemical substances. Khader, Bresgen, & Eckl (2009) suggested that high doses of thymoquinone might cause DNA damage by increasing oxidative stress in hepatocytes, depleting glutathione and reducing antioxidant enzymes. Gurung et al. (2010) reported that, using the Comet assay, thymoquinone did not increase DNA damage in human glioblastoma cell lines at a dose of 25 µM, but significantly increased at a 50 µM dose. They suggested that thymoquinone could induce DNA damage, telomere shortening, and cell death. The cytotoxic and genotoxic effects of thymoquinone (5, 10 and 20 µM) in human peripheral leukocytes were investigated, alone or in combination with doxorubicin (0.15 µg/mL). It was reported that thymoquinone dose-dependently increased apoptotic cell death and DNA damage determined by using the Comet assay, but reduced doxorubicin-induced apoptotic cell death and DNA damage (Al-Shdefat, Abd-ElAziz, & Al-Saikhan, 2014).

Thymoquinone was found to inhibit proliferation and migration of cancer cells by changing some apoptosis-related gene expressions, and it was suggested that thymoquinone might induce apoptosis in cancer cells (Sakalar et al., 2013). Studies claimed that thymoquinone could increase the P53 gene expression level and regulating the Bax/Bcl-2 ratio in SiHa cells (Coutts & La Thangue, 2006) and could occur via the PPAR-g activation pathway (Woo et al., 2012).

According to our results, the cytotoxic effect of etoposide could increase at doses where thymoquinone alone was not toxic in HeLa cells. Thymoquinone achieved this effect at relatively higher (31.25 µM and above) but non-cytotoxic doses, rather than at low doses. Given that thymoquinone has a low bioavailability and a very short half-life, more stable formulations of thymoquinone should be worked on in research studies. There are some limitations of this study. The effects of a triple combination could have been evaluated, by including cisplatin to thymoquinone and etoposide combination, considering the fact that cisplatin and etoposide combination is the most common chemotherapy regimen applied. In this study, in addition to examining the effectiveness of thymoquinone on the cytotoxicity of etoposide in HeLa cells, different possible cellular pathways such as apoptosis, cell cycle checkpoints, and antioxidant defense system could have been examined through more advanced techniques. Although preliminary, our results, demonstrating the complementary role of thymoquinone in improving the therapeutic efficiency of etoposide used in cancer treatment, can be considered to introduce new data to the relevant literature and present promising findings. This present study may be a pioneer for further research.

## CONCLUSION

As a result of this study, it was revealed that thymoquinone can increase the cytotoxic and genotoxic effects of etoposide in cervical cancer cells at high doses and reduce DNA damage at low but not cytotoxic doses, suggesting that thymoquinone might increase the anticancer effect of etoposide at high doses. Although thymoquinone was recognized to have a significant role of in terms of reducing side effects, increasing treatment efficiency and reducing treatment costs in the treatment of various human cancers, current data should be supported by further clinical studies. Moreover, low stability of thymoquinone due to its molecular structure should also be considered, and durable formulations of this molecule should be developed. This issue needs to be handled by further studies.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- S.A.D., H.G.N.Ç.; Data Acquisition- H.G.N.Ç., M.B.K., A.Ç.; Data Analysis/Interpretation- H.G.N.Ç., M.B.K., A.Ç. S.A.D.; Drafting Manuscript- H.G.N.Ç., M.B.K., A.Ç..; Critical Revision of Manuscript- S.A.D., H.G.N.Ç.; Final Approval and Accountability- H.G.N.Ç., M.B.K., A.Ç. S.A.D.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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Original Article

# Effect of flurbiprofen derivative (SGK597) on cell proliferation and apoptosis of breast cancer cell lines

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**ABSTRACT**

**Background and Aims:** The incidence of breast cancer is increasing day by day, especially in women. The search for new drugs against breast cancer is the focus of attention in research. Breast cancer and prostate cancer have remarkable biological similarities. Therefore, the 4-(4-chlorophenyl)-3-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-5-((4-fluorobenzyl)thio)-4H-1,2,4-triazole (SGK597) compound that is suppressing cell proliferation in prostate cancer, was studied in MCF-7 breast cancer and MCF-10A mammary epithelial cell lines.
**Methods:** The WST-8 method was used to determine cell viability and cytotoxicity of SGK597 in MCF-7 and MCF10-A cell lines. The JC-1 test was applied to determine changes in mitochondrial membrane potential. The protein expression levels of Bax, Bcl-2, and c-PARP associated with apoptosis were determined using Western blot analysis.
**Results:** After 24 and 48 hours of incubation of SGK597, the IC50 values were 28.74 µM and 17.28 µM for MCF-7; 65.9 µM and 50.5 µM for MCF-10A, respectively. Mitochondrial membrane potential showed a tendency toward depolarization in MCF-7 cells as a result of increasing concentration of SGK597, while the same tendency was not seen for MCF-10A. As a result of western blot experiments, no increase in the Bax/Bcl-2 ratio and c-PARP expression level was observed, indicating no apoptosis.
**Conclusion:** It was observed that the compound SGK597 suppressed MCF-7 cell proliferation. These results indicate that SGK597 may be a candidate compound for use as an anticancer agent.
**Keywords:** Apoptosis, breast cancer, flurbiprofen, thioether, triazole

## INTRODUCTION

Breast cancer is the most common cancer among women. More than 1.5 million women worldwide are diagnosed with breast cancer each year. The main risk factors for breast cancer in women can be listed as age, family history, and BRCA1 or BRCA2 gene mutations that are thought to be associated with breast cancer (Becker, 2015; Sun et al., 2017). The increase in the recurrence of cancer cases and the serious side effects of chemotherapeutic agents show that there is always a need to develop alternative anticancer drugs (Ali et al., 2012). While traditional chemotherapeutic drugs mostly directly target the DNA of cancer cells, recently designed new anticancer drugs target proteins with abnormal expression in cancer cells (Meegan & O'Boyle, 2019).

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The relationship between chronic inflammation and cancer is known. Therefore, it is thought that drugs that inhibit inflammation may be useful in the treatment or prevention of cancer. Inflammatory stimuli rupture arachidonic acid from phospholipids in the cell membrane via the enzyme phospholipase A2. Two isoforms of the cyclooxygenase (COX) enzyme, COX-1 and COX-2, catalyze the synthesis of various forms of prostaglandin (PG) from arachidonic acid. PGs are known mediators of inflammation. Studies in human breast cancer cell lines have shown that overexpression of COX-2 plays an important role in the pathogenesis of breast cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) have the ability to inhibit the COX enzyme and, accordingly, the synthesis of COX products. (±) (R,S)-Flurbiprofen is one of the non-selective COX inhibitor NSAIDs. By inhibiting the COX activity of the enzyme PG synthetase, flurbiprofen inhibits the production of PG. (McCormick & Moon, 1983). Studies have reported that flurbiprofen and its structural derivatives (Aydın et al., 2013; Çıkla et al., 2013) exhibit anticancer activity. When flurbiprofen was added to chemotherapy in patients with metastatic breast cancer, it was observed that these patients responded better to the treatment (Powles, Alexander, & Millar, 1978). In addition, triazole (Küçükgüzel & Çıkla-Süzgün, 2015; Çıkla-Süzgün & Küçükgüzel, 2021) and thioether derivatives (Çoruh, Çevik, Yelekçi, Djikic, & Küçükgüzel, 2017; Birgül et al., 2020; Han & Küçükgüzel, 2022) have been reported to have anticancer effects. The SGK597 compound is a flurbiprofen triazole-thioether, as in Figure 1, with the chemical structure 4-(4-chlorophenyl)-3-(1-(2-fluoro-[1,1'-biphenyl]-4yl)ethyl)-5-((4-fluorobenzyl)thio)-4H-1,2,4-triazole. This compound was synthesized by Yılmaz et al (2020). Especially MetAP-2, one of the two cytoplasmic methionine aminopeptidases in mammalian cells and known to have high expression in cancer cells, is the target protein for the SGK597 compound. It has been shown that the compound SGK597 inhibits androgen receptor-negative prostate cancer PC-3 and DU-145 and androgen receptor-positive prostate cancer LNCaP cell proliferation with $IC_{50}$ values of 27.1, 6.9 and 106.7 µM, respectively, after 24 hours of incubation (Yılmaz et al., 2020).

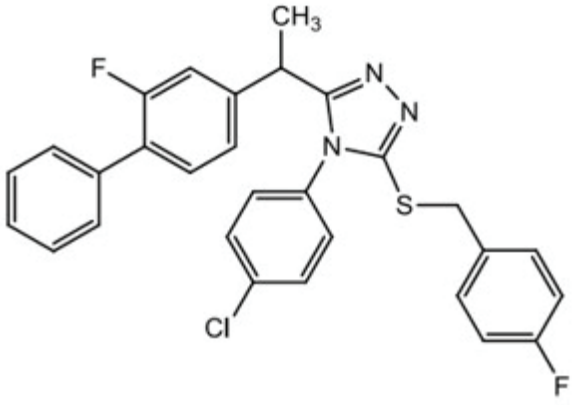


**Figure 1.** Chemical structure of Flurbiprofen derivative SGK597 (Yılmaz et al., 2020).

Prostate cancer is one of the most common invasive cancers in men. It is known that breast and prostate cancer have important biological similarities. There is clear evidence that BRCA1 and BRCA2 mutations carried by patients with a family history of breast cancer have a higher risk of prostate cancer. A family history of breast cancer in first-degree relatives has also been linked to prostate cancer in previous studies (Ren et al., 2019).

With the understanding of the pathophysiology of breast and prostate cancer, new treatment strategies have begun to be developed against breast and prostate cancer (Risbridger, Davis, Birrell, & Tilley, 2010). In an in vivo study, epigallocatechin gallate, which is abundant in green tea, inhibited the growth of human prostate and breast cancer cells and rapidly reduced their size (Liao, Umekita, Guo, Kokontis, & Hiipakka, 1995). Carboxyamidotriazole was discovered to be a selective inhibitor of breast and prostate cancer migration in vitro, and this compound is known to have entered phase III clinical trials in cancer patients (Bradke, Hall, Carper, & Plopper, 2008). Some cinnamic acid derivatives inhibited the growth of prostate and breast cancer cells by inducing apoptosis (Imai, Yokoe, Tsubuki, & Takahashi, 2019). In another in vitro study, a series of hybrid compounds based on tamoxifen, estrogen, and artemisinin were synthesized and some of these compounds were reported to have anticancer activity in human prostate and breast cancer cell lines (Fröhlich et al., 2020). Accordingly, it can be thought that compounds that may have anticancer effects against prostate cancer can also be used against breast cancer.

The aim of this study is to determine the cytotoxic and apoptotic effects of the SGK597 compound applied at different concentrations (0, 10, 25, 50, 75, 100 µM) and periods (24 and 48 h) in MCF-7 breast cancer and MCF-10A mammary epithelial cell lines using the WST-8 test, JC-1 mitochondrial membrane potential test, and Western blot.

## MATERIAL AND METHODS

### Cell culture

MCF-7 breast cancer cells in DMEM medium containing 10% FBS and 1% Pen/Strep; MCF-10A mammary epithelial cells in DMEM/F12 medium containing 5% horse serum, 0.02% EGF, 0.05% hydrocortisone, 0.01% cholera toxin, 0.1% insulin and 1% Pen/Strep were incubated at 37 °C, in an incubator containing 5% $CO_2$. The medium was changed three times a week.

### Preparation of SGK597 stock solution

96 mM stock solution was prepared by dissolving 50 mg of SGK597, supplied as a white powder, in 1 mL of DMSO. This stock was used in lower concentrations to be applied to cells later.

### WST-8 colorimetric test

The effects of SGK597 on cell viability and cytotoxicity in MCF-7 and MCF-10A cell lines were investigated using a CCK-8 kit which is based on the WST-8 colorimetric change. Into 96-well plates, 1500 cells/well were seeded and different concentrations of SGK597 were applied to the treatment group for 24 and 48 hours. Subsequently, the CCK-8 kit was applied according to the manufacturer's protocol (Cell Counting Kit-8, KTC011001, Abbkine). A microplate reader was used to detect absorbance at 450 nm after 4 hours (Synergy H1, BioTek Instruments Inc., USA).

### JC-1 mitochondrial membrane potential test

The JC-1 mitochondrial membrane potential test was carried out in MCF-7 and MCF-10A cell lines. The cells seeded in a 96-well black opaque plate and incubated with SGK597 in different concentrations (10, 25, 50, 75, 100 µM) for 48 hours were stained with JC-1 dye as recommended by the manufacturer (JC-1 Mitochondrial Membrane Assay Kit, 10009172, Cayman Chemical). The green/red fluorescence ratio was used to evaluate the apoptosis of the cells (Mega Tiber, Kocyigit Sevinc, Kilinc, & Orun, 2019).

### Western blot

MCF-7 cell pellets were lysed with a lysis solution which was prepared for each sample using 8 µL of protease inhibitor cocktail, 2 µL of NaF, 190 µL of RIPA lysis buffer (RIPA Lysis Buffer System, sc-24948A, Santa Cruz). Fifty µg of protein from each sample was run for 2 hours in SDS-PAGE under 150 V and the transfer to the membrane was provided at 25 V for 2 hours. Blocking was performed with 5% BSA. The membranes were incubated with primary and secondary antibodies to β-actin, Bcl-2, Bax, and c-PARP which were dissolved in 1X TBS-T with 1% BSA. For the detection, a chemiluminescent substrate solution was used (WesternBright ECL HRP Substrate, Advansta). Protein quantification was performed using the chemiluminescence imaging system Biostep Celvin and TotalLab 1D software.

### Statistics

For the WST-8 colorimetric test and JC-1 mitochondrial membrane potential results, one-way ANOVA analyses were followed by Dunnett's post-hoc tests. For western blot results, Kruskal Wallis analyses were followed by Dunn's post-hoc tests. The analyses were conducted using GraphPad Prism (version 8.0.1, GraphPad Software, CA, USA). The level of significance was accepted for $p < 0.05$.

## RESULTS

### Cell viability and cytotoxicity

The absorbance values for MCF-7 and MCF-10A cells after the WST-8 test are as in Figure 2 and Figure 3, respectively.

The $IC_{50}$ values calculated after the analysis of absorbance values obtained from the WST-8 test for the incubation of MCF-7 and MCF-10A cells with SGK597 at different concentrations and periods are shown in Table 1.

### Apoptosis

Mitochondria are well-known for their significance in the apoptotic process (Ly, Grubb, & Lawen, 2003). During apoptosis, the opening of mitochondrial permeability transition pores results

**Table 1. $IC_{50}$ values in µM for MCF-7 and MCF-10A cells after 24 and 48 hours of incubation with SGK597 compound.**

| SGK597 (µM) | | |
|---|---|---|
| Incubation period | 24h | 48h |
| MCF-7 | 28.74 | 17.28 |
| MCF-10A | 65.9 | 50.5 |

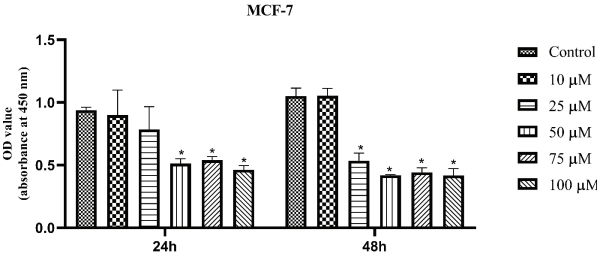


**Figure 2.** Bar chart of the absorbance values for MCF-7 cells after the incubation with SGK597 for 24 and 48 hours (n = 3) with standard deviation error bars. There was a statistically significant difference in the absorbance for the concentrations. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the absorbance for 50, 75, 100 µM at 24 hours of incubation and 25, 50, 75, 100 µM at 48 hours of incubation was significantly different from the control group, *p < 0.05. OD: optical density.

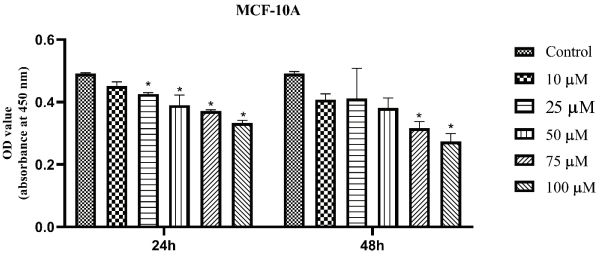


**Figure 3.** Bar chart of the absorbance values for MCF-10A cells after incubation with SGK597 for 24 and 48 hours (n = 3) with standard deviation error bars. There was a statistically significant difference in the absorbance for the concentrations. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the absorbance for 25, 50, 75, 100 µM for 24 hours of incubation and 75 and 100 µM for 48 hours of incubation was significantly different from the control group, *p < 0.05. OD: optical density.

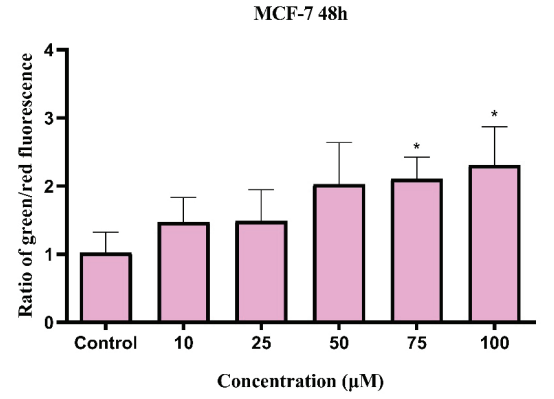


**Figure 4.** Bar chart of the ratio of green/red fluorescence for control and experimental groups in MCF-7 cells that were incubated with SGK597 for 48 hours (n = 3) with standard deviation error bars. For MCF-7 cells, there was a statistically significant difference in the ratio of green/red fluorescence for the concentrations: p = 0.036. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the ratio of green/red fluorescence for 75 µM and 100 µM was significantly different from the control group, *p < 0.05.

in a loss of the electrochemical gradient and a decrease in mitochondrial membrane potential is expected. Green/red fluorescence ratios obtained from JC-1 mitochondrial membrane potential test results at 48 h incubation of MCF-7 and MCF-10A cells with different doses of SGK597 are shown in Figure 4 and Figure 5, respectively. Red fluorescence was obtained in healthy cells and green fluorescence in apoptotic cells.

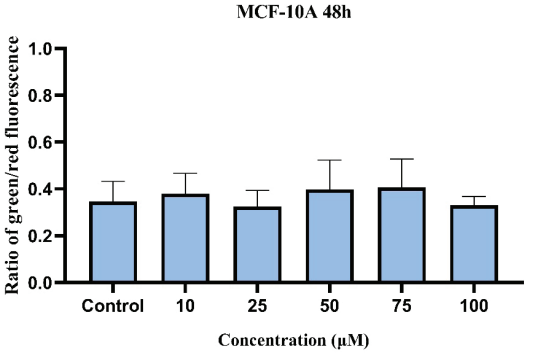


**Figure 5.** Bar chart of the ratio of green/red fluorescence for control and experimental groups in MCF-10A cells that were incubated with SGK597 for 48 hours (n = 3) with standard deviation error bars. For MCF-10A cells, there was no statistically significant difference in the ratio of green/red fluorescence for the concentrations, p = 0.822.

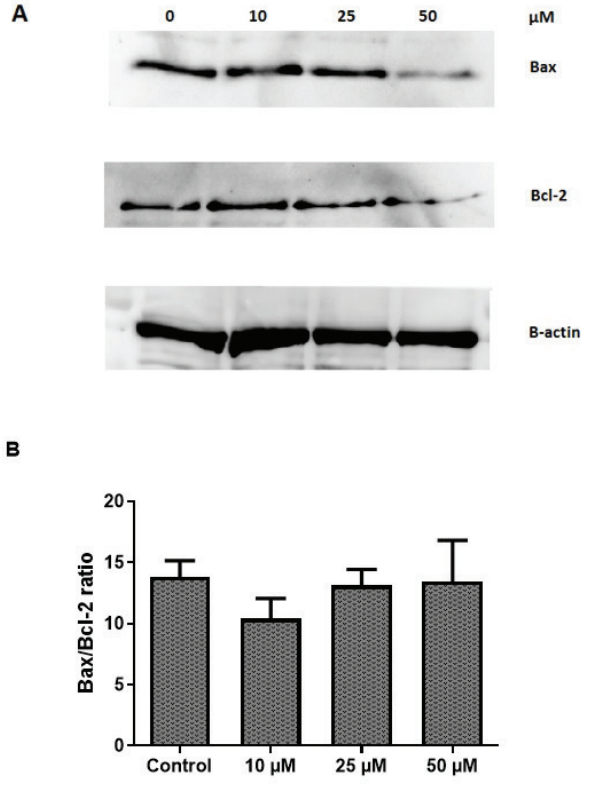


**Figure 6.** Western blot results of Bax/Bcl-2. (A) Bax/Bcl-2 protein expression with β -actin standard in MCF-7 cells that were incubated with SGK597 for 48 hours. (B) Bar chart of band intensities normalized to β-actin (n = 2) with standard deviation error bars. There was no statistically significant difference in Bax/Bcl-2 protein expression levels, p = 0.495.

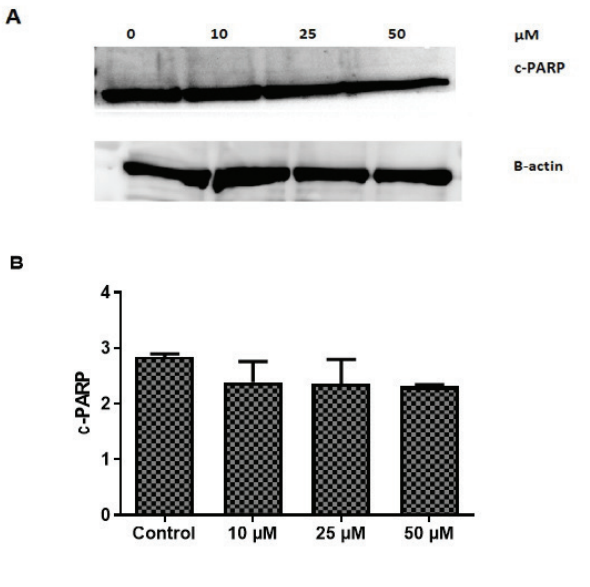


**Figure 7.** Western blot results of c-PARP. (A) c-PARP protein expression with β -actin standard in MCF-7 cells that were incubated with SGK597 for 48 hours. (B) Bar chart of band intensities normalized to β-actin (n = 2) with standard deviation error bars. There was no statistically significant difference in c-PARP protein expression levels, p = 0.343.

## DISCUSSION

It was previously determined that the flurbiprofen derivative SGK597 may have a cytotoxic effect against prostate cancer cells (Yılmaz et al., 2020). Based on previous studies, it is thought that agents showing beneficial effects against prostate cancer may also be effective against breast cancer. On this basis, we studied the cytotoxic effects of SGK597 in breast cancer cell lines. $IC_{50}$ values were calculated by applying SGK597 to MCF-7 and MCF-10A cell lines for 24 and 48 hours. The $IC_{50}$ value determined in prostate cancer cells by Dr. Kücükgüzel et al. was 27.1 µM after a 24-hour incubation period for SGK597. The fact that this value is close to the value determined for MCF-7 of 28.74 µM supports the idea that there may be a relationship between the two types of cancer. The $IC_{50}$ value was much higher in MCF-10A epithelial cells. This suggests that SGK597 applied to MCF-7 will not cause a significant effect on MCF-10A in the same incubation period. However, the $IC_{50}$ value is determined according to all cells that are dead or in the early or late apoptotic stage where cellular functions are interrupted. Therefore, the applied WST-8 test cannot show apoptosis. However, it gives an idea about the viability and metabolic activity of cells.

Although the induction of apoptosis was demonstrated by depolarization of the mitochondrial membrane potential, it was not demonstrated by the Bax/Bcl-2 ratio and c-PARP level. It is expected that the Bax/Bcl-2 ratio and c-PARP expression level will increase in cells undergoing apoptosis. According to the results obtained, the idea that SGK597 does not lead MCF-7 cells to apoptosis is strengthened. Apart from this, although there was no change in the apoptosis-related Bax/Bcl-2 ratio and c-PARP protein expression, the depolarization seen as a result of the JC-1 mitochondrial membrane potential test suggests that

there is some apoptotic stimulation in cells and that SGK597 has the potential to be used as an apoptotic agent in addition to suppressing proliferation. Western blotting is not very sensitive for quantitative evaluation and experiments need to be repeated at least three times. Further tests are needed for the exact determination of apoptotic cell death.

Studies with different proteins may show that the drug is effective on different pathways because the compound SGK597 was designed as a good MetAP-2 inhibitor. It should also be experimentally proven whether the MetAP-2 level changes in the MCF-7 cell line when SGK597 is applied. MetAPs are proteases responsible for removing methionine from the amino terminus of newly synthesized proteins. In eukaryotes, MetAP-1 and MetAP-2 are known to have MetAP activity. Current reports suggest that MetAP-2 plays an important role in the growth of different tumor types due to its role in angiogenesis. It has been reported that MetAP-2 may function as an oncogene. Therefore, this enzyme can be used against cancer cells by reducing its concentration. The TNP-470 compound, known to be a MetAP-2 inhibitor, is known to have entered human clinical trials in the treatment of metastatic breast cancer (Selvakumar et al., 2006; Selvakumar et al., 2009), and it has also been determined to inhibit the growth of MDA-MB-231 triple-negative breast cancer cells (Yamaoka et al., 1993).

In another study, human cervical cancer cell line (HeLa) and liver cancer cell line (HepG2) also showed cytotoxic, genotoxic, and apoptotic effects through the intracellular pathway after flurbiprofen treatment (Bakır et al., 2021). Also, the efficacy of flurbiprofen to suppress the growth of tumor cell lines derived from medulloblastoma and glioblastoma multiform was investigated, and it was revealed that flurbiprofen effectively inhibited the growth of various tumor cells in a dose-dependent manner (King & Khalili, 2001). Similarly, the proliferation suppressive effect of SGK597 was observed in our study. However, this observation needs to be supported by different methods to demonstrate apoptosis.

It is clear that studies on the effect of NSAIDs on breast cancer have led to conflicting results. It is not known whether these differences are due to drug design or a lack of understanding of the action mechanism of NSAIDs on the natural history of breast cancer.

In conclusion, the flurbiprofen derivative SGK597 compound showed cell proliferation suppressive properties in breast cancer cells as well as in prostate cancer cells. This study was a preliminary study for future studies.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- İ.A., P.M.T.; Data Acquisition- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G.; Data Analysis/Interpretation- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G.K.; Drafting Manuscript- İ.A., P.M.T.; Critical Revision of Manuscript- P.M.T., O.O., Ö.Y., S.G.K.; Final Approval and Accountability- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G

**Conflict of Interest:** The authors have no conflict of interest to declare.

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Original Article

# Protective effects of *Brenania brieyi* (De Wild) E.M.A.Petit root bark fractions against inflammatory-mediated hemolysis and dyslipidemia in rats

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## ABSTRACT

**Background and Aims:** The inflammatory response, though protective, is the major cause of debilitating diseases when provoked excessively or if left unresolved. *Brenania brieyi* (De Wild) E.M.A.Petit is widely used in folk medicine for the treatment of inflammatory-related diseases. This study investigated the protective effects of methanol and chloroform root bark fractions of Brenania brieyi on inflammation-induced hemolysis and dyslipidemia.

**Methods:** Anti-inflammatory activity was investigated by inserting 20 mg of autoclaved cotton pellets into forty-five rats randomly distributed into nine groups (n=5), this excluded group 1 (baseline). The extent of hemolysis and dyslipidemia in the inflamed rats was ascertained from hematological parameters, lipid profile, and lipidemic index, while the possible underlying mechanisms of inflammation were determined using standard procedures.

**Results:** Treatment with varying doses of the root bark fractions of *B. brieyi* elicited a significant ($p< 0.05$) decrease in granuloma tissue and an increase ($p<0.05$) in hemoglobin, red and white blood cell count, packed cell volume, and platelets compared with the untreated group 2. A significant ($p<0.05$) decrease in cholesterol, triacylglycerols, and low-density lipoprotein, and a non-significant ($p>0.05$) increase in high-density lipoprotein were observed in almost all the test groups compared with group 2. There was a significant restoration of atherogenic and dyslipidemia indices and inhibition of acetic acid-induced vascular permeability, membrane hemolysis, and platelet aggregation in the fraction-treated groups compared with the control.

**Conclusion:** The findings from this study suggest that *B. brieyi* inhibits exudation and proliferation of granuloma-forming cells and also has the potential to restore the hematological parameters and lipid anomalies to their physiologic state under chronic inflammation. The possible mechanisms of its action could be inhibition of vascular permeability, stabilization of the membrane, or inhibition of platelet aggregation. This justifies the use of the plant in traditional medicine and also demonstrates its potential as a target for the discovery of new anti-inflammatory agents.

**Keywords:** Acute toxicity, Anti-inflammatory activity, *Brenania brieyi,* Chronic inflammation, Hematological parameters, Platelet aggregation, Lipid profile

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## INTRODUCTION

An inflammatory response is a defense mechanism which eradicates noxious stimuli as well as initiating the tissue repair process (Kuum *et al*., 2018; Naher, Aziz, Akter, Rahman, & Sajon, 2019). In a bid to eliminate the injurious agent and restore tissue injury in the body, a network of mediators, cells, and pathways that send chemical signaling cascades are stimulated (Chen *et al.,* 2018; Altan *et al*., 2020). Paradoxically, when this response is not tightly controlled, regulated, or provoked excessively, it leads to inflammatory-induced diseases (Patil & Patil, 2017). Notably, the major predisposing factors to these diseases are not only the excessive generation of reactive oxygen and nitrogen species (Hwang & Thi, 2020), but more importantly, the provoked perturbation of blood homeostasis (Mosser, Hamidzadeh & Goncalves, 2021), and increased hydrolysis of polyunsaturated fatty acids, causing an increase in plasma lipids leading to dyslipidemia (Ogbe, Aghese &Abu, 2020). The increase in plasma lipids, especially triacylglycerols (TAG) and total cholesterol (TC), has been reported as a biomarker for the onset of cardiovascular diseases (CVD) (Aladaileh *et al*., 2019).

Due to the complex etiology of inflammation, the identification of effective therapeutic options has been a great challenge. Currently, in clinical settings, synthetic anti-inflammatory drugs such as steroidal and non-steroidal drugs are prescribed for the management of inflammatory-related diseases (Kuum *et al*., 2018; Alabi et al., 2019; Khan et al., 2021). Although these drugs transiently suppress symptoms and ameliorate inflammation, their chronic usage has severe adverse effects (Patil *et al*., 2019). Interestingly, recent studies have shown that a good percentage of the world's population relies solely on botanical preparations as medicine to meet their health needs (Fernandez-Moriano, Gomez-Serranillos & Crespo, 2016; Oloyede, Lukman & Salamu, 2020) in the management and treatment of numerous diseases, including inflammation (Majouli, Hamdi & Hlila*.,* 2017; Antonisamy et al., 2019; Majumder, Ghosh & Bhattacharya, 2020). So, in the milieu of the discovery of newer anti-inflammatory drug targets, natural products have remained of great interest (Rhetso, Seshadri, Ramnath, & Venkataramegowda, 2021) due to their better safety profile and lower cost in comparison to the increasing side effects and high cost of their synthetic counterparts (Kumar, Gupta & Singh*,* 2016; VasudhaUdupa et al., 2021).

A wide array of extensive studies have investigated the anti-inflammatory and anti-hemolytic effects of several plants on animal models (Dragomanova, Tancheva, Georgieva, & Klisurov, 2019; Patil *et al*., 2019), especially those with known folk remedies, but there is still a paucity of information about the potential of *B. brieyi* in the management of chronic inflammation-induced hemolysis and dyslipidemia. *Brieyi,* a member of the Rubiaceae family of flowering plants, is a herbal plant employed as a folk remedy in the management of several diseases, including swelling, infection, and endocrine disorders (Chukwuma, Nkwocha, Ezeanyika, & Ogugua, 2020a). In addition, a high abundance of phytoconstituents with reported anti-inflammatory activity such as squalene, hexadecenoic acids, 9-octadecanoic acids, eicosanoic acids, and pentadecanoic acids were identified in *B. brieyi* root bark (Odo, Ezeanyika, Ogugua, Joshua, & Okagu, 2017). Hence, this research was carried out to determine the protective effects of methanol and chloroform fractions of the root bark of *B. brieyi* against chronic inflammation in rats subjected to cotton pellet-induced inflammation, a model that could represent the proliferation of macrophages, fibroblasts, and neutrophils in human beings. The effects of the inflammatory cascade on hematological and lipid parameters, which reflect the extent of membrane stability, were ascertained. Additionally, the mechanisms underlying the fractions' actions were further investigated using acetic acid-induced permeability and membrane hemolysis inhibitory effects, as well as anti-platelet aggregatory tests.

## MATERIAL AND METHODS

### Collection and authentication of plant material

The *B. brieyi* root bark used for this study were collected from Njikoka, Anambra State, and identified by Mr. Felix Nwafor, a plant taxonomist in the Department of Pharmacognosy and Environmental Medicine. Voucher specimens with identification numbers PCG/UNN/0327 were deposited in his department's herbarium.

### The procedure for extraction

The root barks of *B. brieyi* were dried at room temperature, pulverized, and extracted with chloroform and methanol in a ratio of 2:1 for 48 hours under cold maceration. It was filtered using filter paper (Whatman No. 4). The filtrate was later separated into two fractions by shaking it in 0.2 mL of distilled water. Using a separating funnel, the fractions were immediately separated into a methanol fraction of *B. brieyi* root bark (MFBB, upper layer) and a chloroform fraction of *B. brieyi* root bark (CFBB, lower layer). The MFBB and CFBB were then evaporated using a rotary evaporator at 45 °C. Both fractions were stored in a refrigerator at 4 °C.

### Animals

Apparently healthy Swiss mice weighing 16.20 g ± 0.04 g and adult Wistar albino rats with an average weight of 120.11 ± 0.03 g, bought from the Animal House of the Faculty of Pharmaceutical Sciences, were used in this study. The animals were kept in a stainless steel cages in a 12 h light and dark cycle, 25 ±1 °C temperature, and given clean water and rodents' feed for at least two weeks before the procedure to acclimatize them to the environment. All the animals used were of different sexes, within a small age range, sourced from the same source, placed under the same environmental conditions, and fed the same rodent meal to eliminate confounding factors that might influence the results. This research work was done in conformity with all international and national approved guidelines on the care and use of laboratory animals as stated by the National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85-23, 1985). Ethical approval for the study was obtained from the Faculty of Biological Sciences Ethics and Biosafety Committee (Ref no: UNN/FBS/EC/1049).

### Acute toxicity ($LD_{50}$)

Acute toxicity and lethality studies of the fractions were determined using the method of Lorke (1983) using 36 mice distrib-

uted into twelve groups (six groups for each fraction) of three mice each being used for the first and second phases of the experiment. In the first phase, 3 sets of mice were orally administered 10, 100, and 1,000 mg/kg body weight of MFBB respectively via a cannula. Lethality and behavioral changes such as dizziness, irritation, jerking, and convulsion were observed for 24 h. This was followed by the administration of 1,600, 2,900, and 5,000 mg/kg b. w. of the same fraction for the other 3 sets in the second phase. Death and behavioral changes were also observed for 24 h after the administration of the test substance. The same procedure was also used to determine acute toxicity for CFBB.

$LD_{50}$ of each extract was calculated using this formula:

Where Do= highest dose that gave no mortality

D100= lowest dose that produced mortality.

**Cotton pellet-induced chronic inflammatory model**

A total of 45 male Wistar rats were randomly grouped into nine groups of five rats each and were implanted with 20 mg of autoclaved cotton pellets according to the method of Mosquera et al. (2011) with the exception of group 1, which served as the baseline. Group 2 was administered normal saline, group 3 was treated with indomethacin (10 mg/kg body weight), groups 4-6, and groups 7-9 received 50, 100, and 200 mg/kg b. w. of MFBB and CFBB, respectively, for seven days. The animals were sacrificed on the eighth day after being anesthetized with chloroform. Blood samples were collected through cardiac puncture by the principal investigator, after which the pellets were carefully removed, dried in an oven at 60 °C for 24 h, and weighed. The blood samples were used for the determination of hematological parameters and lipid profiles. The change in granuloma tissue weight was calculated as follows:

The final weight of the pellet - the initial weight of the pellet.

**Determination of hematological parameters from the serum of rats implanted with a cotton pellet**

Blood samples used for measurement of hematological parameters were transferred into EDTA (anticoagulant) bottles and used immediately to measure the full blood count using a hematology analyzer (Erma PCE 210, Japan).

**Determination of lipid profile from the serum of rats implanted with a cotton pellet**

The following procedures were used to determine the lipid profile: Total cholesterol by the Allain, Poon, Chan, Richmond, & Fu, 1974 method using Quimica Clinical Aplicada (QCA) commercial kits, triacylglycerols was determined with the Randox commercial kit using the method of Albers, Warnick & Chenng (1978), HDL was measured with Quimica Clinical Aplicada (QCA) commercial kits using Albers et al. (1978) methods, while the polyvinyl sulphate method was used to determine the LDL.

**Estimation of atherogenic/dyslipidemia indices**

The following equations were used to calculate the atherogenic/dyslipidemia indices as described by Ogbe et al. (2020).

a. Cardiac risk ratio (CRR) = $\frac{\text{Total cholesterol}}{\text{HDL}}$

b. Atherogenic coefficient (AC) = $\frac{\text{Total cholesterol-HDL}}{\text{HDL}}$

c. Classical ratio (CR) = $\frac{\text{LDL}}{\text{HDL}}$

d. Atherogenic index of plasma (AIP) = $\log \frac{\text{Triglyceride}}{\text{HDL}}$

**Mechanisms of inflammatory reactions**

The following mechanisms of anti-inflammatory activity were investigated: Acetic acid-induced vascular permeability test according to Whittle (1964), the extent of membrane stability by Shinde *et al*. (1999), and anti-platelet aggregatory activity was determined using the Born & Cross (1963) method. The percentage inhibition of the test substances (fractions/ and standard drug) were calculated relative to the control as shown in equations below:

a. Inhibition of vascular permeability (%) = $\left[\frac{AC-AT}{AC} \text{ X } 100\right]$

Where: AC = Absorbance of the control while AT = Absorbance of the fractions/test drug.

b. inhibition of membrane hemolysis (%) = $1 - \frac{OD2-OD1}{OD3-OD1} \text{ X } 100$

Where $OD_1$ = absorbance of test sample unheated, $OD_2$ = absorbance of test sample heated, and $OD_3$ = absorbance of control sample heated.

c. Inhibition of platelet aggregation (%) =

Where : AT = Absorbance of the fractions / test drug while AC = Absorbance of the control.

**Statistical analysis**

The statistical package for social science (SPSS) for windows version 23 (SPSS Inc., Chicago, IL, USA) was used to analyze the data obtained using one-way analysis of variance (ANOVA), and Tukey's *post hoc* test. $p < 0.05$ was taken as the significant threshold. The results were presented as means ± standard deviation.

**RESULTS**

**Acute toxicity study ($LD_{50}$)**

There were no observed behavioral changes or lethality in mice administered 10-1,600 mg/kg b. w of each fraction after 24 h, while sedation, weakness, and dullness were observed in mice given 2,900 and 5,000 mg/kg b. w. of both fractions. Moreover, death was recorded in mice that received 5,000 mg/kg body weight of each fraction within 24 h of administration (Table 1).

**Effects of MFBB and CFBB on cotton pellet-induced granuloma tissue formation**

Cotton pellet-induced formation of granuloma tissue was inhibited in groups 3-9 treated with different doses of the fractions and indomethacin. However, groups 4 and 6 treated with MFBB exhibited a significantly ($p < 0.05$) higher weight of granuloma tissue compared with groups 7 and 9 given the same dose of CFBB (Figure 1).

Results are presented as mean ± SD (n = 5). Mean values having '*' denotes significant difference at $p < 0.05$ compared with negative-control (normal saline).

**Table 1. Acute toxicity study ($LD_{50}$) of the root bark fraction of *B. brieyi*.**

| Dose in mg/kg body weight | Number of deaths recorded with MFBB | Number of deaths recorded with CFBB |
|---|---|---|
| **Phase 1** | | |
| 10 | 0/3 | 0/3 |
| 100 | 0/3 | 0/3 |
| 1000 | 0/3 | 0/3 |
| **Phase 2** | | |
| 1600 | 0/3 | 0/3 |
| 2900 | 0/3 | 0/3 |
| 5000 | 1/3 | 1/3 |

n=3

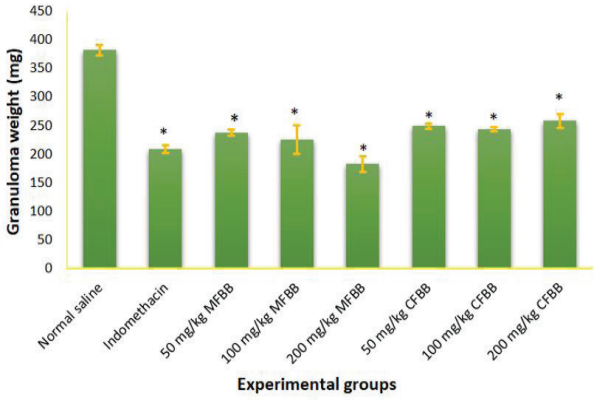


**Figure 1.** Changes in the weight of granuloma tissue formed after treatment.

### Effects of MFBB and CFBB on hematological parameters of rats implanted with a cotton pellet

A significant ($p < 0.05$) decrease in Hb, RBC, WBC, and PCV with a resultant increase in platelet count was observed in group 2 (given normal saline) after cotton pellet implantation compared with group 1. Interestingly, a significant ($p < 0.05$) concentration-dependent restoration of Hb, RBC, WBC, and platelet count occurred in rats administered varying doses of the fractions of the root bark of *B. brieyi* and indomethacin. Varied doses of MFBB were efficacious in restoring RBC, WBC, and platelet counts, compared with CFBB, but the reverse was the case with Hb and PCV (Table 2).

**Key:**

Group 1: Normal rats not implanted with cotton pellets (baseline).

Group 2: Cotton pellet + treatment with 1 ml/kg body weight of normal saline (negative control).

Group 3: Cotton pellet + treatment with 10 mg/kg body weight of indomethacin (standard drug).

Groups 4, 5, and 6 rats were treated with 50, 100, and 200 mg/kg body weight of MFBB, respectively, after cotton pellet implantation.

Groups 7, 8, and 9 were treated with 50, 100, and 200 mg/kg body weight of CFBB, respectively, after cotton pellet implantation.

### Effects of MFBB and CFBB on lipid profile indices of rats implanted with a cotton pellet

Implantation of cotton pellets significantly ($p < 0.05$) altered the lipid profile indices in group 2 when compared with group 1. Interestingly, groups 3, 4, 5, and 6, treated with indomethacin (50 mg/kg) and MFBB (50, 100, and 200 mg/kg) respectively, had a significant ($p < 0.05$) decrease in cholesterol, TAG, and LDL when compared with group 2, except in high-density lipoprotein, which showed a significant ($p < 0.05$) increase only in groups 3 and 6. Moreover, only 200 mg/kg b. w of CFBB was effective in attenuating all the lipid indices significantly ($p < 0.05$) compared with group 2. The MFBB was found to be more potent in decreasing cholesterol and LDL compared with CFBB, whereas the reverse was the case with TAG (Figure 2).

**Table 2. Effects of MFBB and CFBB on concentrations of some serum hematological parameters of rats implanted with a cotton pellet.**

| Groups | Hb (g/dl) | RBC (x $10^6$/l) | WBC (x$10^3$/l) | PCV (%) | Platelets (x$10^6$/l) |
|---|---|---|---|---|---|
| 1 | 25.14±2.22 | 5.44 ±1.37 | 9560.00±219.09 | 45.20±5.63 | 135.00±9.35 |
| 2 | 13.24±1.23# | 3.36 ±1.08# | 7860.00±219.09# | 25.00±3.67# | 110.00±14.58# |
| 3 | 22.44±2.09* | 5.24 ±1.75*# | 6760.00±167.33*# | 48.00±2.92* | 116.00±12.94# |
| 4 | 19.70± 1.08*# | 4.34 ±0.58*# | 9120.00±228.04*# | 34.20±4.60*# | 198.00±12.55*# |
| 5 | 22.38±2.45* | 5.00 ±1.12*# | 12440.0±260.77*# | 36.80±5.54*# | 169.00±4.18*# |
| 6 | 22.94±0.36* | 6.02 ±2.74*# | 14480.0±109.54*# | 44.20±3.49* | 125.00±6.12* |
| 7 | 23.40±1.27* | 3.68 ±1.84*# | 8280.00±109.54*# | 40.20±5.35* | 133.00±12.04* |
| 8 | 23.68±1.61* | 4.72 ±1.18*# | 10040.0±167.33*# | 44.80±3.27* | 150.00 ±7.90*# |
| 9 | 24.88±1.18* | 5.24 ±1.58*# | 9720.00± 178.89* | 46.00±0.71* | 113.00±12.55*# |

Results are presented as mean ± SD (n=5). Mean values with '#' denotes significant difference at $p < 0.05$ compared with baseline while '*' denotes significant difference at $p<0.05$ compared with negative-control.

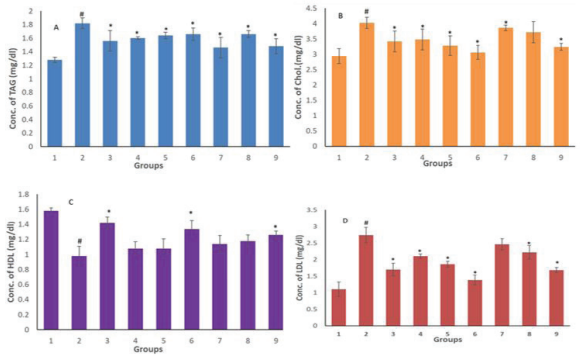


**Figure 2.** Effects of root barks fractions of *B. brieyi* on concentrations of serum lipid profile indices of rats implanted with cotton pellet.

TAG (A), Chol. (B), HDL (C) and LDL (D) stands for triacylglycerol, cholesterols, high density lipoprotein and low density lipoprotein respectively. Values are presented as mean ± SD (n = 5). Mean values with '#' denotes significant difference ($p < 0.05$) compared with baseline while '*' denotes significant difference ($p < 0.05$) compared with negative-control.

**Key:**

Group 1: Normal rats not implanted with cotton pellets (baseline).

Group 2: Cotton pellet + treatment with 1 ml/kg body weight of normal saline (negative control).

Group 3: Cotton pellet + treatment with 10 mg/kg body weight of indomethacin (standard drug).

Groups 4, 5, and 6 rats were treated with 50, 100, and 200 mg/kg body weight of MFBB, respectively, after cotton pellet implantation.

Groups 7, 8, and 9 were treated with 50, 100, and 200 mg/kg body weight of CFBB, respectively, after cotton pellet implantation.

### Effects of MFBB and CFBB on atherogenic /dyslipidemia indices in rats

There was an increase in CRR, AC, CR, and AIP in groups implanted with cotton pellets compared with group 1 (normal rats). Interestingly, significant dose-dependent restoration of dyslipidemia was observed in almost all the fractions treated groups when compared with the untreated control (group 2). The inhibitory effects of the standard drug were found to be comparable with groups administered 200 mg/kg b. w of both fractions. However, the highest inhibitory effects of cardiac risk ratio (CRR), atherogenic coefficient (AC), and classical ratio (CR) were recorded in group 6 administered 200 mg/kg b. w of MFBB (Table 3).

**Key:**

Group 1: Normal rats not implanted with cotton pellets (baseline).

Group 2: Cotton pellet + treatment with 1 ml/kg body weight of normal saline (negative control).

Group 3: Cotton pellet + treatment with 10 mg/kg body weight of indomethacin (standard drug).

Groups 4, 5, and 6 rats were treated with 50, 100, and 200 mg/kg body weight of MFBB, respectively, after cotton pellet implantation.

Groups 7, 8, and 9 were treated with 50, 100, and 200 mg/kg body weight of CFBB, respectively, after cotton pellet implantation

### Effects of MFBB and CFBB on acetic acid-induced vascular permeability in rats

A significant inhibition of vascular permeability, which was in a dose-dependent manner, was observed in the rats administered with both fractions. The inhibitory effect (72%) of group 6, given 200 mg/kg of CFBB, was significantly ($p < 0.05$) higher compared with the 69.3% inhibition observed in group 4, given the same dose of MFBB. However, the percentage inhibition (78%) of vascular permeability in the group given the standard drug, indomethacin, was significantly higher compared with

**Table 3. Effects of MFBB and CFBB on atherogenic/dyslipidemia indices in rats implanted with a cotton pellet.**

| Groups | Atherogenic/dyslipidemia indices in rats | | | |
|---|---|---|---|---|
| | CRR | AC | CR | AIP |
| 1 | 1.86 (54)* | 0.86 (72)* | 0.70 (75)* | 0.09 (133)* |
| 2 | 4.10 (-) | 3.10 (-) | 2.80 (-) | 0.27 (-) |
| 3 | 2.41 (41)* | 1.41 (55)* | 1.20 (57)* | 0.04 (85)* |
| 4 | 3.22 (21)* | 2.22 (28)* | 1.94 (31)* | 0.17 (37)* |
| 5 | 3.04 (26)* | 2.04 (34)* | 1.72 (39)* | 0.18 (33)* |
| 6 | 2.28 (44)* | 1.28 (59)* | 1.03 (63)* | 0.09 (67)* |
| 7 | 3.39 (17) | 2.39 (22) | 2.16 (23)* | 0.11 (59)* |
| 8 | 3.15 (23)* | 2.15 (30)* | 1.88 (33)* | 0.15 (44)* |
| 9 | 2.57 (37)* | 1.57 (49)* | 1.33 (53)* | 0.07(74)* |

Values are presented as the mean of 5 rats. Percentage changes (%) in mean values were calculated relative to control and enclosed in parenthesis. Values with * are significantly ($p < 0.05$) different from control.
CRR, AC, CR, and AIP denote cardiac risk ratio, atherogenic coefficient, classical ratio, and atherogenic index power respectively.

all the groups administered with different doses of both fractions (Table 4).

**Effects of MFBB and CFBB on heat-induced hemolysis of human red blood cells**

The fractions inhibited heat-induced hemolysis of RBC in a reverse concentration-dependent manner. However, MFBB provoked a significantly ($p < 0.05$) higher membrane stabilization potential across all concentrations assayed compared with CFBB and indomethacin (Table 5).

**Effects of root barks fractions of *B brieyi* on platelet aggregation induced by $CaCl_2$**

Both fractions inhibited *in vitro* platelet aggregation induced by $CaCl_2$ in a manner comparable with the standard drug, indomethacin. The highest anti-aggregatory activity was recorded at the highest time assayed (150 sec.). The results in Table 6 also reveal that 200 and 400µg/ml of indomethacin reduced platelet aggregatory response to $CaCl_2$ in a concentration and time-dependent manner. The inhibition of platelet aggregation by the plant was comparable with that of indomethacin.

**Table 5. Effects of MFBB and CFBB on heat-induced hemolysis of human red blood cells.**

| Treatments | Conc. (µg/ml) | % inhibition of HRBC hemolysis |
|---|---|---|
| Control | - | 0 |
| MFBB | 100 | 89.08 |
| | 200 | 84.62 |
| | 400 | 86.32 |
| | 600 | 87.12 |
| | 800 | 83.92 |
| CFBB | 100 | 74.76 |
| | 200 | 71.86 |
| | 400 | 64.36 |
| | 600 | 61.65 |
| | 800 | 61.96 |
| Indomethacin | 200 | 75.36 |
| | 400 | 77.46 |

Results of reported as mean ± SD of triplicate absorbance determination. The inhibition of HRBC hemolysis (%) was calculated relative to control.

## DISCUSSION

The extent of exudation and proliferation as a result of tissue degeneration and fibrosis under chronic inflammatory response is measured by the cotton pellet-induced granuloma model (Kumar et al., 2016; Misra, Varma & Kumar, 2018). Hence, inhibition of granuloma formation by the fractions suggests its potency in offering protection against chronic inflammation, which is recognized as the predisposing factor for the pathogenesis of various forms of cancer (Patil et al., 2019). This suggests that the fractions inhibited abnormal permeability of the vascular tissues and mobilization of inflammatory cells and mediators. It could be possible that the bioactive components present in the plant, which demonstrated antioxidant and anti-inflammatory properties (Chukwuma et al., 2020b; Chukwuma et al., 2021), inhibited the release of inflammatory mediators such as prostaglandin, thereby hindering proliferation of granuloma-forming cells like macrophages, fibroblasts, and neutrophils (Kumar et al., 2016). Lending credence to this also is the high phenolic content found in the plant, which has been reported to inhibit the expression of pro-inflammatory genes (Chukwuma et al., 2020a).

A prolonged inflammatory response is associated with modification of hematological parameters. Hence, monitoring hematological indices under chronic inflammatory diseases helps to ascertain the extent of tissue damage. The observed decreases in Hb, PCV, and RBC counts in groups implanted with cotton pellets reflect the presence of anemia. Also, the excess release of ROS in inflammatory reactions degrades hemoglobin and lipid components of cells. Interestingly, the significant restoration of Hb, RBC, WBC, and PCV in groups treated with the fractions when compared with group 2 might be due to antioxidative compounds found in the fractions which inhibited the release of ROS (Odo et al., 2017; Chukwuma et al., 2020a; Chukwuma et al., 2020b) and hence, preserved the integrity of the cell membrane from hemolysis of RBC. This concurs with the report of Haddouchi, Chaouche, Saker, Ghellai, & Boudjemai (2021) who also studied the antioxidant potential of polyphenolic compounds. Moreover, the increase in WBC count suggests the potential of the plant in maintaining the integrity of the rats' immune system while the observed increase in

**Table 4. Percentage inhibition of vascular permeability by MFBB and CFBB.**

| Groups | Treatments | Dosage (mg/kg) | Absorbance (610 nm) | Inhibition (%) |
|---|---|---|---|---|
| 1 | Control | - | 0.274 ± 0.006 | 0 |
| 2 | Indomethacin | 50 | 0.059 ± 0.005* | 78.47 |
| 3 | MFBB | 100 | 0.128 ± 0.006* | 53.28 |
| 4 | | 200 | 0.084 ± 0.003* | 69.34 |
| 5 | CFBB | 100 | 0.144 ± 0.003* | 47.45 |
| 6 | | 200 | 0.076 ± 0.004* | 72.26 |

Results are presented as mean ± SD n = 5. The absorbance of the treatment groups was used to calculate % inhibition relative to control. Absorbance with '*' is significantly different ($p < 0.05$) compared with the control.

**Table 6. Effects of MFBB and CFBB on platelet aggregation induced by $CaCl_2$.**

| Conc. (µg/ml) | % inhibition of platelet aggregation at different time intervals | | | | | |
|---|---|---|---|---|---|---|
| | 0s | 30s | 60s | 90s | 120s | 150s |
| **MFBB** | | | | | | |
| 100 | 50.37 ± 4.01 | 50.00± 3.20 | 50.13 ±2.71 | 50.52 ± 3.21 | 50.53± 2.34 | 50.53 ± 2.17 |
| 200 | 48.97 ± 3.75 | 48.39 ± 2.89 | 48.52 ± 4.50 | 48.64 ± 2.90 | 48.63± 2.10 | 48.77± 4.00 |
| 400 | 42.49 ± 5.31 | 42.34 ± 3.42 | 42.47± 2.01 | 42.90 ± 1.78 | 42.68± 1.90 | 42.64± 1.21 |
| 600 | 17.08 ± 2.65 | 15.04 ± 1.87 | 15.49± 3.42 | 15.63 ± 2.92 | 17.55± 2.17 | 15.00± 3.10 |
| 800 | 28.67 ± 1.09 | 38.02 ± 3.40 | 27.38 ± 3.11 | 27.59 ± 3.12 | 27.41± 3.20 | 27.51 ± 4.12 |
| **CFBB** | | | | | | |
| 100 | 5.69 ± 0.21 | 7.25 ± 1.23 | 7.23 ± 0.98 | 7.35 ± 2.96 | 6.93± 0.18 | 6.03± 0.97 |
| 200 | 24.05 ± 2.47 | 21.95 ± 2.00 | 21.72 ± 3.17 | 22.22 ± 1.32 | 21.99± 3.19 | 21.43± 4.12 |
| 400 | 34.11 ± 3.33 | 33.33 ± 1.34 | 33.45 ± 2.00 | 33.92 ± 3.45 | 34.27± 2.14 | 34.15± 2.93 |
| 600 | 48.71 ± 4.52 | 49.07 ± 2.05 | 49.20 ± 1.15 | 49.33 ± 5.10 | 49.46± 3.71 | 49.46± 1.87 |
| 800 | 56.46 ± 2.90 | 57.05 ± 3.11 | 54.63 ± 5.12 | 57.34 ± 0.78 | 57.37± 2.09 | 57.40 ± 3.12 |
| **INDO** | | | | | | |
| 400 | 34.96 ± 4.53 | 30.93 ± 2.18 | 27.65 ± 1.26 | 27.02 ± 1.23 | 26.56± 3.11 | 25.49 ± 1.23 |
| 600 | 47.76± 3.90 | 41.46 ± 4.57 | 36.96 ± 2.13 | 36.15 ± 2.30 | 35.17± 1.90 | 35.51 ± 2.17 |

Indo. Stands for indomethacin. Percentage inhibition of platelet aggregation was calculated relative to control.

platelets suggests its wound healing properties since platelets are known to be involved in the healing of damaged tissues (Anyasor, Okanlawon & Ogunbiyi, 2019).

A chronic inflammatory response activates acute phase proteins that alter lipid metabolism, resulting in a decrease in HDL, impairment of reverse cholesterol transport, changes in apolipoproteins, and changes in cholesterol efflux regulatory proteins (Essawy, Abo-elmatty, Ghazy, Badr, & Sterner, 2014; Esteve, Ricart, & Fernández-Real, 2005). Normalization of these lipid anomalies in this study was demonstrated by the decreases in total cholesterol, TAG, and LDL and an increase in HDL after treatment. This potency could be attributed to the antioxidant compounds identified in the plant in the preliminary studies by Odo et al. (2017) which previous studies have reported to be antioxidant molecules (Chakraborty et al., 2021). Notably, squalene found in the fraction is a cardioprotective agent, an enhancer of WBC, and increases fecal excretion which reduces the concentration of cholesterol (Odo et al. 2017). Also, the most abundant compound found in the fraction, 9-octadecanoic acid (oleic acid), helps in preventing atherosclerosis due to its efficacy in lowering LDL (Nkwocha, Odo & Umeakuana, 2019). In the same vein, a previous study by Chukwuma et al. (2021) demonstrated the phospholipase A2 inhibitory effects of the plant, which prevents the breakdown of the lipid membrane. So, this suggests that MFBB and CFBB could be very helpful in reducing the onset of cardiovascular disease since studies have shown that a significant lowering of LDL-cholesterol and a rise in HDL-C are reliable biochemical biomarkers for the prevention of atherosclerosis and ischemic conditions (Ikumawoyi, Awodele, Rotimi, & Fashina, 2016).

Emerging evidence has shown that chronic inflammatory diseases orchestrate the atherosclerotic vasculopathy involved in the pathophysiology of cardiovascular diseases (CVD) (Acay et al., 2014). The use of lipid profiles alone to determine the prevalence and severity of CVD has been questioned. Hence, the use of atherogenic/dyslipidemia indices, mainly atherogenic index of plasma (AIP), which estimate the balance between atherogenic and other non-atherogenic factors, has proven to be a better predictor of CVD than lipid profile (Acay et al., 2014; Ogbe et al., 2020). The observed decrease in CRR, AC, CR, and AIP in this study suggests ameliorating effects of the fractions in averting inflammatory-induced dyslipidemia. The decrease in atherogenic/dyslipidemia indices in this study could be attributed to a decrease in LDL and an increase in HDL. Lipids accumulate in macrophages during inflammation to form lipid foam cells. These cells form fatty streaks when they accumulate in the walls of the arteries, causing atherosclerotic plague (Esteve, Ricart, & Fernández-Real, 2005). Agents that subvert infiltration of inflammatory cells into the adipose tissues help to prevent excessive production of cytokines and adipose lipids which potentiate these lipid metabolism disorders (Esteve *et al*., 2004).

The release of immune cells and mediators in the presence of a stimulus dilates the blood vessels to enhance the mobilization of vascular components to the inflamed region (Chen *et al*., 2018; Altan *et al*., 2020). This study investigated the inhibitory effects of MFBB and CFBB on a vasodilator, acetic acid. Acetic acid stimulates mast cells, which enhances the release of inflammatory agents responsible for dilating blood vessels such as prostaglandins, histamine, serotonin, bradykinin, and leukotrienes (Kumar *et al*., 2016; Patil *et al.,* 2019). The observed inhibition of vascular permeability by MFBB and CFBB suggests

that they could suppress the exudative phase of inflammation, which would avert tissue damage. This potency could also be due to its inhibitory effect on phospholipase A2 and prostaglandin synthase, which hinders the release of inflammatory mediators including A2, PGD2, PGE2, and PG12, involved in vaso-dilation (Chukwuma et al., 2021). Perhaps the bioactive compounds found in the fractions stabilize cell membranes, as shown in their high inhibition of heat-induced membrane stabilization study. Conversely, the plants ability to inhibit membrane hemolysis may be due to their high antioxidant activity, as demonstrated in the plants' *in vitro* and *in vivo* antioxidant studies (Chukwuma et al., 2020a, Chukwuma et al., 2020b). Plants with antioxidant activity have been reported to be key anti-inflammatory drug targets since they prevent the leakage of fluid into the peritoneum, thereby suppressing inflammation. This will also avert the biochemical cascade involved in chronic inflammation, such as granuloma tissue formation.

Furthermore, an increase in thromboxane and platelet-activating factor production, which causes aggregation of platelets, is a marker of inflammation and a pharmacological target in the management of inflammatory diseases (Sokeng, Rokeya, Hannan, Ali, & Kamtchouing, 2013; Gros, Ollivie & Ho- Tin-Noe, 2014). Platelet aggregation helps in cellular hemostasis. However, excessive aggregation of platelets in inflammatory reactions leads to thrombotic diseases (Chukwunelo et al., 2019). Interestingly, both MFBB and CFBB had anti-platelet aggregatory capacity. Platelet aggregation can be inhibited through the inactivation of intracellular signaling pathways or by blocking membrane receptors (Mykola, Ganna & Gennadiy, 2015). This suggests that the fractions inhibited activation of COX-1, thereby limiting the synthesis of thromboxane and platelet-activating factors from arachidonic acid. Hence, the fractions could help in circumventing factors that predispose one to chronic inflammation-induced diseases such as cardiovascular diseases.

## CONCLUSIONS

The results of this study show that the root bark fractions of *B. brieyi* inhibited the exudation and proliferation of granuloma-forming cells, thereby limiting the formation of granuloma tissues. It also demonstrated the potential to inhibit hemolysis and hyperlipidemic aberrations of blood cells and membrane lipids, which could be responsible for normalizing hemolytic and hyperlipidemic anomalies in inflamed rats. This could be due to its ability to inhibit vascular permeability, membrane hemolysis, and platelet aggregation, limiting excessive infiltration of inflammatory exudates that can cause membrane peroxidation and cell damage. This suggests that it has anti-inflammatory activity and efficacy in restoring body homeostasis under inflammation. This justifies the use of the plant in traditional medicine for the management of inflammatory diseases and also opens the window for its usage as a target for the discovery of new anti-inflammatory agents.

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Original Article

# Gene expression profiles for apoptotic and necrotic pathways during *Amanita phalloides* intoxication in mice

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**ABSTRACT**

**Background and Aims:** *Amanita phalloides* is the deadliest toxic mushroom in the world and causes death from acute liver failure. α-amanitin (α-AMA), the most potent toxin, inhibits RNA polymerase II in hepatocytes, stops protein synthesis, and causes hepatotoxicity. However, the information about the mechanisms underlying hepatotoxicity caused by α-AMA is quite inadequate. This study aims to reveal the complex necrotic and apoptotic mechanisms occurring in mouse hepatocytes depending on *A. phalloides* exposure time *in vivo*.

**Methods:** BALB-c male mice were divided into 5 groups (n=7): control, α-AMA-2, α-AMA-12, α-AMA-72, and α-AMA-96 groups. A poisoning model was created by oral administration of *A. phalloides* mushroom extract containing 10 mg/kg of α-AMA to mice and they were sacrificed after 2, 12, 72, and 96 h. Then, *TNF-α, Bax, caspase-3*, and *Bcl-2* gene expression levels in liver tissues were examined by the RT-qPCR method. Time-dependent damage to liver tissues was also evaluated histopathologically.

**Results:** RT-qPCR results showed that proinflammatory cytokine TNF-α mRNA expression levels increased in mouse liver tissues at 2 and 12 h after *A. phalloides* administration compared among the groups. *Bax* mRNA expression levels increased in the 12 and 72 h after *A. phalloides* ingestion. It was observed that *caspase*-3 mRNA expression levels increased in the 72 and 96 h groups compared among the groups, while *Bcl*-2 mRNA expression levels decreased in the 72 and 96 h groups.

**Conclusion:** Our findings showed that necrotic mechanisms develop in the early period after *A. phalloides* mushroom poisoning, and then apoptotic mechanisms are effective. In conclusion, understanding the mechanisms of *A. phalloides*-induced hepatotoxicity will provide important information for new treatment strategies to be developed.

**Keywords:** α-amanitin, *TNF-α, Bax, caspase-3, Bcl-2*, RT-qPCR

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## INTRODUCTION

*Amanita phalloides* (Vaill.) Link species is the deadliest toxic mushroom in the world, resulting in acute liver failure, and is responsible for more than 90% of deaths from mushroom poisoning worldwide (Vetter, 1998). The clinical properties of *A. phalloides* poisoning are the development of liver necrosis, which leads to the development of the hepatorenal phase. Patients gradually lose their liver and kidney functions, and hypoglycemia, delirium, and confusion may develop (Becker et al., 1976). Approximately 20-79% of poisoned patients develop chronic liver disease, but it is unknown how many *A. phalloides* consumed are lethal to humans (Serné et al., 1996; Yilmaz, Ermis, Akata, & Kaya, 2015).

There is typically a 6-24 h delay between ingestion of the mushrooms and the onset of gastrointestinal symptoms (GIS) (latency phase). The first symptoms are GIS complaints and the symptoms may persist for several days. During the next 24-36 h, acute hepatic failure and subsequent multi-organ injury are revealed by both clinical and laboratory measurements (hepato-renal phase) (Escudié et al., 2007; Karlson-Stiber & Persson, 2003). In fatal poisonings, death usually occurs after 5 or 6 days (median time is 6.1 days) (Ganzert, Felgenhauer, & Zilker, 2005). The severity of poisoning seems to be related to the amount of toxin taken in proportion to body weight (Enjalbert et al., 2002; Jan, Siddiqui, Ahmed, Ul Haq, & Khan, 2008). The severity of liver damage, the rate of hepatic regeneration, and an effective treatment of poisoning increase the survival rate of patients (Alldredge B, 2012). Currently, no radical treatment has been found for *A. phalloides* mushroom poisoning (Garcia et al., 2015).

Amatoxins are known as the toxins responsible for fatal mushroom poisoning and are mainly responsible for the severe liver damage observed after *A. phalloides* poisoning (Yilmaz et al., 2015). α-amanitin (α-AMA) is the most toxic substance among amatoxins and it is a bicyclic octapeptide compound with high molecular weight, heat stability, and water solubility (Kaya E, 2012; Wieland & Faulstich, 1978). α-AMA has been the most known and causes hepatocellular failure due to hepatotoxicity. The main toxic mechanism of α-AMA is non-competitive nuclear inhibition of RNA polymerase II in eukaryotic cells, thereby inhibiting protein synthesis. As a transcription inhibitor, α-AMA disrupts/stops many mechanisms in cells. Decreased mRNA levels lead to decreased protein synthesis, the development of necrotic mechanisms, and ultimately cell death (Lindell, Weinberg, Morris, Roeder, & Rutter, 1970; Wieland, 1983).

Apoptosis is the orderly programming of cell death during normal development and is controlled by different intrinsic regulatory pathways. Mechanisms that inhibit DNA transcription, such as α-AMA, induce cell cycle arrest or apoptosis in response to certain cellular stresses. α-AMA is a potent inducer of apoptosis, and apoptotic mechanisms are thought to play an important role in the pathogenesis of hepatic injury during *A. phalloides* poisoning (Y. Arima et al., 2005). *In vitro* studies have shown that apoptosis may play an important role in severe α-AMA-induced liver injury, as observed in canine primary hepatocytes (Magdalan, Ostrowska, Piotrowska, Izykowska, et al., 2010) and human hepatocyte cultures (Magdalan, Ostrowska, Piotrowska, Gomułkiewicz, et al., 2010; Magdalan et al., 2011). It has been reported that α-AMA induces apoptosis by acting synergistically with tumor necrosis factor-α (*TNF-α*), but the underlying mechanisms are not yet known. Also, α-AMA initiates apoptotic mechanisms by inducing p53 protein, which triggers apoptosis (Y. Arima et al., 2005). It has also been suggested that destroying the mitochondrial membrane potential is important in the development of severe hepatotoxicity by α-AMA (Wang et al., 2018). It has been reported that after high-dose α-AMA *in vivo* administration, it increases hepatic pro-inflammatory *TNF-**α*** mRNA levels, apoptosis develops in hepatocytes, and liver damage is prevented in mice treated with anti-TNF-α antibodies (Leist et al., 1997). However, the dependence of α-AMA toxicity on the presence of *TNF-**α*** has not been confirmed in another study using hepatocyte cultures (Magdalan, Ostrowska, Piotrowska, Izykowska, et al., 2010). *TNF-**α*** induces hepatocellular apoptosis but it has also been reported to induce necrosis in *in vivo* models of inflammatory liver injury (Tiegs & Horst, 2022). Induction of necrosis and apoptosis by α-AMA is a complex process and understanding the cellular processes that cause liver injury is clinically crucial.

Today, there is still an increase in cases of poisoning caused by deadly mushrooms and, a radical treatment has not yet been found for *A. phalloides* intoxication (Ertugrul Kaya et al., 2016). Commonly used treatments are carried out only with palliative treatment. The development of effective new therapeutic alternatives is extremely important to improve the survival of patients poisoned with *A. phalloides*. Thus, this study aimed to investigate the complex apoptotic [*Bax* (Bcl-2-associated X protein), *caspase-3* (cysteine-aspartic acid protease-3) and *Bcl-2* (B-cell lymphoma-2)] and necrotic [*TNF-**α*** (tumor necrosis factor-α)] mechanisms at the gene expression levels that occur in mouse hepatocyte cells based on exposure time to α-AMA cytotoxicity. In this way, the molecular mechanisms underlying hepatic damage caused by α-AMA have been attempted to be clarified. Thus, our findings may contribute to developing new treatment strategies for patients poisoned by *A. phalloides*.

## MATERIAL AND METHODS

### *A. phalloides* mushroom collection

*A. phalloides* mushrooms were collected from the forest areas of Gümüşova (Düzce, Türkiye). The collected mushrooms were systematically identified by examining their macroscopic properties (Figure 1).

### *A. phalloides* mushroom extraction

Mushrooms were dried under 50-60°C airflow for 24 h and ground into powder. Ten grams of *A. phalloides* was placed in 150 mL solvent (methanol, water, 0.01 M HCl (5:4:1, v/v/v), homogenized with a sonicator, and incubated for 24 h. Then, the solution was centrifuged for 5 min at 5000 rpm and the supernatant was filtered by syringe filters (0.22 µL) and extracted in 150 mL of 50% methanol for 4 h in a Soxhlet apparatus. The obtained extracts were evaporated in a vacuum evaporator at 50°C until completely dry (E. Kaya et al., 2015).

### Determination of quantity of α-AMA

In the analytical HPLC system, an α-AMA standard (1 mg/mL, Sigma Aldrich, St. Louis, MO, USA) was diluted in $dH_2O$ at 10, 20, 100, 200, 1000, and 2000 ng/mL concentrations, and a 6-point calibration curve (repeated 3 times ) was created. The calibration curve was linear over the desired concentrations ($R^2 > 0.99$). The chromatographic method was performed as reported by Kaya et al (E. Kaya et al., 2015; E. Kaya et al., 2013). Analysis of the mushroom extract was performed on a Reversed-Phase High-Performance Liquid Chromatography system (RP-HPLC, Shimadzu, Japan) and RP-HPLC conditions were as follows: 150 x 4.6 mm, 5 µm particles, C18 column (Agilent Technologies, Palo Alto, CA) with 302 nm at the DAD detector. The mobile phase [0.05 M ammonium acetate (pH 5.5)/acetonitrile (90:10 v/v)] was used with 1 mL/min flow rate. The detection limit was determined as 2 ng/g and the amount of α-AMA was calculated as the mean ± SEM in 1 g of dry mushroom.

Briefly, 1 mL of the *A. phalloides* water extract was applied to the semi-preparative RP-HPLC. From the beginning to the end of the peak at the same retention time as the α-AMA standard, the fraction was collected by the collector (4.6 x 250 mm C18 ODS column with 5 µm particles was used). The obtained fraction was dried in a vacuum evaporator at 50°C, dissolved in 1 mL of 40% methanol, and reapplied to the preparative HPLC system for the second purification. Then, 20 µL of α-AMA was applied to the analytical HPLC system to measure the purity and amount of toxin in that fraction.

The amount of substance was measured by applying the peak areas obtained in the analysis to the equation of the calibration curve. The obtained pure α-AMA was dissolved in 1 mL distilled water after the solvent was evaporated, and quantitative analysis was performed. Based on analysis of the results obtained, *A. phalloides* extracts dissolved in distilled water containing 20 mg of α-AMA were prepared.

**Figure 1.** *Amanita phalloides.*

## Animals and treatments

Male BALB-c mice (weighing 20-30 g) were obtained from Dicle University Health Sciences Application and Research Center (DÜSAM). The permission of Dicle University Experimental Animals Local Ethics Committee (DUHADEK-2021/45) was obtained and all animal experiments were performed according to the instructions of the Local Ethics Committee. The animals were housed under conditions of constant temperature (22±3°C) and humidity (50-55%), a 12 h light/dark cycle, and free access to food and water. After a 1-week adaptation period, the animals were randomly divided into five groups (n = 7).

The animal model of intoxication has been used as a reliable model for α-AMA poisoning, since it shows similar hepatotoxic effects after amatoxin administration in humans (Tong et al., 2007; Zhao et al., 2006). In addition, toxin concentrations and experimental design in the *in vivo* study were designed from a clinical perspective, based on available information on clinical toxicity practices and α-AMA pharmacokinetics. The experimental design is shown in Figure 2. *A. phalloides* mushroom water extract was given orally through stomach gavage at a concentration of 10 mg/kg α-AMA (Garcia et al., 2015; Park et al., 2021; Wieland & Faulstich, 1978). Mice were starved for 24 h before the start of the experiments. In the control group, a single dose of 1 mL of 0.9% physiological saline was administered to the mice via orogastric gavage at 0 min. In the α-AMA-2, α-AMA-12, α-AMA-72, α-AMA-96 groups, *A. phalloides* mushroom water extract, which included 10 mg/kg α-AMA, was

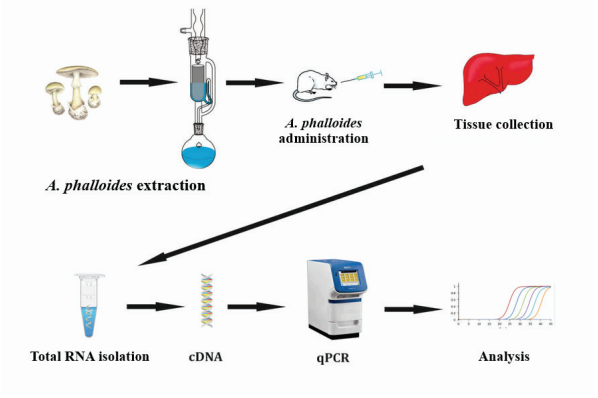


**Figure 2.** Experimental Design.

**Table 1. Primer sequences**

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| ***Bax*** | GGATGCGTCCACCAAGAAG | GGAGGAAGTCCAGTGTCCAGCC |
| **Bcl-2** | TGAGTACCTGAACCGGCATCT | GCATCCCAGCCTCCGTTAT |
| ***caspase-3*** | TGCAGAACAAAACCTCAGT | TGTCTCTCTGAGGTTGGCTG |
| ***TNF-α*** | AAATGGGCTCCCTCTCATCAGTTC | TCTGCTTGGTGGTTTGCTACGAC |
| ***GAPDH*** | ACTCCACTCACGGCAAATTC | TCTCCATGGTGGTGAAGACA |

administered to the mice via orogastric gavage at 0 min. The animals were sacrificed and their livers were taken at 2, 12, 72, and 96 h, respectively. At the end of the experiments, the mice were sacrificed under anesthesia [Ketamine (90mg/kg) + Xylazine (10mg/kg)]. The liver tissues were stored at -80°C for RT-qPCR analysis. A part of the liver was also fixed in 10% zinc-formalin solution for 24 h and then embedded in paraffin for routine histopathologic analysis.

### Quantitative real-time polymerase chain reaction (RT-qPCR) assay

To investigate the molecular mechanisms of apoptotic and necrotic pathways caused by *A. phalloides*, *TNF-α*, *Bax*, *caspase-3* and *Bcl-2* gene expression levels were measured by Real Time-Polymerase Chain Reaction (RT-qPCR, Applied Bioscience StepOnePlus™, Foster City, CA). RNA purification was performed from liver tissues stored at -80°C. Total RNA was isolated from tissues using RiboZol™ (VWR-Amresco, USA) according to the manufacturer's instructions. The quantity and quality of RNA samples were measured using a microvolume spectrophotometer (Nano-Drop 2000C, Thermo Scientific, USA). Genomic DNA contamination was removed with the DNAse-I digestion enzyme (Thermo Fisher, USA). cDNA synthesis was performed using the cDNA Synthesis Kit (Thermo Fisher, USA) according to the manufacturer's instructions. The sequences of primer pairs used for qPCR analysis were designed using Primer3 software and based on the sequences in the NCBI database (Table 1). For RT-qPCR amplification, a 20 µL volume of reaction mix was prepared with 10 µL of 2X SYBR Green Master Mix, 0.2 µm primer, and 1 µL of cDNA. Real-time PCR thermal cycling conditions were performed by initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, 1 min reassembly at 60°C, and an extension period for 30 sec at 72°C. Melting curve analysis was performed by increasing the temperature by 1°C at each step from 55°C to 95°C. Samples without cDNA were used as a negative control. The housekeeping gene (GAPDH) expression was used as an internal control for normalization. For each cDNA sample, PCR amplifications were performed in triplicate and relative mRNA expressions were evaluated by the comparative Ct method ($2^{-\Delta\Delta CT}$) described by Schmittgen & Livak (Schmittgen & Livak, 2008).

### Histopathological assessment

For light microscopic evaluation, liver tissue samples were fixed in 10% formaldehyde and embedded in paraffin. Then, 5-µm-thick sections were cut from the paraffin-embedded samples, mounted on slides, and stained with hematoxylin and eosin (H-E). The tissue samples were examined using a light microscope. The sections were evaluated for liver damage such as inflammation, sinusoidal dilatation, necrosis, congestion, and pyknotic nucleus.

### Statistical analysis

The One-way ANOVA was performed with the IBM SPSS Statistics 24.0 (IBM Inc, Chicago, IL, USA) statistical software. Post hoc Tukey test was used for between-group comparison of *Bax*, *Bcl-2*, *caspase-3*, and *TNF-α* gene expressions. The data of this study are given as the mean ± SEM. A value of $p<0.05$ was accepted as statistically significant among groups.

## RESULTS

The retention times for β-AMA, α-AMA, and γ-AMA in the RP-HPLC chromatogram were 4.818, 6.219 and 12.577 min respectively. The RP-HPLC chromatogram of α-AMA analysis is given in Figure 3.

### Time-dependent expression of *TNF-α*, *Bax*, *caspase-3*, and *Bcl-2* genes after *A. phalloides* administration

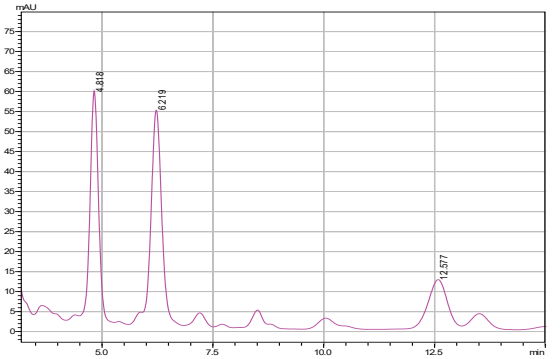


**Figure 3.** Analytical HPLC chromatograms of α-AMA.

The results of RT-qPCR clearly showed that pro-inflammatory cytokine *TNF-α* mRNA expression was increased in α-AMA-2 ($p < 0.01$) and α-AMA-12 ($p < 0.05$) groups after *A. phalloides* administration (Figure 4), suggesting that exposure to *A. phalloides* induces inflammation in hepatocytes. *TNF-α* expression at the mRNA levels decreased gradually at α-AMA-72 and α-AMA-96 groups after *A. phalloides* administration compared to the α-AMA-2 group.

While *Bax* mRNA expression level was upregulated in the α-AMA-12 and α-AMA-72 groups compared to the control

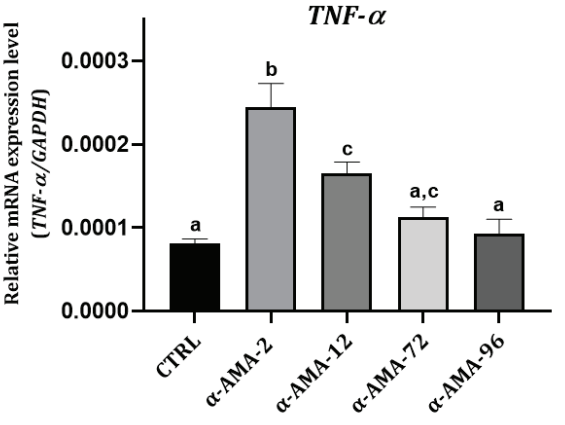


**Figure 4.** Time-dependent expression of *TNF-α* gene after *A. phalloides* administration. Mice were treated with *A. phalloides* extract including 10 mg/kg α-AMA. *TNF-α* gene expression was analyzed by RT-qPCR. Different letters mean significant differences between groups. [b]$p < 0.01$ compared to control, α-AMA-72 and α-AMA-96 groups; [c]$p < 0.05$ compared to control, α-AMA-2 and α-AMA-96 groups.

group and α-AMA-2 ($p < 0.01$), expression of the *Bax* gene was downregulated in the α-AMA-96 group compared to the α-AMA-12 and α-AMA-72 groups ($p < 0.01$) (Figure 5). These results indicate that apoptosis is induced 12 h after *A. phalloides* administration compared to that in healthy untreated mice. However, *Bax* mRNA expression level decreased at 96 h after *A. phalloides* administration.

Additionally, it was observed that the pro-apoptotic marker *caspase-3* mRNA expression level decreased in the α-AMA-2 group compared to the control group, but it was not statistically significant. Administration of *A. phalloides* significantly enhanced the mRNA expression of the *caspase-3* in the α-AMA-72 ($p < 0.05$) and α-AMA-96 groups ($p < 0.01$) (Figure 6).

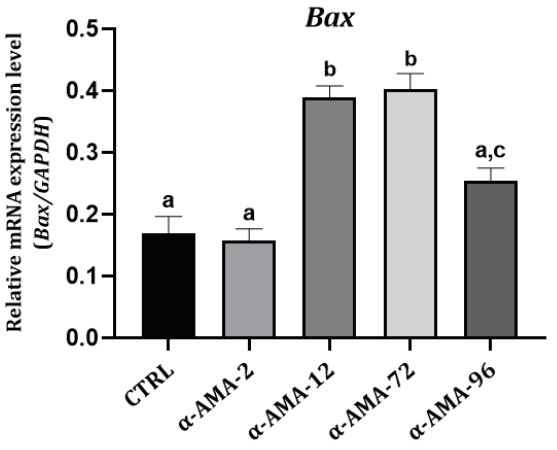


**Figure 5.** Time-dependent expression of *Bax* gene after *A. phalloides* administration. Mice were treated with *A. phalloides* extract including 10 mg/kg α-AMA. *Bax* gene expression was analyzed by RT-qPCR. Different letters mean significant differences between groups. [b]*p < 0.01* compared to control, α-AMA-2 and α-AMA-96 groups; [c]*p < 0.05* compared to α-AMA-2 groups.

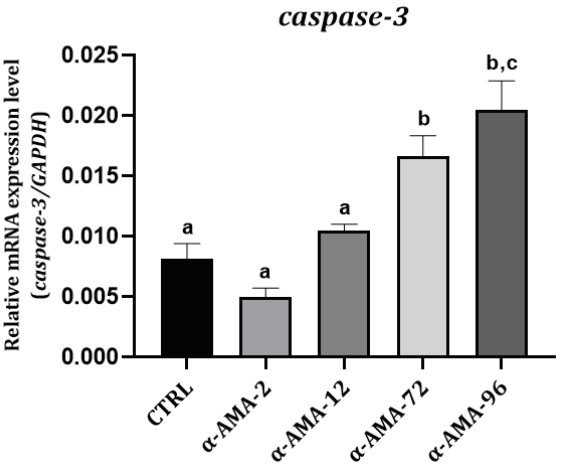


**Figure 6.** Time-dependent expression of *caspase*-3 gene after *A. phalloides* administration. Mice were treated with *A. phalloides* extract including 10 mg/kg α-AMA. *caspase-3* gene expression was analyzed by RT-qPCR. Different letters mean significant differences between groups. [b]*p < 0.05* compared to control, α-AMA-2 and α-AMA-12 groups; [c]*p < 0.01* compared to control, α-AMA-2 and α-AMA-12 groups.

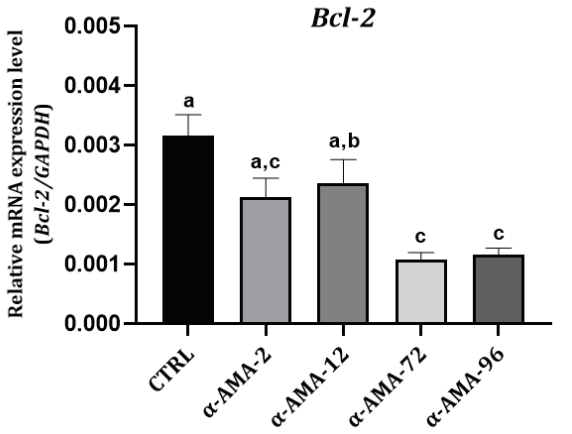


**Figure 7.** Time-dependent expression of *Bcl-2* gene after *A. phalloides* administration. Mice were treated with *A. phalloides* extract including 10 mg/kg α-AMA. *Bcl-2* gene expression was analyzed by RT-qPCR. Different letters mean significant differences between groups. [a]*p < 0.01* compared to α-AMA-72 and α-AMA-96 groups; [b]*p < 0.05* compared to α-AMA-72 and α-AMA-96 groups.

There were only decreased expression profiles for the anti-apoptotic marker *Bcl-2* in the α-AMA-72 and α-AMA-96 groups, compared among the groups ($p < 0.05$ and $p < 0.01$) (Figure 7).

### Time-dependent histopathological examinations after *A. phalloides* administration

No pathological observations were seen for liver tissue in the control group. Hepatic cells and the central vein exhibited normal histological appearances as shown in Figure 8-a. In the α-AMA-2 group, sinusoidal dilatation and hepatocytes with the pyknotic nucleus were observed (Figure 8-b). The liver tissues of the α-AMA-96 group showed prominent portal and lobular changes including vascular congestion, inflammation, necrosis, sinusoidal dilatation, and hepatocytes with pyknotic nucleus (Figure 8-c). Histopathological changes observed in the α-AMA-2 group were milder than in the α-AMA-96 group (Figure 8: a, b, c: H&E staining; x20 magnification).

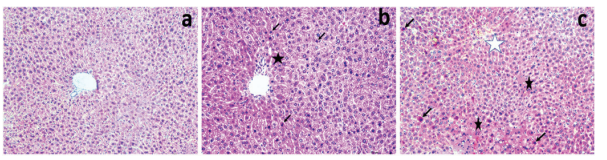


**Figure 8.** Histopathologic assessments. Mice were treated with *A. phalloides* extract including 10 mg/kg α-AMA. **a)** Liver tissue showed normal histological appearance with hepatic cells and central vein in the control group. **b)** Sinusoidal dilatation (black asterisks) and hepatocytes with pyknotic nucleus (black arrows) were observed in the α-AMA-2 group. **c)** In the α-AMA-96 group severe necrosis with the disappearance of nuclei (black asterisks), sinusoidal dilatation (white asterisks), and hepatocytes with pyknotic nucleus (black arrows) were observed. a, b, c: H&E; x20.

## DISCUSSION

There is no clinical antidote for *A. phalloides* poisoning and symptomatic treatment is the only option (Garcia et al., 2015). α-AMA, the most potent toxin in *A. phalloides,* irreversibly inhibits RNA polymerase II in hepatocytes and inhibits protein synthesis. The liver is the most affected by α-AMA because of the high protein synthesis and regeneration. Hepatic damage is exacerbated by the accumulation of α-AMA in liver tissues and undergoing enterohepatic circulation (Faulstich, Talas, & Wellhöner, 1985; Smith & Davis, 2016). In a study by Arima et al., it was shown that administration of α-AMA (2 μg/mL) for 24 h significantly induced p53 in fibroblast and HCT116 human colon carcinoma cells (Yoshimi Arima et al., 2004). In addition, it has been reported that α-AMA induces P53 protein to bind to anti-apoptotic Bcl-2 and Bcl-XL proteins independent of RNA polymerase inhibition and triggers apoptosis by causing cytochrome c to migrate from mitochondria to the cytosol (Y. Arima et al., 2005). Magdalan et al. reported that apoptosis induced by α-AMA in canine primary hepatocyte cells may contribute to the pathogenesis of severe liver damage, particularly in the early stages of poisoning (Magdalan, Ostrowska, Piotrowska, Izykowska, et al., 2010). Likewise, it has been reported that p53 and caspase-3-dependent apoptosis develops in hepatocyte cells exposed to α-AMA (2 μM) (Magdalan et al., 2011). On the other hand, in another *in vitro* study, L-amino-acid oxidase isolated from *A. phalloides* increased the Bax/Bcl-2 ratio by inducing caspase-dependent apoptosis in Jurkat cells, and then increased caspase-3 and caspase-9 protein expression (Pišlar, Sabotič, Šlenc, Brzin, & Kos, 2016). It has been revealed that the L-amino-acid oxidase enzyme also plays a role in the apoptotic mechanisms developing in *A. phalloides* poisoning.

Recent studies have shown that inflammatory cytokines such as TNF-α may induce local inflammatory mechanisms and ultimately lead to liver damage (Li et al., 2020). Fatal acute liver failure after *A. phalloides* ingestion can cause hepatocyte necrosis by inhibiting the synthesis of structural proteins (Angioi et al., 2021). Patient symptoms and clinical analyses show that acute tubular necrosis occurs with renal failure. In animals, 48 h after intravenous administration of α-AMA (0.327 mg/kg), tubular necrosis was observed in BALB/c mice (Zhao et al., 2006). Besides its role in inflammatory mechanisms, TNF-α has an important role in directly activating the extrinsic apoptotic pathway (Zhang et al., 2013). *TNF-α* mRNA levels were shown to increase concomitantly with hepatocyte apoptosis after administration of α-AMA (3 mg/kg i.p.) to mice (Leist et al., 1997). These studies show that *TNF-α* plays a role in both necrotic and apoptotic mechanisms after *A. phalloides* poisoning. The current study found that pro-inflammatory cytokine *TNF-α* mRNA levels increased in the early stage after *A. phalloides* administration (Figure 4), and increasing *TNF-α* mRNA levels tended to decrease at 72 and 96 h. These results indicate that caspase-independent necrotic mechanisms may play a role through the pro-inflammatory cytokine *TNF-α* in the early stages of poisoning. Activation of caspase-independent necrotic pathways may also trigger necroptotic mechanisms. Further studies on genes related to necroptosis will contribute to the emergence of necrotic mechanisms caused by α-AMA in liver tissues. Therefore, hepatic inflammation developing in the acute phase may be effective in the pathogenesis induced by *A. phalloides*, but the effect of *TNF-α* on the apoptotic and necrotic mechanisms remain unclear. More studies are needed to define the crosstalk between inflammation, apoptosis and necrosis mechanisms in *A. phalloides* poisoning.

In the present study, pro-apoptotic *caspase-3* mRNA expression level was significantly increased at 72 and 96 h after *A. phalloides* administration, however, no significant change was observed in *caspase-3* mRNA levels after 2 and 12 h (Figure 6). Deregulation of caspases triggers cell death (McIlwain, Berger, & Mak, 2013). Increased *caspase-3* mRNA levels result in the rapid induction of apoptosis, and our results show that apoptosis is elevated 72 h after *A. phalloides* administration. These results may indicate the late onset of apoptosis (Zhou et al., 2017). Thereby, our findings suggest a critical role of *caspase-3* in *A. phalloides*-induced hepatotoxicity. In addition, it was observed that pro-apoptotic *Bax* mRNA levels increased 12 and 72 h after *A. phalloides* administration, while anti-apoptotic *Bcl-2* mRNA levels decreased after 12 and 72 h (Figure 5, Figure 7). As the *Bax/Bcl-2* ratio indicates the balance between the pro-and anti-apoptotic mRNA levels (Barbosa, Machado, Skildum, Scott, & Oliveira, 2012), our results suggest that apoptotic mechanisms play an effective role approximately 12 h after *A. phalloides* ingestion.

The severity of liver injury and the rate of hepatic regeneration are important factors that determine the survival of individuals. In the early study on hepatotoxicity in *A. phalloides* poisoning, massive hepatic central lobular cell necrosis was reported (Fineschi, Di Paolo, & Centini, 1996). Likewise, it was observed that mice poisoned with α-AMA (0.6 mg/kg i.p.) developed higher percentages of hepatonecrosis compared to the control group (Tong et al., 2007). In a study by Kaya et al., vacuolar degeneration was observed at 1 and 6 h in mouse liver tissues after α-AMA (1 mg/kg i.p.) administration, while Councilman-like structures and pycnotic cells were observed after 24 h (E. Kaya et al., 2014). Similarly, in a study by Garcia et al., it was reported that α-AMA (0.33 mg/kg i.p.) caused significant hepatic cellular edema, cytoplasmic vacuolization and interstitial inflammatory cell infiltration in mouse liver tissues 24 h after administration (Garcia et al., 2015). In our study, the results were similar to the literature. Sinusoidal dilatation and pyknotic nucleus were observed 2 h after *A. phalloides* ingestion (Figure 8-b). After 96 h, it showed prominent portal and lobular changes, including vascular occlusion, inflammation, necrosis, sinusoidal dilatation, and hepatocytes with pycnotic nuclei (Fig. 8-c).

## CONCLUSION

In the current study, the mRNA expression of *Bax*, *caspase 3*, *Bcl-2*, and *TNF-α* were examined to investigate time-dependent apoptotic and necrotic mechanisms occurring in mouse liver tissues after *A. phalloides* administration. Pro-apoptotic, anti-apoptotic, and pro-inflammatory mRNA expression profiles evaluated by RT-qPCR show that *A. phalloides* induces necrotic mechanisms in the early phase of intoxication and then causes cell death through apoptotic mechanisms. Consequently, we have proven that apoptotic mechanisms play an essential role in the pathogenesis of *A. phalloides*-induced hepatotoxicity, and necrosis is also essential in the pathogenesis

of *A. phalloides*-induced hepatotoxicity during the early stage after ingestion. Our results have demonstrated for the first time that investigation of time-dependent apoptotic and necrotic mechanisms in mice will provide critical knowledge for further studies on the signaling pathways underlying hepatotoxicity from *A. phalloides*.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- S.K., Z.A., E.K., F.Ö.; Data Acquisition- S.K., Z.A., E.K., F.Ö., S.İ.; Data Analysis/Interpretation- S.K., Z.A., E.K., M.B., F.Ö.; Drafting Manuscript- S.K., Z.A., F.Ö., S.İ.; Critical Revision of Manuscript- E.K., M.B.; Final Approval and Accountability- S.K., Z.A., E.K., F.Ö., M.B., S.İ.

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Financial Disclosure:** Scientific Research Projects Coordinator of Dicle University (DUBAP:DUSAM.21.002)

**Ethics committee approval:** Animal experimental procedures were conducted in accordance with animal study protocols approved by the Dicle University Animal Experiments Local Ethics Committee (DUHADEK-2021/45).

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Original Article

# Comparative assessment of different nanobodies that inhibit the interaction of B7-1/2 with CD28 as a potential therapeutic target for immune-related diseases by molecular modeling

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**ABSTRACT**

**Background and Aims:** Active T cells are central players in the self-defense system as well as in immune-related diseases. Being crucial for T cell activation, the interaction of B7-1/2 with CD28 is associated with T cell activation-related diseases such as alloreactivity in transplantation and autoreactivity in autoimmune disorders. Nanobodies are the recombinant variable and single-domain smallest antigen-binding fragments. The focus of this study is to investigate the interactions between B7-1/2 and eight antibodies at the molecular level utilizing computational methods, and to guide the best nanobody for in-vitro and in-vivo studies about immunosuppressive

**Methods:** After receiving the 3D models of agents via Robetta, molecular docking techniques were used to compare the binding modes and affinities of six nanobodies and two FDA-approved fusion protein models against B7-1/2(CD80/CD86).

**Results:** According to our *in silico* outputs, we selected the top of model clusters from HADDOCK 2.4 (Z-Score of CD80/CD86:-2.7 to -1.3/-2.1 to -2.1) and distinguished that 1A1 and 1B2 have higher affinities than Belatacept and Abatacept for the percentage of a calculation scale.

**Conclusion:** Our findings suggest that selected nanobodies show higher affinity by interacting with the CD80/86 epitope regions and provide helpful insights into the design and improvement of further computational investigations of nanobody modeling.

**Keywords:** Immunosuppression, Immune-related diseases, Nanobody, B7 Antigens, Molecular modeling

## INTRODUCTION

Activated T cells are significant players in immune responses. The activation of T cells is dependent on antigen provided by APCs (antigen-presenting cells) through the MHC (major histocompatibility complex)-TCR (T cell receptor) interaction, which is the first signal and antigen-specific pathway required for T-cell activation. However, the MHC-TCR interaction is hardly sufficient for T-cell activation owing to the low affinity of the TCR for the specific MHC-peptide complex, so there is a second signaling pathway that requires T-cell activation (Abbas et al., 2019). The second pathway stabilizes the weak MHC-TCR interaction with stronger non-specific protein interactions and leads to an increase in the antigen presentation capacity and T cell activation ability of

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APCs (Janeway Jr et al., 2001). The B7-1/2(CD80/CD86)-CD28 is one of the most prominent second signaling interactions. The interaction component is CD28, which is expressed on all naïve T cells, interacting with the proteins B7-1(CD80) and B7-2(CD86) as the co-stimulatory signal or interacting with CTLA4 as the inhibitory signal (Goronzy & Weyand, 2008). In this regard, Green et al. highlighted the significance of B7-1/,2-CD28 and its interaction associated with the deficient germinal center formation and impaired cytotoxic lymphocyte functions in CD28-deficient mice (Green et al., 1994). In conclusion, these features of the B7-1/2-CD28 interaction are compulsory for T-cell-mediated immune responses.

The role of autoreactive T lymphocytes in autoimmune diseases is pivotal. T cell mediated-autoreactivity responses, which are significant signals of B7-1/2-CD28, commence with the presentation of the body's own antigens to T cells (Khan & Ghazanfar, 2018). Abatacept, a CTLA-4 recombinant fusion protein, is a well-reported agent in experimental autoimmunity such as systemic lupus erythematosus (Crepeau & Ford, 2017). Also, Abatacept is the first FDA-approved drug with the ability to block the B7-1/2-CD28 pathway and is prescribed for distinct autoimmune diseases such as psoriasis and rheumatoid arthritis (Ansari et al., 2017).

Alloreactive T lymphocytes mediate acute graft rejection. Alloreactivation of T cells is contingent on antigen presentation. The B7-1/2-CD28 pathway is a positive stimulator of antigen presentation and also enhances T cell alloreactivity, therefore, inhibiting that pathway has gained importance. In previous studies, Abatacept was tested to block B7-1/,2-CD28 in non-human kidney transplant models (Larsen et al., 2005). However, Abatacept failed to prolong graft survival due to the insufficient blockade of B7-1 and B7-2 proteins (Ansari et al., 2017). Subsequently, Belatacept, a higher avidity and selectivity fusion protein than Abatacept as binding to B7-1 and B7-2, was developed (Larsen et al., 2005). Like Abatacept, Belatacept is FDA-approved for the prevention of acute kidney transplant rejection. It has been reported that Belatacept is a safer agent due to both its non-nephrotoxic and nephroprotective properties compared to other immunosuppressive agents (Noble et al., 2019).

Nanobodies (NBs), recombinant variable domains of heavy chain-only antibodies, are in the range of 12–14 kDa molecular weights (Jovčevska & Muyldermans, 2020). Due to their small size, NBs exhibit better tissue penetration than conventional monoclonal antibodies with the features of unique solubility, high stability, ease of production, and quick clearance from the blood (Sun et al., 2021). These properties are regarded as very promising and NBs could become potential therapeutic agent candidates for autoimmune disorders such as against TNF and IL-6 in rheumatoid arthritis and, IL-17 in Psoriasis in phase-1 and phase-2 clinical trials (Jovčevska & Muyldermans, 2020).

Several scientific papers and patents containing information on therapeutic agents indicate that various agents, such as fusion proteins and NBs, are designed to target the B7-1/2-CD28 interaction. These include the FDA-approved fusion protein Belatacept for kidney transplants and the FDA-approved fusion protein Abatacept for rheumatoid arthritis. These therapeutic agents, which can be designed with various engineering technologies, might have a different ability to bind B7-1 and B7-2 proteins, even in terms of variations in amino acid sequences. For instance, even though the amino acid sequence of Belatacept differs from Abatacept by only two amino acids, there is a significant affinity difference between them (Larsen et al., 2005). Therefore, there is a demand to assess the most effective candidates among various therapeutic agents.

In silico analysis of agent-target, protein interactions play a substantial role in augmenting the yield of drug research and facilitating the development of new therapies before experimental and clinical approvals (Song et al., 2013).

In the current study, the molecular docking process was conducted under the inspiration of experimental and clinical findings. This study is the first attempt to show the interaction of B7-1 and B7-2 with eight agents by analyzing docking poses. Moreover, it would also be highly advantageous to assess various immunosuppressive nanobodies before preclinical pharmacokinetic investigations. The main aim of this study is to recommend the best nanobody for *in vitro* and *in vivo* studies about immunosuppressive therapy by comparing the binding affinity of eight agents to B7-1 and B7-2. This is a comprehensive study that uses the predicted structure of agent models to simulate interaction with target antigens throughout molecular modeling.

## MATERIALS AND METHODS

### Pre-preparation for docking procedure obtaining the 3D structure of protein and peptide

First, the crystal structure of human T-lymphocyte activation antigen CD80(PDB ID:1DR9)/ CD86(PDB ID:1I85) in PDB format was downloaded from PDB (Protein Data Bank) at http://www.rcsb.org/. All NBs, Belatacept, and Abatacept sequence information including Complementarity-determining regions (CDRs) were obtained from https://patentscope.wipo.int (see supplement file 1). Then, each of the amino acid sequences was subjected to the Robetta (Raman et al., 2009; Song et al., 2013). In this process, RoseTTAFold was used. All settings were left as default and generated five 3D-structure models having selected the most accurate with comparative assessment according to the confidence score that indicates the accuracy of model protein in terms of predicted GDT (1.0 good, 0.0 bad). RoseTTAFold is a method that is based on the principle of simultaneously considering patterns in protein sequences which show how a protein's amino acids interact with each other. The method also shows the possible three-dimensional structure of a protein. In this construction, primer, 2D, and 3D information flow back and forth, permitting the network to collectively sense the connection between chemical parts of a protein and its folded structure. The epitope information of CD80/CD86 was fetched from Immune Epitope Database (Vita et al., 2012).

### Energy minimization and assessment of model protein structures

The energy minimization of 3D model protein structures was subjected to the minimization method in chimera 1.14 (Pet-

tersen et al., 2004). The default was the steepest descent:100 with 0.02 step sizes, without fixing any atoms, followed by 10 steps of conjugate gradient steps with 0.02 step size (Å) minimization. To control the quality of the model peptides, we evaluated the analysis of structural quality using Qualitative Model Energy Analysis (QMEANDisCo) (A. Waterhouse et al., 2018). In addition, Ramachandran plots were drawn to assign key secondary structures to specific regions in the plot.

### Visualization of molecular modeling simulations using Jalview and PyMOL

The primary structure of CD80/CD86 was colored to exhibit the epitope regions by the Jalviewprogram (A. M. Waterhouse et al., 2009), which is an application designed for the sort of deep sequence analysis required when investigating novel protein or RNA sequence families to figure out how their sequences associate with structure and function (see supplement files). The PyMOL (Schrödinger, LLC, 2015) software is a molecular visualization system, utilized to illustrate the tertiary structure of antigen-NBs and to analyze the molecular modeling results at an atomic level. All complexes of protein-peptides modeling were obtained via the HADDOCK 2.4 web server which is an integrative platform for the docking of biomolecular complexes. It was adjusted to the default settings and provided active residues (epitope regions on CD80/CD86 and CDRs on nanobodies) for docking on both molecules.

## RESULTS

The understanding of the 3D structures of target proteins is critically essential for plausible protein engineering. Until recently, there has been some convincing evidence that the role of CD80/CD86 in blocking interaction with CD28 as a treatment for Immune-Associated Diseases (Crepeau & Ford, 2017; Khan & Ghazanfar, 2018). Although Belatacept and Abatacept are the fusion proteins currently used for this therapy, some peptide agents such as NBs might also promise targets as potential therapeutic agents thanks to their more efficient properties (Jovčevska & Muyldermans, 2020).

In Table 1, the confidence scores of 3D models of agents are given and they display the accuracy of models in terms of predicted GDT scores (with 1.0 being good, 0.0 being bad). Besides, we evaluated the model peptides in the analysis of quality estimate as well as the outputs of Ramachandran plots using the QMEAN assessment tool (A. Waterhouse et al., 2018). Accordingly, all predicted model proteins are of a quality to be subjected to the docking procedure. All CDR positions of the listed antibodies in Table 1 with their sequences are available, as well as target regions of proteins and peptides for the molecular docking in supplement file 1.

### Docking outputs of CD80/CD86 with each of the nanobody models and fusion proteins

This study attempts to investigate the binding mode and affinity between CD80/CD86 epitopes and CDRs of six model NBs compared to Abatacept and Belatacept peptides that block the interaction of CD80/CD87 and CD28 proteins by the analysis of polar contacts between the peptide chains. Herein, we performed molecular modeling to design the appropriate

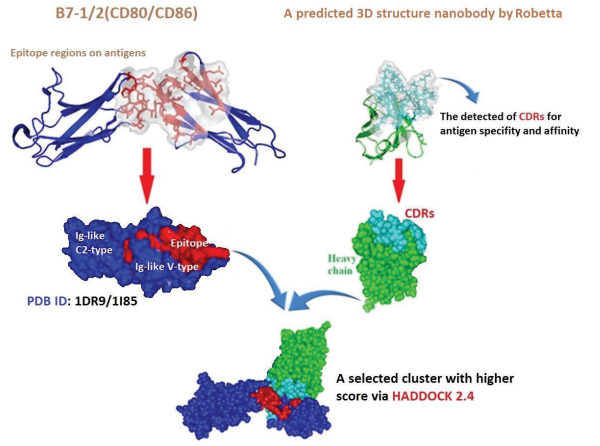


**Figure 1.** The description of the molecular modeling of B7-1/2 and model nanobodies.

**Table 1. The table presents the epitope region (107-131aa) of CD80 interactingwith the residuesof the CDRs of the model NBs and reference peptides and Robetta confidence scores in the structural assessment of models. Polar contact residues in the CDRs are indicated in red font.**

| | Robetta confidence score | Length(aa) | Fetched CDR positions | | |
|---|---|---|---|---|---|
| | | | $CDR_1$ | $CDR_2$ | $CDR_3$ |
| **Belatacept** | 0.68 | 357 | - | - | - |
| **Abatacept** | 0.66 | 357 | - | - | - |
| **CD8086PMP1A1** | 0.86 | 125 | IDAMG | SIGRSGNSATNVDSVKG | ATRRAYLPIRIRDYIY |
| **CD8086PMP1E11** | 0.86 | 124 | YSAIG | YISSSDGSTYYADSVEG | GGPFTVSTMPWLANY |
| **CD8086PMP2B4** | 0.88 | 120 | IYTMG | AITSGGSTNYADSVKG | IAHEEGVYRWDE |
| **CD8086PMP2B10** | 0.94 | 118 | DNTMN | SLSIFGATGYADSVKG | GPVRRSR LEY |
| **CD8086PMP1B2** | 0.89 | 123 | SYVMG | AIIGRDIGTYYADSVKG | DSRSRLSGIRSAYDY |
| **CD8086PMP1C7** | 0.87 | 123 | DYAAG | AINWSGGSTYYADSVKG | GWGRTTVLADTVVY |

**Table 2. The table indicates the epitope region (59-74aa) of CD86 interacting with the residues of the CDRs of the model NBs and reference peptides and Robetta confidence scores in the structural assessment of models. Polar contact residues in the CDRs are indicated in red font.**

| | Robetta confidence score | Length(aa) | Fetched CDR positions | | |
|---|---|---|---|---|---|
| | | | $CDR_1$ | $CDR_2$ | $CDR_3$ |
| **Belatacept** | 0.77 | 357 | - | - | - |
| **Abtacept** | 0.75 | 357 | - | - | - |
| **CD8086PMP1A1** | 0.86 | 125 | IDAMG | SIGRSGNSATNVDS-VKG | ATRRAYLPIRIRDYIY |
| **CD8086PMP1E11** | 0.86 | 124 | YSAIG | YISSSDGSTYYADSVEG | GGPFTVSTMPWLANY |
| **CD8086PMP2B4** | 0.88 | 120 | IYTMG | AITSGGSTNYADSVKG | IAHEEGVYRWDE |
| **CD8086PMP2B10** | 0.94 | 118 | DNTMN | SLSIFGATGYADSVKG | GPVRRSRLEY |
| **CD8086PMP1B2** | 0.89 | 123 | SYVMG | AIIGRDIGTYYADSVKG | DSRSRLSGIRSAYDY |
| **CD8086PMP1C7** | 0.87 | 123 | DYAAG | AINWSGGSTYYADS-VKG | GWGRTTVLADTVVY |

**Table 3. The table presents the statistics of top clusters from molecular docking results. The top clusters are the most reliable according to HADDOCK 2.4. The Z-score of each cluster designates how many standard deviations from the mean these clusters are placed in terms of score (lower is better). The polar contacts formed in the docking complex are between the residues from the epitope of CD80 and CDRs of NBs. In the write-up of the residues, the first one is in the epitopes and the next one is in the CDRs and each contact between residues is separated by "/".**

| Model Antibody | HADDOCK score | Target Domain(Ig-like V-type) of CD80(107-131) | RMSD from the overall lowest-energy structure | Z-Score | Residues from Epitope of CD80 and CDRs of NBs | Contact Distance (Å) |
|---|---|---|---|---|---|---|
| 1A1 | -82.6 +/- 7.7 | RPSDE-GTYECVVLK YEKDAFKREHL | 1.4 +/- 0.9 | -2.7 | Y121-Y104,L105/K123-L105/D124-R53,S54,S57 | 2.3-2.5/2.1/1.7-2.3 |
| 2B4 | -88.4 +/- 3.6 | RPSDE-GTYECVVLK YEKDAFKREHL | 0.7 +/- 0.5 | -2.2 | K120-S53/E122-H100/ D124-Y105,G103/Y121-S56/K120,K127-N58/ R128-D61/E129-K65 | 2.0-2.4/1.9-2.5/1.7-2.6/1.5-1.6/1.8-2.0 |
| 1C7 | -73.5 +/- 2.6 | RPSDE-GTYECVVLK YEKDAFKREHL | 14.0 +/- 0.1 | -2.2 | E122,K123-K65/R128-V105 | 1.7-2.6/2.0 |
| 1E11 | -71.4 +/-5.6 | RPSDE-GTYECVVLK YEKDAFKREHL | 0.5 +/- 0.3 | -1.8 | D124-S105/K127-S54/ R128-N112,L110/ Q67,K70-Y113 | 1.8/1.6/1.8-2.5/1.7-2.6 |
| 2B10 | -85.9 +/- 8.9 | RPSDE-GTYECVVLK YEKDAFKREHL | 0.8 +/- 0.5 | -1.8 | E122-R102/R128-R104, R102/ E115-R102 | 1.8-2.3/1.8-2.1/1.7-2.2 |
| 1B2 | -83.4 +/- 5.3 | RPSDE-GTYECVVLK YEKDAFKREHL | 1.4 +/- 1.0 | -1.8 | K70-D55/R128-D55,R54/ R63,Y65-Y59/Y121,K123-R108/ D124-R101,R103 | 1.6/1.7-2.5/1.9-2.4/1.9-2.0/1.6-1.7 |
| Belatacept | 5.0 +/- 14.7 | RPSDE-GTYECVVLK YEKDAFKREHL | 1.4 +/- 1.0 | -2.0 | E111,T113-H2/ R128-Y90 / L131-A7 / E133-K93 / R63-D41/Y65- A40,S42 | 1.6-1.9-2.8/1.6-2.4/1.9/1.7-1.8 |
| Abatacept | 20.1 +/- 7.0 | RPSDE-GTYECVVLK YEKDAFKREHL | 15.4 +/- 0.6 | -1.3 | Y121-A7/ D124-V8,L10/ R128-Q80 | 2.3/1.7,1.9/2.3-2.2 |

**Table 4. The table provides that polar contacts formed in the docking complex are between which residues from the epitope of CD86 and CDRs of nanobodies.**

| Model Antibody | HADDOCK score | Target Domain(Ig-like V-type) of CD86(59-76) | RMSD from the overall lowest-energy structure | Z-Score | Residues from Epitope of CD147 and Antibody CDRs | Contact Distance (Å) |
|---|---|---|---|---|---|---|
| 1B2 | -85.2 +/- 3.2 | DQ ENLV-LNEVYLG-KEKFD | 0.3 +/- 0.2 | -2.1 | K72-E46,F47,S63/D76-Y60 | 2.3/1.5-2.4 |
| 1A1 | -118.4 +/- 0.9 | DQ ENLV-LNEVYLG-KEKFD | 0.6 +/- 0.4 | -2.0 | K74-A58/D76-S54,G55,N56,S57/F75-S57 | 1.7/1.8-2.6/1.7-2.5 |
| 2B10 | -83.7 +/- 4.7 | DQ ENLV-LNEVYLG-KEKFD | 5.7 +/- 0.2 | -1.9 | K74-T57/D76-S53,R101 | 2.2/1.7-1.8 |
| 1E11 | -87.4 +/- 3.7 | DQ ENLV-LNEVYLG-KEKFD | 10.3 +/- 0.2 | -1.5 | H79-E65 /K74,S77-Y59/M120-Y31,S53/H113-S54 | 1.7/2.1-2.2/2.2-2.4 |
| 1C7 | -83.8 +/- 2.4 | DQ ENLV-LNEVYLG-KEKFD | 0.7 +/- 0.4 | -1.4 | N62-T109 / Y69-Y60/D76-S57,T58/S77-R102 | 1.7-2.4/2.5/2.1/1.9 |
| 2B4 | -85.0 +/- 6.5 | DQ ENLV-LNEVYLG-KEKFD | 6.7 +/- 0.2 | -1.1 | D76-T52,G55,S56/Y69-T57,K64/S78-H100 | 1.7-2.6/1.8-2.4/2.6 |
| Belatacept | -32.0 +/- 2.4 | DQ ENLV-LNEVYLG-KEKFD | 1.2 +/- 1.2 | -1.8 | Q60,N62-Q43/H79-T45/Y69-L58/K72-Y52 | 2.8-2.4/2.3/2.3/2.0 |
| Abatacept | -18.7 +/- 6.7 | DQ ENLV-LNEVYLG-KEKFD | 13.7 +/- 0.3 | -1.5 | Q60-S70/E61-S70,S71/N62-E57/E67-T67/K72-R14,G15,Q80/E73-Q80 | 2.0/2.3,1.9/2.1/2.1 |

tertiary structure of the six patented nanobody models and opted for both Belatacept and Abatacept, which are FDA- approved drugs, as reference peptides.

The docking scores and the residues of polar contacts between CD80/CD86 and peptide models are listed in Tables 3 and 4. As can be seen from Tables 3 and 4, residues formed polar bonds with CDRs of peptide models from the epitopes of CD80 and CD86 are mentioned. Considering Belatacept and Abatacept as the variants of CTLA-4, the Ig-like V-type domain, where CTLA4 interacts with CD80/86, was identified as the active binding site for the molecular docking. Although CDR1,2,3 of nanobody models are actively processed for molecular docking in Tables 1 and 2, no polar bond formation was observed in the CDR1 region.

The docking scores between target CD80/CD87 and NBs are listed in Tables 3 and 4. Based on the Z-score value, the best clusters among all cluster results of CD80/CD87 and nanobody models were selected. In this respect, during submitting to the HADDOCK web server, since the obtained epitopes and CDR regions were prioritized as active residues (directly involved in the interaction), docking results might be more dependable. Overall, all clusters might achieve good outcomes, even if the predicted peptide model structures when an uncertain epitope region and variable CDRs are revealed.

## DISCUSSION

All docking processes are linked to the structural validity and reliability of 3D model components at an atomic level. Contemplating the antibodies own a sufficient conserved framework that accurately is predictable CDRs, and the development of algorithms used in component modeling is progressing, appropriate modeling techniques for NBs or antibodies are capable to constitute fairly proper structures (Leem et al., 2016; Weitzner et al., 2017). The NBs have VHH domains and lack VL domains but are still immensely stable. The absence of the VL domain indicates nanobodies possess a hydrophilic side as well (Siontorou, 2013).

As presented by the output of the cluster analysis in Tables 3 and 4, our data might indicate that all NBs have a reasonable affinity for the CD80/CD86. In the list of the diverse polar interactions depending on the distance between the atoms of the residues, our findings showed that the CDRs of all NBs actively interact with the surface epitope residues of CD80/CD86 enacting main roles. Among six NBs, 1A1 targeting CD80 and 1B2 targeting CD86 have one of the highest performance and affinity according to the Z-scoring scale (-2.7 and -2.1). 1A1 also exhibits one of the highest avidity as it targets both CD80 and CD86 compared to Belatacept and Abatacept and the other NBs. This highest value for each CD80 and CD86 depends on both distinct the antigens epitopes on antigens and different CDRs of nbs. Most of the interacting region in the epitope CD80 is YEKD and the common contact residue is D124 from YEKD (121-124 residues) (see part A in Figure 4).

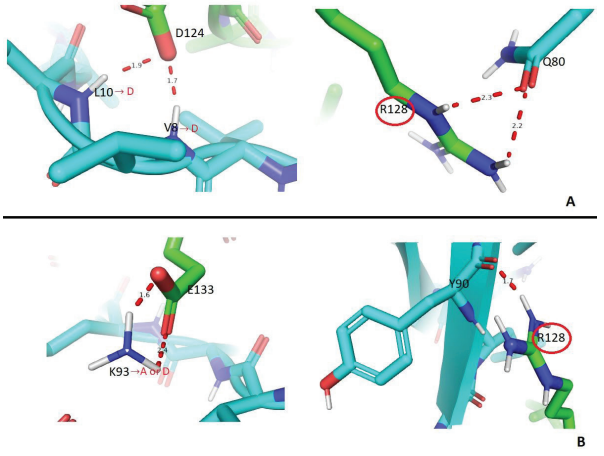


**Figure 2.** Cluster analysis results of reference fusion peptides by CD80. The docking complex is represented in a surface image, colored by (Abatacept and Belatacept in parts A, and B in blue color and CD80 in green). Abatacept (part A) and Belatacept (part B) commonly interact with the residue R128 on the CD80 epitope. Mutated residues of Abatacept are L10, V8→D, while Belatacept is solely K93→A or D.

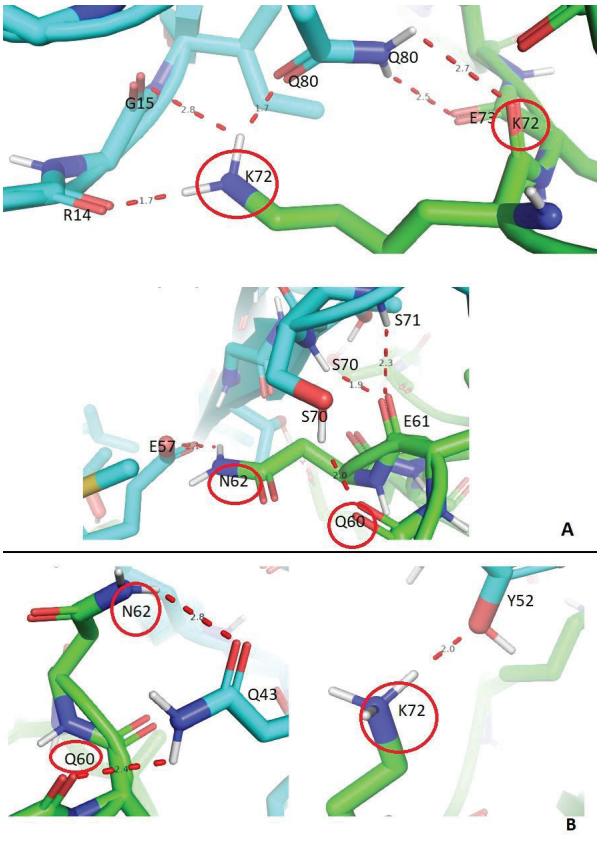


**Figure 3.** The Cluster analysis results of reference fusion peptides by CD86. The docking complex is depicted in a surface image, colored by (Abatacept and Belatacept in parts A, and B in blue color, and CD86 in green). Abatacept (part A) and Belatacept (part B) commonly interact with the residue Q60, N62, and K72 on the CD86 epitope.

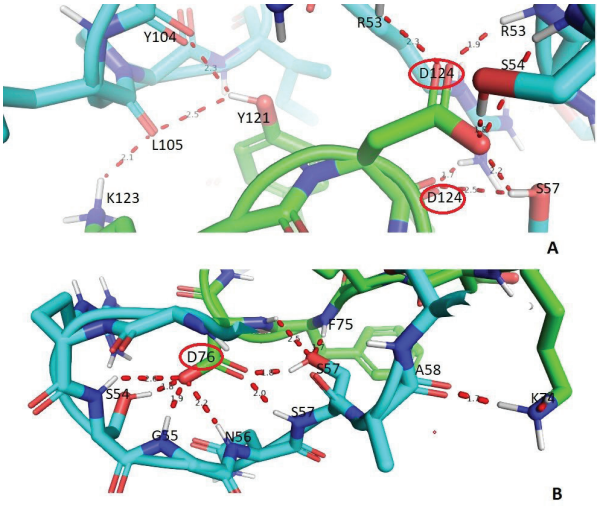


**Figure 4.** Part A: The most interacting region in the epitope CD80 is YEKD (121-124) and the common contact residue with model NBs is D124. Part B: In the 1A1 docking model with CD86, residue D76 in CD86 formed many polar bonds with 1A1. The docking complex is depicted in a surface image, colored by NBs in parts A, and B in blue color and CD80/86 in green).

The epitope regions of CD80/86 are positioned in the Ig-like V-type domain. In the docking complexes of NBs targeting the Ig-like V-type domain, intermolecular polar bonds were formed with the residues of K120, Y121, E122, K123, D124, and R128 on the CD80-epitope, while the residues of N62,Y69, K72, K74, F75,D76 on CD86-epitope. The most common one of these binding residues of CD80 is R128 (see Figure2), while for CD86 the most common residue is D76 (see part B in Figure4). In this regard, in the 1A1 docking model, residue D76 formed many polar bonds, suggesting that it plays a significant role in affinity.

The epitope regions of CD80/86 antigens targeted in this study were also analyzed in previous studies (Mifsud et al., 2021; van Balen et al., 2020). In another study, it was shown from which residues the CD28-CD86 protein complex performs forming the interface (Krupa Pawełand Spodzieja & Sieradzan, 2021), and reported that six residues from CD86 binding to CD28 at the interaction interfaces are significant for the stability of the complex. In our results, N62, V64, E67, and Y69 of the six residues exhibited significant affinity in the interaction CD86 with nanobodies and reference peptides, and one of the most common residues at the interface of CD80-Abatacept and Be-

latacept was N62 (see Table 4 and Figure 3). In this context, the model nanobodies interacting with CD80 may effectively inhibit CD80-CD28 interaction. As mentioned previously, preventing T cell-mediated autoreactivity responses, the crucial signal of CD80/86-CD28 (Khan & Ghazanfar, 2018) seems to be an essential molecular approach for the cure of autoimmune diseases.

The molecular reason why Belatacept, a variant of the CTLA-4-Ig-like V-type domain, has higher avidity for both CD86 and CD80 than Abatacept is due to two amino acid changes (L104E and A29Y) (Larsen et al., 2005). This is in agreement with our in silico results. Only these two amino acid changes display a conformational modification in the 3D structure of Belatacept compared to Abatacept (Supplement file X) and additionally affect the docking consequence with having a higher affinity score. Nevertheless, in our docking results, no polar bond formation was observed with these altered amino acids (L104E and A29Y) to epitopes of CD80 and CD86. Our results additionally denoted, as shown with a red circle in Figure 2, that the CD80 epitope forms polar bonds with the Abatacept and Belatacept (CTLA4 variants) via the residues of V8, L10, and K93 Experimental mutagenesis studies of V8,L10, and K93, V8 → D, L10→ D and K93→A or D display that these mutations cause strongly reduced interactions with CD80 and CD86 (Ramagopal et al., 2017). Thus, possible polymorphisms and mutations in these residues may play a role in the pathogenesis of various alloreactive and autoreactive disorders.

The results, as shown with a red circle in Figures 2 and 3, indicate the residue R128 on the CD80 epitope that commonly interacts in the Abatacept and Belatacept in the complexes, just as Figure 3 exhibits the residues Q60, N62, and K72 in the Abatacept and Belatacept in the greater part of which interaction poses in the complexes. The formation of multiple polar bonds with K72 may determine its effect on the affinity and binding mode with CD86. In this context, we observed that 1B2, which has the most affinity score with CD86, also formed multiple polar bonds with K72.

Overall consequences of this study indicate that 1A1 has an affinity for CD80 and CD86 and is higher than the FDA-approved Belatacept currently in clinical use in renal transplantation. Thus it is a potential candidate for *in vitro* and *in vivo* immunosuppressant therapy investigations. In addition, the affinity of 1B2 and 2B10 to CD80 and CD86 is significantly higher than Abatacept, the first FDA-approved fusion protein in the treatment of autoimmune diseases and clinical use in rheumatoid arthritis. For this reason, 1B2 and 2B10 might be potential candidates in the treatment of autoimmune diseases.

## CONCLUSION

In conclusion, to evaluate the affinity of antigen-peptide, we examined the mechanisms of interaction between nanobody models and CD80/86 and found that the interactions between them are mainly achieved by polar bonds. We found that in the CD80 epitope, most of the region interacting with NBs is the YEKD(121-124), which of the D124 residue that commonly interacts with NBs. Also, we reported that the most prevalent interacting residue in the CD86 epitope was D76, and 1A1 has one of the highest performance and affinity according to the HADDOCK scoring scale. Additionally, we found that 1B2, the agent with the highest affinity for CD86, made multipolar bonds with the K72 residue, and we showed that the K72 residue in the CD86 epitope binds with Abatacept, and Belatacept as well. The nanobodies 1A1 and 1B2 as a result of affinity tests might be potential candidates in future immunosuppressive therapy studies. In short, our *in silico* approaches may contribute a source for quick and cost-effective *in vitro* affinity maturation of nanobody. The reader should bear in mind that the study is based on the preliminary molecular docking findings. The results of the study should be validated by molecular dynamics simulation followed by *in vitro* and *in vivo* studies.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- H.İ.B., N.B., G.Y.; Data Acquisition- H.İ.B., N.B., G.Y.; Data Analysis/Interpretation- H.İ.B., N.B.; Drafting Manuscript- H.İ.B., N.B., G.Y.;Critical Revision of Manuscript- H.İ.B., N.B.; Final Approval and Accountability- H.İ.B., N.B., G.Y.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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**Original Article**

# Evaluation of some *o*-benzenedisulfonimido-sulfonamide derivatives as potent antimicrobial agents

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**ABSTRACT**

**Background and Aims:** The discovery of new antimicrobials to overcome antimicrobial resistance has always been an important topic for sustainable world health. Since the sulfonamide carrying heterocyclic compounds present a number of advantages as biologically active compounds, in our work reported herein, a small collection of previously synthesized *o*-benzenedisulfonimido-sulfonamide derivatives were assayed to determine antimicrobial profiles against ten different microorganisms in search of finding promising new antibacterial/antifungal agents.
**Methods:** Eight compounds and their standards were tested against seven bacterial and three fungal strains, including members of Gram-positive, Gram-negative bacteria, and *Candida spp*., using the microbroth dilution method to measure their MIC (minimum inhibitory concentration) values.
**Results:** All assayed molecules showed different inhibitory effects on ten different targets, with considerable MIC values. Particularly, compound **2** exhibited better antimicrobial activity against the largest number of assayed microorganisms.
**Conclusion:** Further modification and development of *o*-benzenedisulfonimido-sulfonamide derivatives and additional *in vitro* studies against putative targets may result in new antimicrobial drug candidates in the near future.
**Keywords:** *o*-benzenedisulfonimide, sulfonamide, antibacterial agents, Gram-positive bacteria, Gram-negative bacteria, antifungal agents, *Candida spp*.

## INTRODUCTION

Antimicrobial resistance is one of the most challenging worldwide health and development threats facing human beings. Misuse or overuses of antimicrobials are leading to untreatable infections caused by multi- and pan-resistant bacteria (also known as "superbugs"), viruses, fungus, and other microorganisms. According to World Health Organization (WHO), investing in research and development of new antimicrobials is an essential part of strategic global action plans on antimicrobial resistance (WHO 2021; WHO 2015).

Heterocyclic compounds have always attracted much attention for drug discovery because of their versatile chemical structures with various pharmacological potentials. Phthalimides are a class of cyclic imides involved in a huge number of promising biological properties, such as antihyperlipidemic (Alaa et al., 2011), analgesic (Banarouei, Davood, Shafaroodi, Saeedi, &Shafiee, 2019), anticonvulsant (TabatabaeiRafiei et al., 2020), anti-inflammatory (Abdel-Aziz et al., 2020), anticancer (Oliveira et al., 2021), antiviral (Mandić et al., 2020), antitubercular (Phatak et al. 2019), and antimicrobic (Singh et al. 2015; Holanda et al., 2020). In addition, chlorthalidone

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(diuretic), lenalidomide (immunomodulator), thalidomide and pomalidomide (multiple myeloma treatment), phosmet (insecticide) and apremilast (phosphodiesterase 4 (PDE4) inhibitor) are examples of clinically used phthalimide derivatives (**Figure 1**).

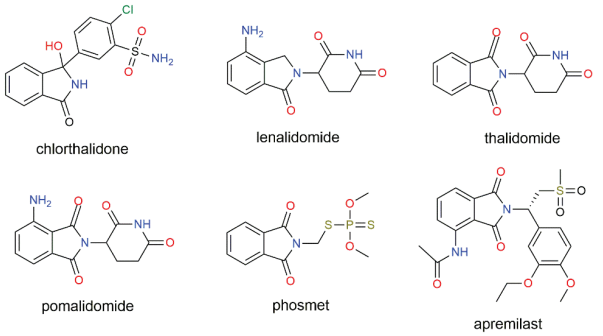



**Figure 1.** Clinically used phthalimide-based drugs.

Similarly, sulfonamides have been reported to show a broad pharmacological profile (Apaydın&Török, 2019; Azevedo-Barbosa, Dias, Franco, Hawkes, &Carvalho, 2020) for example, antibacterial (Nunes, Manaia, Kolvenbach, &Corvini, 2020), antifungal (Pippi et al., 2020), antiviral (Supuran, Innocenti, Mastrolorenzo, &Scozzafava, 2004), diuretics (Turza, Borodi, Miclaus, &Kacso, 2020), anticancer (Wan, Fang, Chen, Deng, & Tang, 2021), carbonic anhydrase inhibitor (Angeli et al., 2021; Hewitt et al., 2021; Petreni et al., 2021), anti-tubercular (Chen et al., 2021), antimalarial (Karpina et al., 2021), and so on. There area number of pharmacological agents that belong to different therapeutic classes derived from sulfanilamide, which is accepted as the first modern chemotherapeutic drug discovered by Domagk as an antibacterial prodrug named Prontosil (Scozzafava et al., 1999; Greenwood, 2010; Kalgutar, Jones, &Sawant, 2010). Moreover, sulfonamides are the most broadly used antibiotic class throughout the world and in clinical use since 1968 (Connor, 1998). They act as inhibitors of the dihydropteroate synthase (DHPS), which is a crucial enzyme for bacterial folic acid synthesis, resulting in the blocking of DNA replication in bacteria. Unlike bacteria, mammals are not able to synthesize their folate and must get folate from their diet, therefore the biosynthetic pathway of bacterial folate production is a selective and attractive target for antimicrobial therapy (Bermingham&Derrick, 2002; Capasso&Supuran, 2014).

To date, many sulfonamide-bearing heterocycles have been reported as strong antimicrobial agents against Gram-negative/positive, and even bacterial strains and fungus which developed multidrug resistance (Sayed, Kamal El-Dean, Ahmed, &Hassanien, 2018; Verma et al., 2020). In this work, to take advantage of both structures, sulfonamide, and phthalimide, we investigated the antibacterial activity of a series of sulfonamide compounds incorporating *ortho*-benzenedisulfonimid moieties (**1**-**6**) which were previously synthesized and evaluated as carbonic anhydrase inhibitors (CAIs) by Güzel-Akdemir and co-workers (**Figure 2**) (Güzel-Akdemir, Akdemir, Isik, Vullo, &Supuran, 2013). In our efforts to discover new potent antimicrobial agents, here we report that six compounds were assayed against a panel of seven bacteria and three fungi. MIC values of the molecules were determined and their antimicrobial profiles were discussed.

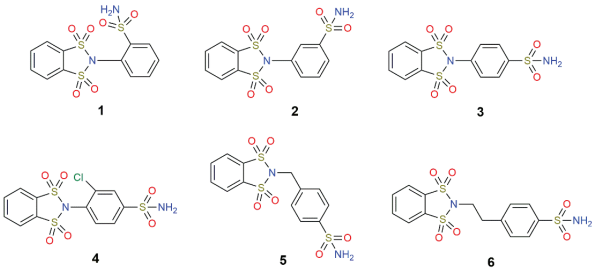



**Figure 2.** Potent antimicrobial *o*-benzenedisulfonimido-sulfonamide derivatives **(1-6).**

## MATERIAL AND METHODS

### Chemistry

Commercial sources of chemicals used and properties of utilized devices for characterization and analyzing of compounds were reported in work previously published work by Güzel-Akdemir's group (Güzel-Akdemir et al., 2013).

### Synthesis of o-benzenedisulfonimide derivatives (1–6)

A solution of *o*-benzenedisulfonyl chloride in 20 mL of dry dichloromethane ($CH_2Cl_2$) was prepared and added to a mixture of a substituted benzenesulfonamide derivative in 25 mL of dichloromethane ($CH_2Cl_2$) including 1.0 mL of $Et_3N$ in 60 min. (see **Scheme 1**). Then, the mixing process was continued at room temperature overnight. The obtained crude product was washed first with aqueous HCl, 5% $NaHCO_3$, and then water, and after that dried with anhydrous $MgSO_4$. The yielded mixture was filtrated and the excess solvent was evaporated, recrystallized from EtOH. (Güzel-Akdemir et al., 2013).

### Antimicrobial activity studies

Antibacterial activity of six molecules were studied *in vitro* with microbroth dilution method against *Staphylococcus aureus* ATCC 29213 (meticillin susceptible *Staphylococcus aureus*, MSSA), *Enterococcus facealis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 12228 and *Proteus mirabilis* ATCC 14153. Antifungal activity was assayed *in vitro* against *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750. The evaluation of antibacterial and antifungal activity was done using microbroth dilution method according to CLSI (Clinical Laboratory Standards Institute) guidance (CLSI 2000, CLSI 2006). As a test medium, for bacteria a Mueller-Hinton broth, and for yeast a Roswell Park Memorial Institute (RPMI-1640) medium were used. Serial two-fold dilutions were prepared beginning from 5000 to 4.9 µg/ml in the medium. The inoculum was produced utilizing a 4-6 h broth culture of each bacteria type, and 24 h culture of yeast strains set to a turbidity equivalent to 0.5 McFarland standard, diluted in broth medium to obtain an eventual concentration of 5 x $10^5$cfu/ml for bacteria, and 5 x $10^5$cfu/ml for yeast in the test plate. To prevent evaporation, plates were protected with plastic bags. Incubation of trays including Mueller-Hinton broth was performed at 35°C for 18-20 h and for the trays including RPMI-1640 medium at 35°C for 46–50 h. In addition, dimethyl sulfoxide (DMSO), used as a solvent in our experiments, was measured against each test strain for its antibacterial or antifungal effects.

Antibacterial or antifungal activity was taken into account in the evaluation of these results .MICs of the newly synthesized compounds were determined. The MIC was described as the lowest concentration of compound which gives the total inhibition of visible growth.

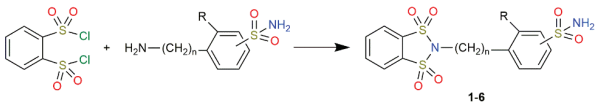
## RESULTS AND DISCUSSION

### Chemistry

As outlined in **Scheme 1**, the reaction of *o*-benzendisulfonyl chloride with suitable aminosulfonamides yielded various benzenesulfonamides (*-ortho*, *-meta* and *-para* substituted derivatives) with alkyl chains of different lengths between the benzenesulfonamide and the *ortho*-benzenedisulfonamide structures. The structures and characterization of previously synthesized compounds **1**-**6** were confirmed by analytical and spectral data (Güzel-Akdemir et al., 2013).

### Antimicrobial activity

Our six-membered small collection of *o*-benzenedisulfonimido-sulfonamides was evaluated for their antibacterial and antifungal potency against members of Gram-negative/positive bacteria, and *Candida spp.*, as outlined in **Table 1**. As reference antimicrobials, ciprofloxacin for antibacterial assays, and fluconazole for antifungal assays were studied. Also, it is founded that reference antimicrobials MIC values were within the CLSI quality control limits. All tested compounds showed antibacterial or antifungal activity in a broad range of MIC values (**Table 1**).Compound **2**, especially, showed better activity against tested bacteria, and fungal strains. These results suggest that compound **2** might be a better therapeutic investigation option among others. But the obtained MIC values of the compounds are still fairly high, so further studies are needed.



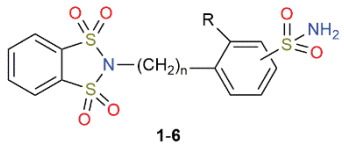

**Scheme 1.** General synthesis pathway of *o*-benzenedisulfonimido-sulfonamide derivatives **(1-6)**.

Among the tested derivatives, compound **2** with *–meta* sulfonamide moiety at phenyl ring and no additional alkyl chain between linked to *o*-benzenedisulfonimid structure showed the best antimicrobial activity. Particularly, compound **2** had the lowest MIC values against the bacterial strains *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, and fungal strain *C. tropicalis* ATCC 750. Compared to the *-ortho* and *-para* analogs of **2**, namely compound **1** and **3**, the *-meta* sulfonamide substitution seems to be beneficial for antimicrobial activity. The remaining derivatives **4**, **5**, and **6** showed a similar antimicrobial potency against tested strains with each other and compounds **1** and **3**. Accordingly, an additional alkyl chain as a linker or a chlorine substitution at phenyl ring made no remarkable changes for the MIC values of the aforementioned compounds. But the chlorine substitution at phenyl ring (compound **4**) has improved the antibacterial activity a little against the *S. aureus* ATCC 29213.

## CONCLUSION

In summary, we investigated the potential antimicrobial activity of previously synthesized six *o*-benzenedisulfonimido-sul-

**Table 1. Antimicrobial activities of compounds 1-6.**




**1:** n= 0, R= H, *o*-$SO_2NH_2$ **4:** n= 0, R= Cl, *p*-$SO_2NH_2$
**2:** n= 0, R= H, *m*-$SO_2NH_2$ **5:** n= 1, R= H, *p*-$SO_2NH_2$
**3:** n= 0, R= H, *p*-$SO_2NH_2$ **6:** n= 1, R= H, *p*-$SO_2NH_2$

| | | Compounds and MIC[a] value µg/ml | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** | **Reference antimicrobials** |
| **Microorganisms** | *S. aureus* ATCC 29213 | 625 | 312.5 | 2500 | 625 | 1250 | 1250 | 0.25 (Ciprofloxacin) |
| | *E. faecalis* ATCC 29212 | 1250 | 625 | 2500 | 1250 | 1250 | 1250 | 0.5 (Ciprofloxacin) |
| | *E. coli* ATCC 25922 | 1250 | 1250 | 2500 | 1250 | 1250 | 1250 | 0.125 (Ciprofloxacin) |
| | *K. pneumoniae* ATCC 4352 | 1250 | 1250 | 2500 | 1250 | 1250 | 1250 | 0.5 (Ciprofloxacin) |
| | *P. aeruginosa* ATCC 27853 | 1250 | 1250 | 2500 | 1250 | 1250 | 1250 | 0.5 (Ciprofloxacin) |
| | *S. epidermidis* ATCC 12228 | 1250 | 1250 | 2500 | 1250 | 1250 | 1250 | 0.125 (Ciprofloxacin) |
| | *P. mirabilis* ATCC 14153 | 1250 | 1250 | 2500 | 1250 | 1250 | 1250 | 0.5 (Ciprofloxacin) |
| | *C. albicans* ATCC 10231 | 625 | 625 | 1250 | 625 | 625 | 625 | 0.5 (Fluconazole) |
| | *C. parapsilosis* ATCC 22019 | 1250 | 625 | 2500 | 625 | 1250 | 1250 | 0.5 (Fluconazole) |
| | *C. tropicalis* ATCC 750 | 1250 | 625 | 2500 | 1250 | 1250 | 1250 | 1.0 (Fluconazole) |

[a] MIC: Minimum inhibitory concentration of the compounds required to suppress a visible growth

fonamide derivatives against ten different bacterial and fungal strains with *in vitro* assays. For all the tested compounds, antibacterial and antifungal activities were obtained and compound **2** with *-meta* sulfonamide substitution without an alkyl spacer between two main structures of molecule showed more promising antimicrobial activity. It is possible to develop more effective antimicrobial candidates by using *o*-benzenedisulfonimido-sulfonamides in different substitution patterns as a key structure, and it is also a prospective idea that further *in vitro* tests may be performed to investigate their potential against different microorganisms.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- Ö.G.A., K.D.Y., F.N.Y., B.Ö.Ç.; Data Acquisition- Ö.G.A., K.D.Y., F.N.Y., B.Ö.Ç.; Data Analysis/Interpretation- Ö.G.A., K.D.Y.; Drafting Manuscript- Ö.G.A., K.D.Y., G.Y.;Critical Revision of Manuscript- Ö.G.A., K.D.Y., F.N.Y., B.Ö.Ç.; Final Approval and Accountability- Ö.G.A., K.D.Y., F.N.Y., B.Ö.Ç.

**Conflict of Interest:** The authors have no conflict of interest to declare

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Original Article

# Diapin®: A food supplement with diverse therapeutic potentials

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**ABSTRACT**

**Background and Aims:** For many centuries herbs and spices have traditionally been used to treat or manage a variety of diseases. The formulation of food supplements containing single or multiple herbs or spices is now popular. These formulations provide biochemical, pharmacological and medicinal benefits due to their diverse phytochemical constituents.
**Methods:** In the present study, Diapin® – a food supplement containing *Olea europaea* L. leaves extract, *Cinnamomum cassia* (L.) J. Presl stem extract, *Nigella sativa* L. seed oil, *Cocos nucifera* L. oil and vitamin D3 – dissolved in absolute ethanol was evaluated for α-amylase, α-glucosidase, acetylcholinesterase, elastase, neuraminidase, adenosine deaminase and arginase inhibitory activity.
**Results:** The supplement strongly inhibited neuraminidase ($IC_{50}$ = 0.272 ± 0.007 mg/mL), while adenosine deaminase, acetylcholinesterase, elastase and arginase were moderately inhibited (with an $IC_{50}$ of 4.562 ± 0.052, 5.396 ± 0.563, 5.783 ± 0.058 and 6.800 ± 0.067 mg/mL respectively). The less inhibition activity was on α-amylase and α-glucosidase ($IC_{50}$ = 9.593 ± 0.582 and 14.010 ± 2.280 mg/mL respectively).
**Conclusion:** The pharmacological activities of Diapin® can be attributed to its opulent phytochemical composition. The present findings support the folkloric claim of Diapin® supplement having antidiabetic, anticancer, anti-inflammatory, antimicrobial, anti-ageing, and immune bolstering properties, in addition to the mitigation of Alzheimer's disease and the alleviation of neurological dysfunction.

**Keywords:** Diapin®, *Olea europaea*, *Cinnamomum cassia, Nigella sativa*, *Cocos nucifera*, Enzyme inhibition, Extracts

## INTRODUCTION

Food supplements, also known as dietary supplements, are diet based formulations (from a singular or combined source) containing vitamins, minerals, essential fatty acids, amino acids or even fibre. Beside these, plant based food supplements contain bioactive compounds that include phenols, terpenoids, thiols, saponins, glycosides, amines, essential oils etc. Thus, they exert pharmacological and therapeutic effects above and beyond their nutritional functions (Garcia-Alvarez *et al*., 2014). Supplements are usually sold as tablets, capsules or in liquid forms. They are easily administered or consumed orally, either before, during or after regular meals. Since they are food based, it is claimed that they have minimal or no adverse effects. In general terms, no clear cut difference exist between food supplements and nutraceuticals. Nevertheless, nutraceuticals (also bioceutical, or sometimes

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functional foods) are more or less food based pharmaceutical alternatives, believed to have biochemical or physiological significance (Hardy, 2000; Kalra, 2003). They are categorised as food supplements and/or food additives by the Food and Drug Administration (FDA) of the United States. Despite the wide use/acceptability of food supplements and nutraceuticals, doubt about the declared benefits and pharmacological effects of these products remains a serious concern. More so, the safety and quality of such products are significant issues (Hasler, 2005).

Olive (*Olea europaea* L.) is a predominant plant in the Mediterranean region (Waterman & Lockwood 2007). The plant fruits are rich sources of unsaturated fatty acid, vitamin E, minerals and carbohydrate. In addition, its fruits and leaves are composed of biologically active phytochemicals, with diverse therapeutic benefits (Jilani, Cilla, Barberá, & Hamdi, 2016; Guo *et al*., 2018). Hitherto, the therapeutic and/or medicinal benefit of the Mediterranean diet has been attributed to the olive rich component of the diet (Obied *et al*., 2012; Roman, Jackson, Gadhia, Roman, & Reis, 2019). The health benefits are said to include higher life expectancy and decreased occurrence of degenerative diseases (Vogel *et al*., 2014; Morris *et al*., 2015; Guo *et al*., 2018; Roman *et al*., 2019). Therefore, the therapeutic benefits of olives cannot be over emphasized.

Cinnamon (also cassia) is a spice obtained from the inner back of a plant species of the genus *Cinnamomum*. The spice is chiefly composed of cinnamaldehyde and many essential oils (including eugenol), the compounds responsible for aromatic and flavouring properties (Jayaprakasha & Rao, 2011). This spice has a history from ancient time, and it is both sacred and highly priced (Gray & Miller, 1970). Cinnamon is reported to have a lowering effect on total cholesterol and triacylglycerols (Maierean *et al*., 2017), and it is said to aid digestion as well as to have a controversial effect on both diabetes (Leach & Kumar, 2012) and glycated haemoglobin levels (Akilen, Tsiami, Devendra, & Robinson, 2012; Leach & Kumar, 2012; Costello *et al*., 2016).

Black seed (*Nigella sativa* L.) has been historically used as an essential herb. In accordance with prophetic sayings, Muslims believe it can cure all diseases except death (Al-Bukhari, 1976). In addition to protein, carbohydrate and unsaturated/essential oils, the plants is rich in active phytochemical such as thymoquinone, thymohydroquinone, dithymoquinone, p-cymene, carvacrol, 4-terpineol, t-anethol, sesquiterpene longifolene, α-pinene and thymol. Moreover, nigellicimine, nigellicimine-N-oxide, nigellidine, nigellicine and alpha-hederin are found in trace amount. Vitamins, provitamins (carotene, stigmasterol among other sterols) and minerals (copper, zinc, phosphorus and iron) are found in the plant seed (Al-Jassir, 1992; Nickavar, Mojab, Javidnia, & Amoli, 2003). A review by Ahmad *et al*. (2013) indicates that black seeds have tremendous pharmacological benefits such as antimicrobial, anti-schistosomiasis, antioxidants, anti-inflammatory and anticancer effect. The plant was also reported to have antidiabetic effect, to act as a vasodilator in the lung, and to protect gastrointestinal, hepatic and renal tissues.

Coconut (*Cocos nucifera* L.) is a member of the palm falmily Arecaceae, whose edible fruit is widely consumed due to it fat composition and milk (Lebrun, Grivet, & Baudouin, 2013; Nayar, 2017). The fresh fruits contains high amount of saturated fats, and lower amounts of carbohydrates and proteins. It is reported to contain significant amount of micronutrients that includes selenium, zinc, copper, iron, manganese and phosphorus (Naik, Raghavendra, & Raghavarao, 2012). Despite containing appreciable levels of antioxidant elements, excessive and chronic consumption of coconut is associated with high risk of cadiovascular diseases due to its high levels of saturated fats. This is manifested as high levels of LDL cholesterol and lauric acid in the blood (Neelakantan, Seah, & van Dam, 2020).

Diapin® (NatiVital) is a Turkish food supplement made from *O. europea* L. leaves extract, *Cinnamomum cassia* (L.) J. Presl stem extract, *N. sativa* L. seed oil, *Cocos nucifera* L. (coconut) oil and vitamin D3 (cholecalciferol). It is believed to have ample therapeutic benefits, thus prompting the present research. The present study is aimed at investigating the inhibitory effects of Diapin® on the activity of some important metabolic enzymes (viz; α-amylase, α-glucosidase, acetyl cholinesterase, elastase, neuraminidase, angiotensin-converting enzyme and arginase).

## MATERIALS AND METHODS

### Sample preparation

Diapin® capsule was pierced, and the content emptied into a weighed empty beaker. A stock solution of 50 mg/ml was prepared by dissolving the obtained contents in absolute ethanol via sonication. Thereafter, serial dilution was prepared from the stock and used for enzyme inhibition study.

### Enzyme inhibition assay

The inhibitory effect of Diapin® on activities of α-amylase, α-glucosidase, acetylcholinesterase, elastase, neuraminidase, arginase and adenosine deaminase were determined according to the method of Bhutkar & Bhise (2012); Tao, Zhang, Cheng, & Wang, 2013; Ingkaninan, Temkitthawon Chuenchon, Yuyaem, & Thongnoi, 2003; Moon, Yim, Song, Lee, & Hyun, 2010; Myers *et al*., (1980); Corraliza, Campo, Soler, & Modolell, 1994, and Blum & Schwedt (1998) respectively. The findings of this research are expressed as mean ± standard deviation of three replicate values. Percentage enzyme inhibition activities of the inhibitors were used to calculate half maximum inhibitions ($IC_{50}$) for individual enzymes, via regression analysis data. The lower the $IC_{50}$ values, the higher the inhibition activity.

## RESULTS

The effect of Diapin® on the activities of α-amylase, α-glucosidase and acetylcholinesterase are presented in Table 1. Diapin® inhibited α-amylase, α-glucosidase and acetylcholinesterase with an $IC_{50}$ of 9.593 ± 0.582, 14.010 ± 2.280 and 5.396 ± 0.563 mg/mL respectively. These inhibition activities were below that of the corresponding standard inhibitors of the enzymes. Acarbose inhibited α-amylase and α-glucosidase with an $IC_{50}$ of 0.059 ± 0.002 and 0.177 ± 0.010 mg/mL, while 1,2,3,4-tetrahydroacridin-9-amine hydrochloride (tacrine) inhibited acetylcholinesterase with a very low $IC_{50}$ of $8.833\times10^{-4} \pm 2.943\times10^{-5}$ mg/mL.

**Table 1. Inhibitory effect of Diapin® on α-amylase, α-glucosidase and acetylcholinesterase activities.**

| Enzyme | Extract/ Standard | Concentration (mg/mL) | Inhibition (%)* | $IC_{50}$ (mg/mL)* |
|---|---|---|---|---|
| α-Amylase | Diapin® | 30.000 | 91.614±1.929 | |
| | | 20.000 | 88.476±0.943 | 9.593±0.582 |
| | | 10.000 | 61.892±2.729 | |
| | | 1.000 | 16.356±1.061 | |
| | Acarbose | 0.050 | 43.042±1.994 | 0.059±0.002 |
| | | 0.030 | 28.620±0.875 | |
| | | 0.010 | 12.458±1.179 | |
| | | 0.005 | 8.642±0.927 | |
| α-Glucosidase | Diapin® | 50.000 | 88.001±1.412 | 14.010±2.280 |
| | | 40.000 | 69.464±1.082 | |
| | | 30.000 | 64.168±1.880 | |
| | | 20.000 | 58.044±2.032 | |
| | Acarbose | 0.050 | 16.092±1.249 | 0.177±0.010 |
| | | 0.030 | 13.291±0.300 | |
| | | 0.010 | 6.791±0.755 | |
| | | 0.005 | 4.390±0.458 | |
| Acetylcholinesterase | Diapin® | 5.000 | 45.284± 3.070 | 5.396±0.563 |
| | | 2.500 | 35.814±2.812 | |
| | | 1.000 | 27.659±2.086 | |
| | | 0.500 | 12.101±0.396 | |
| | Tacrine | 0.003 | 90.721±0.152 | $8.833\times10^{-4}$±$2.943\times10^{-5}$ |
| | | 0.001 | 79.376±1.122 | |
| | | 0.0001 | 33.597±0.623 | |
| | | 0.00001 | 9.807±0.858 | |

*Mean ± SD of triplicate values

As shown in Table 2, Diapin® had a high inhibitory effect on neuraminidase that corresponds to a low $IC_{50}$ of 0.272 ± 0.007 mg/mL, while quercetin inhibited neuraminidase with an $IC_{50}$ of 0.013 ± 0.001 mg/mL. On the other hand, Diapin® had a lower inhibitory effect of adenosine deaminase ($IC_{50}$ = 4.562 ± 0.052 mg/mL), elastase ($IC_{50}$ = 5.783 ± 0.058 mg/mL) and arginase ($IC_{50}$ = 6.800 ± 0.067 mg/mL). The standard inhibitors, erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA), ursolic acid and quercetin inhibited these enzymes with an $IC_{50}$ of $0.053\times10^{-3}$ ± $0.002\times10^{-3}$, 2.907 ± 0.146 and $7.619\times10^{-3}$ ± $1.243\times10^{-5}$ mg/mL respectively (Table 2).

## DISCUSSION

For centuries, herbs and spices have been used as food additives or food supplements due to their immense therapeutic/ health benefits. They are widely used in folk medicinal practices, and are proven to contain pharmacologically active compounds. The inhibition of enzymes is among the major techniques employed by modern medicine for the treatment of disease and infection, as well as for the management of metabolic diseases. In the present study, a Turkish food supplement, Diapin® was investigated for its inhibitory effect on α-amylase, α-glucosidase, acetyl cholinesterase, elastase, neuraminidase, arginase and adenosine deaminase activity.

α-Amylase and α-glucosidase are involved in the degradation of food based carbohydrate in the intestine. They break carbohydrates down into molecular components, which are readily absorbed into the blood stream. The normal activity of these enzymes, accompanied by deranged insulin action (i.e. insulin deficiency or insulin resistance) can be detrimental to normal metabolic processes (Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996). For instance, the activity of these enzymes leads to high postprandial blood glucose in diabetic patients. The excess unutilised blood glucose is then channelled to alternative pathways such as aldose reductase and sorbitol dehydrogenase due to inefficient insulin action. These fallouts are accompanied by increased osmotic pressure, non-enzymatic glycation of macromolecules, and the accumulation of oxidants and advance glycation products. Hence, precipitating diabetic complications such as cataract, kidney failure, heart diseases, autoimmune reactions, increased oxidative stress, neural complications etc. (Villarreal, Reyes, Angelo, Reines, & Ramo, 2011). Therefore, inhibiting the activity of these enzymes plays a vital role in controlling postprandial blood glucose level and attenuating the progression of diabetic complication (Kim, Kwon, & Son, 2000; Zhen *et al*., 2017).

Previous studies have shown that several herbs and species, including components of the food supplement (Diapin®) used in the present study, exhibit antidiabetic properties. Temiz & Temur (2019) demonstrated that the extract of olive leaves significantly inhibited intestinal α-amylase and α-glucosidase of streptozotocin-induced diabetic rats. In addition, the levels of insulin increased, while those of blood glucose and glycated haemoglobin decreased. Reports by Komaki *et al*. (2003) and Nickavar & Yousefian (2011) revealed that olive extracts directly have inhibitory effects on the activities of α-amylase. In

**Table 2. Inhibitory effect of Diapin® on neuraminidase, adenosine deaminase, elastase and arginase activities.**

| Enzyme | Extract/ Standard | Concentration (mg/mL) | Inhibition (%)* | $IC_{50}$ (mg/mL)* |
|---|---|---|---|---|
| Neuraminidase | Diapin® | 0.500 | 82.669±0.488 | 0.272±0.007 |
| | | 0.250 | 49.678±2.511 | |
| | | 0.100 | 25.956±0.614 | |
| | | 0.050 | 13.751±1.101 | |
| | Quercetin | 0.040 | 93.720±1.272 | 0.013±0.001 |
| | | 0.020 | 60.959±3.091 | |
| | | 0.010 | 45.433±2.174 | |
| | | 0.005 | 37.440±1.824 | |
| Adenosine deaminase | Diapin® | 10.000 | 91.599±0.162 | 4.562±0.052 |
| | | 5.000 | 63.005±0.162 | |
| | | 2.000 | 27.464±1.131 | |
| | | 0.500 | 11.632±0.323 | |
| | EHNA | $0.06\times10^{-3}$ | 52.784±0.798 | $0.053\times10^{-3}\pm0.002\times10^{-3}$ |
| | | $0.04\times10^{-3}$ | 44.631±0.204 | |
| | | $0.02\times10^{-3}$ | 39.475±1.135 | |
| | | $0.01\times10^{-3}$ | 34.940±0.488 | |
| Elastase | Diapin® | 5.000 | 45.260±0.306 | 5.783±0.058 |
| | | 4.000 | 35.474±0.917 | |
| | | 3.000 | 33.028±0.306 | |
| | | 1.000 | 16.616±2.212 | |
| | Ursolic Acid | 1.000 | 28.030±0.309 | 2.907±0.146 |
| | | 0.100 | 22.854±0.945 | |
| | | 0.010 | 17.424±1.856 | |
| | | 0.001 | 12.879±0.619 | |
| Arginase | Diapin® | 10.000 | 87.451±0.432 | 6.800±0.067 |
| | | 8.000 | 58.538±0.654 | |
| | | 5.000 | 45.169±1.732 | |
| | | 4.000 | 6.631±0.283 | |
| | Quercetin | 0.010 | 64.975±1.547 | $7.619\times10^{-3}\pm1.243\times10^{-5}$ |
| | | 0.008 | 56.419±0.066 | |
| | | 0.006 | 28.154±0.458 | |
| | | 0.004 | 14.404±0.458 | |

*Mean ± SD of triplicate values

another study, aqueous extract of olive leaves were shown to inhibit maltase and sucrose, as well as intestinal glucose uptake and transport (Kerimi *et al*., 2019). In addition to preventing the digestion of carbohydrate, standardised olive leaf extract (20% oleuropin) has been shown to effectively increase the activity of pancreatic beta cells in obese Australia men (de Bock *et al*., 2013). Furthermore, the strong antioxidant action of the standardised olive extract protects pancreatic cells from diabetic induced oxidative damage, which might occur due to increasing levels of hydrogen peroxide and reactive oxygen (Cumaoglu *et al*., 2011). Moreover, this standardised extract has been demonstrated to attenuate the formation of advanced glycation products- a secondary complication in diabetes that distort the structure and function of biomolecules, and damage tissues as well (Kontogianni *et al*., 2013). Cinnamons are also reported to have antidiabetic effects. However, the specific antidiabetic mechanism and the efficacy of *C. cassia* is still doubted (Vanschoonbeek, Thomassen, Senden, Wodzig, & van Loon , 2006; Costello *et al*., 2016). Several studies have expatiated the antidiabetic potentials of black seeds. In experimental animals, the seed extract is observed to induce a reduction in intestinal glucose absorption and blood glucose level, while increasing insulin level and glucose tolerance (Kanter, Meral, Yener, Ozbek, & Demir, 2003; Meddah *et al*., 2009). Studies by Adekola *et al*., 2017 reveal that extract of coconut testa is capable of inhibiting both pancreatic α-amylase and α-glucosidase. Mohammed *et al*. (2017) found that blood glucose levels were markedly reduced upon administration of aqueous coconut oil extract to alloxan-induced diabetic rats. Vitamin D3 is also reported to strongly inhibit α-glucosidase (Peng, Zhang, & Zen,2016). The aforementioned reports support the findings of the present study as well as the folkloric claim that Diapin® has antidiabetic effect. However, in the present study, the antidiabetic effect of the aforementioned plants mixture could not be proven through the enzyme inhibition mechanisms of a-glucosidase and a-amylase.

In addition to the deposition of β-amyloid in brain and nervous tissue, the excessive activities of cholinesterases (acetylcholinesterase and/or butyrylcholinesterase) plays an important role in the development of Alzheimer's disease (Rao, Sridhar, & Das, 2007). The inhibition of these enzymes alter their catabolic activities, consequently retaining high systemic levels of their substrates. These are some of the basic strategies used for

the management of dementia and other related neurological diseases (Heinrich & Teoh, 2004). Studies have shown that olive leaves and cinnamon bark extract are capable of subsiding the intensity of Alzheimer's disease via promoting autophagy (Cordero, Garcia-Escudero, Avila, Gargini, & Garcia-Escudero, 2018), mitigating the formation and deposition of β-amyloid (Frydman-Marom *et al*., 2011) or by inhibiting cholinesterase activity (Omar, Scott, Hamlin, & Obied, 2018; Park *et al*., 2018), thereby aiding cognitive function. Similarly, acetylcholinesterase is reported to be inhibited by both black seed oil extract (Kannan, Ittiyavirah, & Harindran, 2019) and coconut extract (Nafar & Mearow, 2014; Mirzaei, Khazaei, Komaki, Amiri, & Jalili, 2019) or to improve cognitive functions by decreasing plaque formation and neuron death. Conversely, altered levels of vitamin D are reported in people with Alzheimer's disease (Shah *et al*., 2012; Johansson *et al*., 2013), and the administration of this vitamin tends to downplay the progression and symptoms of the disease as well as the activity of acetylcholinesterase (Annweiler, Karras, Anagnostis, & Beauchet, 2014). The findings of the present study, in addition to the aforementioned reports, suggest that Diapin® supplementation may play a vital role in the attenuation of plaque formation, progression of dementia as well as retaining adequate systemic levels of acetylcholine.

Neuraminidases have a substantial effect on the pathogenesis and virulence microorganisms (Rothe, Rothe, Roggentin, & Schauer, 1991). These enzymes hydrolyse neuraminic acid and its derivative, thus aiding pathogens-host cell interaction, virion progeny elution, aggregation and motility (von Itzstein, 2007; McAuley *et al*., 2017). The outcome of the present study indicated that Diapin® is a good inhibitor of neuraminidase ($IC_{50}$ = 0.262 ± 0.012 mg/mL). This finding is in line with previous reports that demonstrated the antimicrobial effect of the various components of Diapin® which are: *O. europaea* L. leaves extract (Pereira *et al*., 2007; Korukluoglu, Sahan, Yigit, Ozer, & Gucer, 2010; Liu, McKeever & Malik, 2017). *C. cassia* stem extract (Munazza, Najam-us-Sahar, Deeba, & Farhan, 2016), *N. sativa* seed oil (Bakathir & Abbas, 2011) and *C. nucifera*oil (Silva *et al*., 2013; Hovorková, Laloučková & Skřivanová, 2018). Moreover, the hypothesised effect of the Mediterranean diet against COVID-19 and other respiratory syndromes (Angelidi, Kokkinos, Katechaki, Ros, & Mantzoros, 2021; Baeta, Bagina, & Canilhas, 2020; Tamer, Fayed, Ayman, & Ibrahim, 2020) may not be unconnected to the anti-neuraminidase activity of the diet - as displayed by Diapin® (a supplement composed of herbs commonly used in the Mediterranean diet).

Adenosine deaminase is an important enzyme of purine metabolism. Its primary function is the irreversible deamination adenosine to inosine (Losey, Ruthenburg, & Verdine, 2006). This enzyme is also believed to be associated with normal immune function, neurotransmission, epithelial cell differentiation and gestation (Moriwaki, Yamamoto, & Higashino, 1999). Deficient level/activity of adenosine deaminase is linked to pulmonary fibrosis, while its over expression or hyperactivity is observed in some disease conditions such as autoimmune dysfunction (e.g. arthritis, psoriasis and sarcoidosis), cancer, ischemia, haemolytic anaemia and AIDS (Blackburn & Kellems, 2005). Thus, inhibiting this enzyme may help in the management of these diseases, and/or alleviate their symptoms. Cubukcu, Durak, Kocaoglu, & Durak, (2018) demonstrated that the adenosine deaminase activity of cancerous gastric tissue was strongly inhibited by aqueous olive leaves extract. Likewise, nanoparticles of the extract were shown by Farhan *et al*.(2016) to inhibit this enzyme in sera of arthrosclerosis patients. Another study reveals the cytotoxic effect of the extract on cancer cells (Korkmaz, Sarimahmut, Ozel, & Ulukaya, 2016). Similarly, *N. sativa* (Shafiq, Ahmad, Masud & Kaleem, 2014; Gholamnezhad, Rafatpanah, Sadeghnia, & Boskabady, 2015) and *C. cassia* extract are proven to exhibit cytotoxic effects or prevent mutations (Ngoc *et al*., 2014). These reports support the claim of Diapin® having both anti-inflammatory, anticancer and autoimmune stabilizing effects.

Elastase are protein proteases responsible for the degradation of elastic- a connective tissue protein critical for elasticity in association with collagen (Bieth, 2001). These enzymes are associated with the degradation and recycling of extracellular tissue matrixes, which in turn aid tissue repair, wound healing and re-epithelialization processes. Moreover, they play an important immunological role through degradation of the outer membrane protein A Gram-negative bacteria (e.g. *E. coli*) and the Shigella virulence factors. However, elastase may instigate the progression of inflammatory anomalies, heart diseases, cancer, fibrosis, as well as virulence factor of some microbes (Girish, Kemparaju,Nagaraju, & Vishwanath, 2009; Alam, Newby,& Henriksen, 2012). Findings suggest that elastase inhibitors could mitigate inflammatory responses and decrease the release of inflammatory cytokine (Alam, Newby, & Henriksen, 2012), as well as lung cancer metastasis (Moroy, Alix, Sapi, Hornebeck, & Bourguet, 2012). The outcome of the present study indicates that Diapin®inhibits elastase activity. This finding is in agreement with previous reports that demonstrate elastase inhibitory activity of *O. Europaea* (Battinelli *et al*., 2006; Angelis *et al*., 2020) and *N. sativa* (Kacem & Meraihi, 2006), as well as the wound healing and anti-inflammatory activities of *C. nucifera* (Zakaria *et al*., 2006).

Arginase is an enzyme of ureagenesis that catalysis the conversion of L-arginine into L-ornithine and urea (Wu & Morris, 1998). This ureohydrolase is abundant in liver, kidney and prostate, and to a lesser extent in the brain, macrophages and lactating mammary glands (Morris, 2002). The arginase II isozyme is believed to be co-expressed with its substrate competitor - nitric oxide synthase - in the genitals and other smooth muscle tissue. The activity of nitric oxide synthase on the other hand is correlated with the bioavailability of nitric oxide, a molecule that induces nitric oxide-dependent smooth muscle relaxation and is capable of acting as an oxidant as well. Over expression/activity of arginase and inhibition of nitric oxide synthase is accompanied by competitive depletion of arginine pool, and the ultimate depletion of nitric oxide respectively. This is believed to precipitate erectile dysfunction and decreased smooth muscle relaxation (Cama *et al*., 2003; Christianson, 2005; Kim *et al*., 2009). Moreover , elevated activity of arginase is reported in asthma, oxidative stress induced diabetes (Kiss *et al*., 2014), chronic obstructive pulmonary disease (van den Berg, Meurs, & Gosens, 2018) and in cystic fibrosis (Maarsingh, Khazaei, Koma-

ki, Amiri, & Jalili, 2008). Therefore, inhibitors of arginase such as Diapin® may have positive implications on the aforementioned maladies.

## CONCLUSION

The present study demonstrates that Diapin® inhibits the activities of α-amylase, α-glucosidase, acetyl cholinesterase, elastase, neuraminidase, adenosine deaminase and arginase. The previously reported antioxidant, anti-inflammatory and beta-cell enhancing activity of Diapin® is an added advantage to its inhibitory potentials. Its broad pharmacological and biochemical activities are attributed to the rich phytochemical composition of the herbs, spices, vitamins/provitamins (i.e. *O. europaea* L. leaves extract, *C. cassia* (L.) J. Presl stem extract, *N. sativa* L. seed oil, *Cocos nucifera* L. oil and vitamin D3) contained in the supplement. Moreover, the present findings support the folkloric claim of Diapin® supplement having antidiabetic, anticancer, anti-inflammatory, antimicrobial, anti-aging, and immune bolstering properties, and that it alleviates neurological dysfunction and mitigates Alzheimer's disease.

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**Original Article**

# Effects of vitamin D on proliferation, invasion and energy metabolism of MCF-7 breast cancer cell line

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**ABSTRACT**

**Background and Aims:** 1,25(OH)2D3 (vitamin D) is a pleiotropic hormone with anti-proliferative, pro-apoptotic, and pro-differentiation effects on various cell types, which suggest anti-cancer activity in addition to its classical regulatory action on calcium and phosphate metabolism.
**Methods:** We aimed to put forward the effects of vitamin D in various concentrations and time intervals on cell proliferation and invasion of human estrogen receptor-positive breast cancer (MCF-7) cells by real-time cell electronic sensing system (xCELLigence). A determined dose of the $IC_{50}$ was applied on samples taken from cell lysates and analyzed the levels of the energy. We also aimed to clarify how vitamin D effects the activity of the protease uPA and their relations with each other.
**Results:** Vitamin D showed a cytotoxic effect on MCF-7 cells in a time and dose dependent manner, with dose of $IC_{50}$ found to be 140 nM. ATP, ADP, and AMP levels, as well as uPA activities were respectively increased in vitamin D treatment group compared to the control group for the first 24 hours while decreasing at 48, 72, and 96 hours. We determined that 70 and 140 nM vitamin D were decreased in invasion of MCF-7 cells compared to control cells.
**Conclusion:** We observed that proliferation and invasion of breast cancer cells were inhibited by vitamin D treatment on a dose and time dependent manner, and also vitamin D supplementation decreased uPA activity and energy levels. Further studies on the mechanisms of vitamin D and the formulation of none-hypercalcemic analogues in featured are needed.
**Keywords:** Activity of uPA, Energy levels, Invasion, Proliferation, Vitamin D

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## INTRODUCTION

Breast cancer is the second most common cancer among cancer-related deaths in women. Growth factors, age, diet, genetic factors, and changes hormonal regulation play a role in breast cancer progression (Kamińska, Ciszewski, Łopacka-Szatan, Miotła, & Starosławska, 2015). Therefore, studies are ongoing to research the treatment of breast cancer. In recent years, there have been studies indicating that vitamin D can be taken as a supplement in cancer treatment. The active form of vitamin D not only regulates calcium metabolism (Anderson, 2017), but also has many functions, such as the regulation of immune response, cell proliferation, and differentiation in metabolism. It plays a critical role on many diseases, such as osteoarthritis, diabetes, cancer, cardiovascular diseases, and tuberculosis (Uitterlinden, Fang, Van Meurs, Pols, & Van Leeuwen, 2004). uPA, uPAR, plasminogen activator inhibitor-1 (PAI-1), and plasminogen activator inhibitor-2 (PAI-2) take place in the urokinase plasminogen activator (uPA) system. uPA system plays an important role in tumor invasion and metastasis by causing degradation of tumor stroma and basement membrane. It has been reported that a high activity of uPA in the primary tumor is associated with poor survival in breast cancer patients (Duffy & Duggan, 2004; Schmitt et al., 1997).

Cancer cells can proliferate rapidly and convert glucose into lactate in an anaerobic environment, since the amount of ATP obtained from glucose is not sufficient. This effect supports the accumulation of nucleosides and amino acids with increased glucose intake and therefore facilitates energy production (Pavlova & Thompson, 2016). In our study it was aimed to clarify the effects of vitamin D supplementation according to the metabolic critical points of MCF-7 breast cancer cells. ATP, ADP, and AMP levels were determined to evaluate the energy metabolism and the capacity of invasion, and uPA activity was demonstrated to explain the status of the invasion of the cancer cells. The difference of our study from current studies is that there are no cell culture experiments in breast cancer cells in which vitamin D supplementation and their capacity of uPA and invasion and energy status have been evaluated so far. Studies in which vitamin D is given externally make up the majority of clinical studies, therefore this aspect makes our studies important.

## MATERIAL AND METHODS

### Cell culture

MCF-7, human breast cancer cell line, gained from the ATCC (Manassass,VA, USA), was cultured in DMEM (Dulbecco's Modified Eagle Medium), containing 10% fetal bovine serum (FBS) and 1% penicilin/ streptomycin, respectively. Cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$–95% air.

### Cell viability assay

1α,25-Dihydroxyvitamin $D_3$ (calcitriol) was purchased from Sigma Aldrich (Missouri, USA). It was dissolved in ethanol. The cytotoxic effect of vitamin D on breast cancer cells was determined by a real time cell analyzer (xCELLigence, ACEA Biosciences, Inc, CA, USA). The cells (10000/well) were seeded into 16-well plates for 24 h. After seeding, based on previous studies (Mathiasen, Lademann, & Jäättelä, 1999), a range for vitamin D dose has been established and in order to determine the proliferative and toxic range, with a total of eight different doses being applied in a wide range to attain the sigmoidal curve. The cells were thus treated with various concentrations of vitamin D (10, 25, 50, 125, 250, 500, 1000, and 2500 nM). MCF-7 cells were monitored every 15 min for a period of up to 81 h via xCELLigence system. The values of the electrode impedance were represented as the cell index. The concentration of vitamin D that inhibits 50% cell viability ($IC_{50}$) was determined according to sigmoidal curve.

### Determination of protein concentration

The cells were seeded in a 5. $10^5$ arrangement within the T-25 flasks. After 24 hours, cells were divided into two groups: 1-control group and 2- vitamin D treated group, which was formed according to the value of $IC_{50}$ (140 nM) that we found in the assay of cell viability. Cells were washed with PBS, culture supernatant was removed, and ripa lysis buffer (Saint Louis.U.S.A.) was added to cell lysates to measure protein, uPA, and energy levels. The protein concentration was analyzed with Lowry protein assay method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin (BSA) was used, which is a standard in protein analysis (Melbourne, Germany). This assay was performed at 700 nm against a reagent blank via colorimetric method with a spectrometer Perkin Elmer Lambda 25 UV / Vis, U.S.A.) Finally, the samples were calculated as mg/mL.

### Determination of uPA enzyme activity

The samples (control and vitamin D treated groups), blank, and uPA standards were added into the well. For calibration and linearity studies of uPA (R&D systems, U.S.A.) standard curve and linearity equation of uPA standards were obtained at dose ranges of 10-2500 ng/ml. Firstly, Tris-HCl and plasminogen (R&D systems, U.S.A) were added to all samples, they were incubated at 37 ºC for 2 hours. Then plasminogen activator substrate (Chromogenix, Canada, U.S.A.) were added into the well, which was then shook at 37 ºC for 6 hours. The uPA values of the samples were measured at 405 nm via colorimetric method. In order to calculate the uPA activity as IU/mg protein, total protein concentration (mg/mL) was divided by uPA concentration (IU/mL).

### Measurement of energy levels of cells

The energy levels of the cells was measured using the method established by a study conducted by Cimen et al. in 2004 (Cimen, Turkozkan, Unlu, & Erbil, 2005). Mobile phase was prepared by degassing the solution containing 160 mM $KH_2PO_4$ and 100 mM KCl, and energy values of the cells were measured in HPLC (AGILENT 1200, Santa Clara, U.S.A.) with GES C18 column (VertiSep™ 4,6x150 mm, 5 µm, Thailand). It was determined that ATP, ADP, and AMP peaks according to the retention times of ATP, ADP, and AMP standards, respectively (). ATP, ADP and AMP standards were prepared at different concentrations and were first injected into the system subsequently.

### Determination of invasion capacity in vitamin D treated cells

Firstly, we added matrigel (BD Biosciences, Germany) to each well of the upper chamber of the 16-well cell invasion/mi-

gration (CIM)plate for 4 hours incubation. Then, DMEM was placed in the lower chamber, before the upper and lower chambers were combined. Into the upper chamber, we then added 20,000 cell/each well and a different concentration of plasminogen. The ideal amount of plasminogen for invasion of MCF-7 cells was determined from this experiment, and subsequent experiments were performed based on these values. In the second experiment of invasion assay, different doses of vitamin D (28-70-140 nM) with plasminogen were treated in MCF-7 cells. Changes in cell invasion capacity were observed on CIM-plates with the xCELLigence® device inside the incubator every 15 min for 72 hours.

### Statistical analysis

Statistical analyzes were made with SPSS 18.0 package program. The Kolmogorov-Smirnov test was performed to examine whether the MCF-7 cell line proliferation, energy levels, uPA activity, and invasion capacity data, which were treated with various doses of vitamin D, fit the normal distribution. Accordingly, it was seen that the data showed a normal distribution. Then, One Way Anova analysis of variance was performed in repeated measurements to examine the differences between dose groups and times in an interactive fashion. The difference in times in each dose group was analyzed with the Post Hoc Dunnett test. Energy level, uPA activity, and invasion capacity data at different time intervals were evaluated with the "t" test. The statistical difference was accepted as $p \leq 0.05$.

## RESULTS

### Effect of vitamin D treatment on MCF-7 cell proliferation

The dose- and time-dependent effect of vitamin D on MCF-7 cell growth was analyzed and cell growth rates were monitored for 82 hours at 10, 25, 50, 125, 250, 500, 1000, and 2500 nM concentrations of vitamin D. It was observed that vitamin D inhibited cells compared with the control group in a dose and time dependent manner, as shown in Figure 1 below.

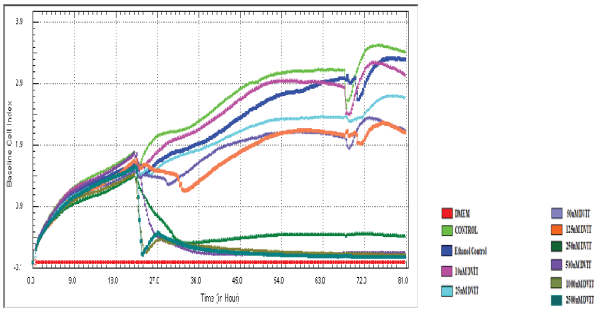


**Figure 1.** The effect of vitamin D treatment to MCF-7 at different concentrations and time dependent manner on cell viability.

The $IC_{50}$ value was calculated as 140 nM ($r^2$= 0.99) from the proliferation curve graph of the vitamin D treatment on MCF-7 cells. This value was calculated by taking the logarithms of all administered dose groups at 48 hours, except the control and ethanol groups, and plotting a sigmoidal curve against the cell index value with this value.

### ATP, ADP, AMP levels of vitamin D treatment cells

We evaluated the energy status effects of vitamin D in MCF-7 cells on according to various time intervals. The levels of ATP were higher at 24th hour forvitamin D application group compared to the control group, with decreases observed at 48, 72, and 96 hours ($p \leq 0{,}001$). ADP levels for vitamin-D-treated group were increased ($p \leq 0.05$) compared to the control group at 24 hours, and decreased respectively at 48, 72, and 96 hours ($p \leq 0.001$). AMP levels decreased at an application time of 48, 72, and 96 hours compared to the control group ($p \leq 0.001$). All of these values are provided in Figure 2 below.

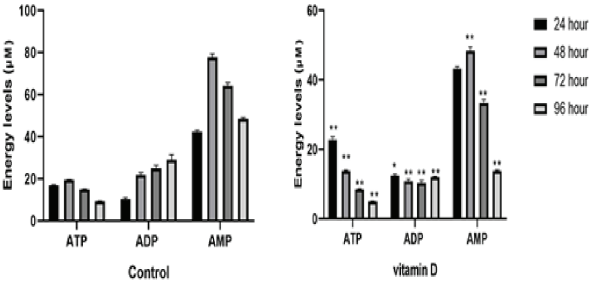


**Figure 2.** Comparison of the effect of 140 nM vitamin D treatment on energy level at different times (*$p \leq 0.05$ and **$p \leq 0.01$, treatment group vs control).

### The measurement of uPA activity in vitamin D treated and control group cells

The effect of vitamin D treatment on uPA activity of MCF-7 cells was evaluated considering different times of application. In our study, when the vitamin D administered group was compared with the control group at 24th hour, the uPA level increased ($p \leq 0.05$), with decreases observed at the 48th, 72nd ($p \leq 0.05$), and 96th hours ($p \leq 0.001$). Comparison of the effect of vitamin D on uPA activity at different times is given in Figure 3 below.

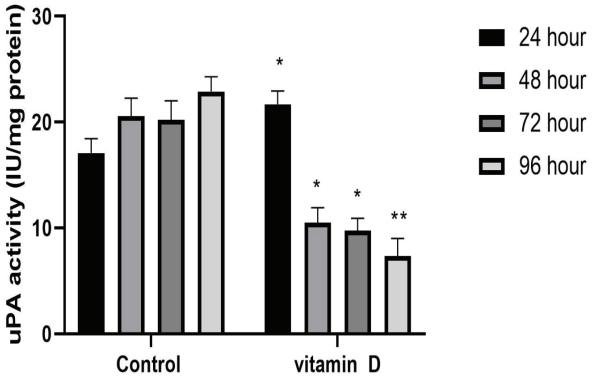


**Figure 3.** uPA activity of control and 140 nM vitamin D treated groups (*$p \leq 0.05$ and **$p \leq 0.001$, treatments groups vs control group).

### Invasion capacity of vitamin D treated and control group cells

Since the invasion capacity of the MCF-7 cell line is low, we firstly increased the invasion capacity by giving different doses of plasminogen to the cells, as shown in Figure 4a. Following

this, we determined appropriate dose of plasminogen as 10 µg/mL.

The effect of different doses of vitamin D at the 72nd hour (48 hours after treatment of vitamin D) on the invasion of MCF-7 cells was compared with the control group. There was a significant difference between the dose groups (70 and 140 nM) compared to the control group ($p \leq 0.05$). As can be seen from Figure 4b, vitamin D decreases the invasion capacity of cells in a dose and time-dependent manner.

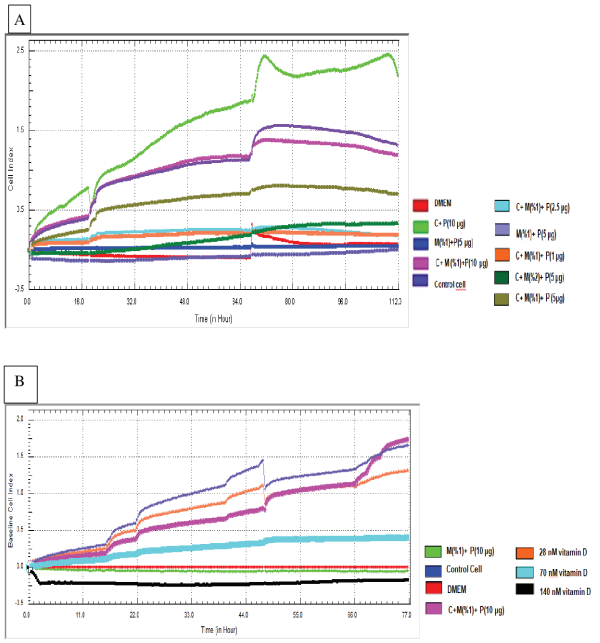


**Figure 4.** A. Time dependent invasion capacity of MCF-7 cells at different amounts of plasminogen and matrigel (C: cell, M: matrigel, P: plasminogen) B. the effect of vitamin D on invasion in MCF-7 cells.

## DISCUSSION

The effects of vitamin D supplementation in cancer have been mostly shown in clinical studies. Supplementation of vitamin D constitutes an alternative to the use of chemotherapy and hormone therapy with anti-estrogens, especially in the treatment of breast cancer. Both in vitro and in vivo studies have shown that vitamin D compounds can inhibit breast cancer. Some evidence suggests that vitamin D deficiency enhances the risk of cancer development and/or progression (Welsh, 2021).

The first studies were conducted on epidemiological effect of vitamin D. It has been postulated that epidemiological studies on the relationships between breast cancer and vitamin D were primarily ecologically based (Robsahm, Tretli, Dahlback, & Moan, 2004).

Garland et al. (1991) showed the relationship between lowest area which receives intense sunlight versus the incidence and mortality of breast cancer in the USA. Therefore, they suggested that there is a relationship between vitamin D and sunlight. A strong inverse relationship was established between breast cancer mortality and sunlight ($r=-0.80$ $p \leq 0.0001$) ( Garland et al., 2006) and also exposure to sunlight and breast cancer incidence (Lim et al., 2006; Porojnicu et al., 2007).

Furthermore, when the studies on receiving vitamin D as a supplement are evaluated, the research of Rossi et al. (2009) draws particular attention. The study was performed on 2,569 patients aged 23-74 years who were diagnosed with breast cancer as well as 2,588 healthy women aged 20-74 years, asking about their weekly diets for two years. The study determined that intake of vitamin D >3.57 µg or 143 IU appeared to have a protective effect against breast cancer. In addition, a study supported the protective effects of vitamin D on pre-menopausal women with breast cancer (Abbas, Chang-Claude, & Linseisen, 2009).

Veldhuis et al. (2011) included a total of 885 women in their study, 112 (12.7%) of which were found to have various types of cancer. The prevalence of breast (n = 56, 50%) cancer in women with low 25-Hydroxyvitamin $D_3$ (25-OHD) (≤50 nmol/L) was higher than in women with high 25-OHD levels (≥ 50 nmol/L). It has been indicated that the prevalence of breast cancer is increased in osteoporotic women with low 25-OHD (≤50 nmol/L) serum levels.

The cell culture experiments were carried out to explain the effectiveness of vitamin D based on a molecular perspective. In vitro study of MCF-7, the ratio of apoptosis to proliferation (A/P) was determined. It has been reported that 1.25 $D_3$ levels are related to an increased A/P (apoptosis/proliferation) rate (Veldhuis et al., 2011). Furthermore, mechanisms underlying the anti-proliferative actions of 1.25 $D_3$ have been identified. Some data support the concept that the anti-tumor effects of vitamin $D_3$ compounds on ER (estrogen receptor)-sensitive human breast cancer cells are associated with estrogen-mediated disruption of mitogenic and viable signaling. Flanagan et al. (2003) found that 100 nM vitamin D supplementation inhibited the cell number and invasion of breast cancer. Although the types of cell lines are different than those used in our studies, the results are similar from the view of the inhibited effects of vitamin D supplementation on invasion of breast cancer.

According to a study working in the same cell culture line as our study, three main vitamin D metabolic enzymes have been found in malignant breast tissue including 25-hydroxylase, 1alpha-OHase, and 24-OHase. The study revealed that MCF-7 cells are expressed 24-hydroxylase. The ability to form inactive vitamin D metabolite with the 24 OH'lase enzyme might be a major action that tumor cells will use to protect themselves against the antiproliferative and calcitriol induced-apoptosis (Diesing, Cordes, Fischer, Diedrich, & Friedrich, 2006).

The presence of VDR (vitamin D receptor) has been identified in the MCF-7 cell line in 2009 by Sertznig et al. In vivo, osteosclerotic metastasis developed more rapidly as vitamin D-deficient mice had extensive defects in their tibia compared with mice with adequate vitamin D levels. The tumor area has been shown to be increased by 55.8% in vitamin D-deficient mice. Also, MCF-7 cells express genes of VDR, 1α- and 24-hydroxylase and play critical roles in the vitamin D signaling pathway and metabolism (Ooi et al., 2010).

In a study comparing 25(OH)$D_3$ and 1,25-$D_3$, $10^{-7}$ -$10^{-9}$ M 25(OH) $D_3$ caused insignificant growth inhibition in the proliferation of MCF-7 cells, whereas treatment of 25-$D_3$ with a concentration of $10^{-7}$ and $10^{-8}$ M was found to inhibit cell growth significantly (Friedrich et al., 2006).

In two metastatic subtypes of human MCF-7 breast tumor cells, QW-1624F2-2 (1,25-$(OH)_2D_3$ synthetic analogue) inhibited breast tumor cell proliferation and invasion, promoted cell differentiation, and induced apoptotic cell death. It is thought that QW-1624F2-2 affects the blocking of 24-hydroxylase, thus protecting 1,25-$(OH)_2D_3$ and its analogs from 24-hydroxylation, prolonging their biological life, facilitating the effective treatment of breast cancer, and enabling the use of chemotherapeutic agents at low levels (Sundaram et al., 2006).

Although the active form of vitamin $D_3$, 1,25-dihydroxy vitamin $D_3$, has anti-invasion and anti-migration properties in pre-clinical studies, it has not yet been fully implemented into clinical practice due to its hypercalcemic side effects. Therefore, vitamin D analogues have been developed to reduce hypercalcemia. In a study by Chiang et al. (2014), it was reported that MART-10, a vitamin D analog, is 1,000 times more active than vitamin D in suppressing MCF-7 cell growth. In addition to MART-10, MMP-13 are more active than vitamin D in preventing cell invasion and migration in MCF-7. The MCF-7 cells were treated with $10^{-7}$ and $10^{-6}$ M, with vitamin D inhibiting the invasion of MCF-7 cells by 46±5% and 62±6%, respectively. MCF-7 cells were treated with $10^{-8}$ and $10^{-7}$ M MART-10 was found to be at least 10 times more effective than vitamin D in preventing MCF-7 invasion. In our study, the invasion capacities of 28, 70, and 140 nM vitamin D at the 72$^{nd}$ hour were reduced by 29%, 78% and 94%, respectively, compared to the control group. When we compared our results with Chiang et al (2014), findings about the reduction of invasion capacity are similar with our results. The differences in the values may be due to the method used in analysis of invasion capacity in both studies.

In a study conducted on different cell lines, non-malignant MCF-12A and malignant MCF-7, MDA-MB-231 epithelial breast cells were treated for six days at increasing concentrations of 1.25-$D_3$. MCF-12A cells (growth inhibition 60% by 100 nM treatment for six days) were shown to be more sensitive than MCF-7 cells (growth inhibition 40% by 100 nM treatment for 6 days) ($p \leq 0.001$). Malignant MDA-MB-231 cells were not susceptible to growth inhibition on treatment with 1,25-$D_3$ ($p \leq 0.05$). The cell viability test was measured with the neutral red dye assay and the same results were obtained with the cell growth (Brosseau, Pirianov, & Colston, 2010).

Marchionatti et al. (2009) applied different doses of 1.25$(OH)_2D_3$±Menadione concentrations for 96 hours. While 1 or 10 nM $D_3$ alone or combined with 5 µM menadione did not inhibit MCF-7 cell growth, 100 nM $D_3$ alone or combined with different doses of menadione was found to inhibit MCF-7 cell growth. In our study, the appropriate dose of vitamin D was found to be close to the mentioned literature.

In a different vitamin D- combined study, to test whether resveratrol (RES) improves cellular sensitivity at lower doses of 1,25$(OH)_2D_3$, T47D cells were treated with vitamin D in the presence of 4nM RES or absence of RES for five days. It was observed that vitamin D at 1 nM did not alone reduce cell growth, but it reduced cell numbers by approximately 40% when combined with RES. Similarly, 10 nM vitamin D reduced cell numbers by 25% in the absence of RES and by 50% in the presence of RES. In the presence of RES, 10nM vitamin D alone was as effective as 100 nM vitamin D in growth inhibition (Wietzke & Welsh, 2003).

Proietti et al. (2011) treated MCF-7 breast cancer cells with combined vitamin $D_3$ and melatonin, and demonstrated the synergistical proliferative inhibition with the completion of cell growth for 144 hours.

Besides our proliferation study, we also analyzed uPa activity as an invasion marker. As it is well known that extracellular matrix proteases (such as uPa, ADAM, MMP, TIMP, RECK) are complex and heterogeneous enzymes play an important role in many pathological processes including cancer. They can alter various biological processes, such as angiogenesis, growth factor bioavailability, cytokine modulation, cell migration, proliferation, invasion, and apoptosis. Highly-invasive cancer is usually characterized by an abnormal activity of certain intracellular or extracellular molecules, such as protein kinases, phosphatases, transcription factors, and proteolytic enzymes. The expression of both urokinase-type plasminogen activator (uPA) and its receptor (uPAR) were correlated with an invasive cancer cell phenotype and a poor prognosis (Sliva, 2004). Duffy and Duggan (2004) stated that uPA and PAI-1 are among the strongest prognostic factors in node-negative patients, and a combined evaluation of these factors would make a more powerful prognostic criteria rather than alone. In a study comparing urokinase system factors, high uPA levels were associated with low efficacy of tamoxifen treatment (Meijer-van Gelder et al., 2004). In our study, uPA levels decreased on the 48$^{th}$ (10.5±1.41 versus 20.5±1.68 IU/mg protein), 72$^{nd}$ (9.75±1.17 versus 20.22±1.79 IU/mg protein), and 96$^{th}$ (7.34±1.66 versus 22.89±1.39 IU/mg protein) hours in the vitamin D-treated compared to the control groups. We demonstrated that the reduction of invasion by vitamin D treatment might be mediated with the reduction in uPA activity.

So et al. (2013) observed that Geminin (vitamin D analog) in MCF10DCIS cells inhibited MCF10DCIS xenograft tumor growth. They stated that the vitamin D analog is more effective in cell invasion than vitamin D. Kim et al (2014) investigated the effectiveness of pepper seed extract (PSE) on the invasion and migration of breast cancer cells using a Boyden chamber. PSE application suppressed the invasion of MDA-MB-231 and MCF-7 cells in a dose-dependent manner. They observed that the invasion of MDA-MB-231 and MCF-7 cells was reduced by 27% and 32.3%, respectively, at a concentration of 50µg/mL.

In a study with grape seed extract (GSE), it was observed that high concentrations of grape seed extract inhibited cell proliferation and apoptosis. Conversely, low GSE concentration inhibits the activity of uPA, MMP-2 (matrix metalloprotease-2) and MMP-9. Thus, it resulted in the reduction of cell migration and invasion in MCF-7 and MDA-MB- 231 cell lines (Dinicola et al., 2014).

Another remarkable aspect of our study is the results of energy charge with ATP, ADP, and AMP values, of which we evaluated the effect of vitamin D on energy metabolism at different time intervals. It is a critical point that the energy charge explains a sensitive intracellular mechanism of the cells through the regulation of enzymatic reactions in the utilization of ATP.

Kaur et al. (2013) examined 6-Mercaptopurine (6-MP) and dasatinib combination on changing ATP concentration in MCF-7 and MDA-MB-468 breast cancer cells, NCI-H23 and NCI-H460 non-small cell lung cancer cells, and A498 and 786-O kidney cancer cells. In their results, a significant decrease was observed in the ATP concentration of breast and lung cancer cells, while kidney cancer cells were resistant to this combination compound. Zoledronic acid (Zol), a bisphosphonate group compound, is an antitumoral compound used in the chemotherapeutic treatments of breast cancer patients with bone metastases. Fehm et al. (2012) reported that Zol significantly reduced the ATP concentration of breast cancer cells. One of the important semi-synthetic plant alkaloids, Vinorelbine, was used in the chemotherapeutic treatment of metastatic breast cancer. It was reported that Vinorelbine decreased the ATP concentration by 42%, and that MCF-7 cell lines were more sensitive to Vinorelbine supplementation than MDA-MB-435 (Ning et al., 2011).

Sucha et al. (2013) investigated the effect of α-Tomatin on the ATP concentration of MCF-7 human breast cancer cells. The study found that α-Tomatin significantly reduced the ATP concentration in MCF-7 cells compared to the control group, depending on dose and time interval.

In our study, there was a decrease in energy levels in the vitamin D administered group compared to the control group for 48 (13.76±0.25 versus 19.48±0.17 μM), 72 (8.46±0.21 versus 15.00±0.21μM), and 96 (4.97±0.13 versus 9.31±0.18 μM) hours. Although treatment differed between the studies, our findings have the same results as the mentioned studies in the terms of reduction of ATP levels.

## CONCLUSION

We observed that vitamin D is anti-proliferative, causes a decrease in ATP, ADP, and AMP nucleotides, which indicates the energy level, and is anti-invasive and effective in the uPA system. We demonstrated the reductive effects of vitamin D on invasion via uPA. However, more extended studies are required to demonstrate how different proteases influence this process. Further studies are also needed to evaluate vitamin D analogues as an anticancer agent with strong anticancer effects and low calcemic activity.

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**Conflict of Interest:** The authors have no conflict of interest to declare.

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Original Article

# Investigation of enzyme inhibition potentials, and antioxidative properties of the extracts of endemic *Stachys bombycina* Boiss.

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**ABSTRACT**

**Background and Aims:** The genus *Stachys* L., is represented by around 300 species worldwide. More than 120 taxa, almost 60 of which are endemic, are widely distributed in Turkey, particularly in the eastern and southern regions. Stachys species have traditionally been used for many diseases such as asthma, rheumatism, cough, genital tumors, ulcers, diabetes, hemorrhoids, kidney stones, and various mental disorders. Among the species, *S. bombycina* Boiss., namely "arıçayçesi" in Turkish, is one of the near-threatened endemic perennial herbs.

**Methods:** The antioxidant activity of methanol and water extracts of *S. bombycina* was examined utilizing in vitro techniques, including radical scavenging, such as DPPH, and ABTS, an iron-chelating assay, and the total phenol (TPC) and flavonoid contents (TFC). The extracts were also investigated on enzyme inhibition effects using *in vitro* spectrophotometric method. HPLC analysis was also used for the determination of the phytochemical profiles of the extracts.

**Results:** Based on our results, the methanol extract of *S. bombycina* demonstrated higher DPPH and ABTS radical scavenging activity with the $IC_{50}$ value of 605.7 ± 1.04 and 19.40 ± 0.37 µg/mL, respectively, than the water extract. Otherwise, the water extract was found to have a higher iron chelating activity ($IC_{50}$= 917.9 ± 3.55 µg/mL) than the methanol extract. The highest TPC of the water extract was determined as 81.07 ± 4.71 µg GAE/mg, although the methanol extract had more TFC at 46.93 ± 1.94 µg QE/mg. In addition, high anti- BChE activity was observed ($IC_{50}$= 58.09 ± 1.18 µg/mL) in the water extract. In addition, ellagic acid was defined as a major component in the methanol extract, while caffeic acid was detected as the main compound in the water extract.

**Conclusion:** Consequently, the current study is the first to report the antioxidant and enzyme inhibitory properties of *S. bombycina*. According to our findings on *S. bombycina*, this work can contribute to the development of bioactive agents from natural sources. Moreover, further investigations still need to be conducted on the discovery of the phytoconstituents of *S. bombycina* responsible for the bioactivity, as well as its potential various biological activities.

**Keywords:** *Stachys bombycina*, Enzyme inhibition, Antioxidant activity

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## INTRODUCTION

There are around 290 species of *Stachys* L. (Lamiaceae) throughout the world. These species are widely grown in the Mediterranean, southern Asia, South Africa, and the Americas (Yılmaz, Daşkın, & Kaynak, 2010). Turkey is a country with an endemism rate of 48% in terms of *Stachys* species (Kirkan, 2019). For millennia, the plants from the genus *Stachys* have traditionally been utilized for the treatment of genital organ malignancies, splenic disease, inflammatory disorders, ulcerations, and cough (Tomou, Barda, & Skaltsa, 2020; Tundis, Peruzzi, & Menichini, 2014). Bioactive compounds, phenolic components including phenolic acids, iridoids, and flavonoids, as well as fatty acids are the principal groups of secondary metabolites in these species belonging to the genus (Duru, Çakır, Harmandar, Izumi, & Hirata, 1999). Several studies have found that extracts from *Stachys* spp. exhibit anti-inflammatory, cytotoxic, antibacterial, and antioxidant activities (Háznagy-Radnai et al., 2008; Háznagy-Radnai et al., 2012; Kukić, Petrović, & Niketić, 2006; Saeedi, Morteza-Semnani, Mahdavi, & Rahimi, 2008).

*S. bombycina* Boiss. is an endemic species to Turkey, and can only be found in abundance in the provinces of Antalya, Muğla, and Mersin (Delazar et al., 2005). This species has been explored for phytochemical and biological activity, but no research on antioxidant and enzyme inhibitory activitieshas been performed on the extracts as far as we know (Kucukbay, Ozgul, Kucukbay, & Akcicek, 2011).

Alzheimer's disease (AD) is a neurodegenerative disorder that causes cognition, memory and behavior problems and is quite common form of dementia., and therefore, gradually, is one of the world's most critical chronic geriatric illnesses. Acetylcholinesterase (AChE) inhibitors are used to treat AD. Cholinesterase inhibitors have been found to be abundant in medicinal plants. Plant materials have been an important source in the search for cholinesterase inhibitors due to the secondary metabolites. These compounds are known for different chemical structures, and there are many scientific studies on them.

Diabetes mellitus (DM) is a metabolic condition characterized by high blood sugar levels caused by the pancreatic failure to make adequate insulin for the organism or the body's failure to properly utilize the insulin effectively. Several anti-diabetic drugs are available on the market, and they are made from natural and/or synthetic sources. However, due to inefficiency, cost, and side effects, the present medications have limitations in their use (Saravanakumar et al., 2021). The extracts and essential oils of various *Stachys* species have previously been tested for antidiabetic activity (Bahadori, Maggi, Zengin, Asghari, & Eskandani, 2020; Bursal, Taslimi, Gören, & Gülçin, 2020; Kang et al., 2017).

Tyrosinase is a copper-containing enzyme that participates in the production of melanin, which protects the dermal layer from the sun. However, excessive accumulation on the skin causes skin problems such as blemishes, skin cancer, and melasma. Therefore, inhibition of the tyrosinase enzyme that produces melanin helps to avoid not only disorders caused by excessive pigmentation, but also neurological diseases (Gou et al., 2017).

As far as we know, there is very little information on the phytochemical components, including phenolics and flavonoids, biological activities, as well as the usages of *S. bombycina*. Therefore, the objective of this research was to examine the antioxidant activity of methanol, and water extracts of *S. bombycina* aerial parts, as well as the inhibitory activity of tyrosinase, AChE, BChE, α-amylase, and α-glucosidase. In addition, we analyzed the extracts for their phytochemical profiles regarding phenolic compounds by HPLC-DAD.

## MATERIAL AND METHODS

**Plant Material:** Before the extraction process, the fresh aerial parts of wild *S. bombycina* were gathered in the west of Antalya, in Yarikpinar canyon, on 12.04.2018. Identification of the plant material was confirmed by Prof. Dr. Hayri Duman, from the Faculty of Science, Gazi University. The plant sample of the specimen was held in Selcuk University, the Herbarium KNYA number 26911.

**Extraction:** After powdering and drying, 10 g of material from the *S. bombycina* sample was macerated with methanol. After filtering the combined filtrates and extracting them three times, they were concentrated until drieness with a rotary evaporator to give a methanol extract. Then the residue of the plant material was subjected to maceration with distilled water three times. After filtering, the water extract was lyophilized to dryness. The extracts were kept at -20 °C until used for the experiments.

### TPC and TFC determination

The TPC and TFC were determined using Folin-Ciocalteu (gallic acid as standard) and aluminum chloride (quercetin as standard). The methods are based on our previously published research (Eruygur & Ayaz, 2021).

### Phenolic compounds quantitative analysis by High-Performance Liquid Chromatography (HPLC)

To investigate phytochemical profiles of the methanol and water extracts, chromatographic analysis was conducted by HPLC (Agilent Technologies, Wilmington, DE, USA). The wavelength of the DAD detector was adjusted at 280 nm generally used for the simultaneous determination of different phenolic compounds. For analysis, 25 mg of dry crude extract was diluted in 1 ml of methanol, and a sample volume of 10 µl was injected. The analysis of separations was performed at 30 °C on column C18 (ACE 5,250 x 4.6mm; 5µm; 0.8 ml/min). The mobile phase was composed of water with 0.1% acetic acid (A), methanol with 0.1% acetic acid (B), and acetonitrile with 0.1% acetic acid (C). A gradient elution program for a mixture of A, B, and C was applied at 0-8 min (A: B: C; 80:12: 8). The mobile phase polarity was gradually decreased with 75:15:10, 70:18:12, 65:20:15, 50:35:15, and 25:60:15 at 8-45 min, and then programmed back to the initial elution program (80:12:8) for the reconditioning of the column for 5 min. The samples and mobile phase were filtered utilizing a 0.22 µm filtration apparatus (Millipore Corp., Billerica, MA). Each sample was analyzed in triplicate.

### Determination of antioxidant activity

To investigate DPPH radical scavenging activity, experimental procedures were conducted as previously stated (Clarke, Ting,

Wiart, & Fry, 2013). For determining the ABTS scavenging activity, the method applied by Re et al. (Re et al., 1999) was used with minor modifications. The metal chelating test was based on a spectrophotometric measurement of iron-ferrozine absorbance at 562 nm (Chai, Mohan, Ong, & Wong, 2014).

### Enzyme inhibitory activity

To evaluate the anticholinesterase activity (AChE, and BChE) of the samples, they were processed as mentioned by Ellman's protocol (Ellman, Courtney, Andres Jr, & Featherstone, 1961) with slight modification. α-Glucosidase inhibition properties of the extracts were assessed by the 96-well plate technique (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013). The Caraway-Somogi iodine/potassium iodide design was used to investigate the α-amylase inhibition capabilities as reported previously (Özek, 2018). The tyrosinase enzyme inhibition effect was determined using an original technique as previously mentioned (Jeong et al., 2009).

### Statistical analysis

GraphPad Prism 8.0 was used to conduct the data analysis. The report was produced as a mean of three parallel determinations with standard deviation. To evaluate the statistical significance, one-way ANOVA (Tukey test) and Student's t-test were utilized. The results were regarded as significant when the p-value was less than 0.05.

## RESULTS

### HPLC analysis of phenolics

HPLC–DAD was used to examine the phytochemical profiles of the methanol and water extracts with respect to various phenolic acids and flavonoids discovered (Table 1). The main constituents of the methanol extract were detected as ellagic acid (92.807 µg/mg), chlorogenic acid (11.817 µg/mg), salicylic acid (3.182 µg/mg), and caffeic acid (1.875 µg/mg) as seen in Figure 1. However, caffeic acid (3.306 µg/mg), catechin (0.411 µg/mg), and quercetin (0.596 µg/mg) were the more predominant phenolic compounds in the water extract (Figure 2).

### Antioxidant activity

When phenolics were compared to flavonoids, the level of phenolics was found to be greater in the extracts. The TPC was higher in the water extract (81.07 µg gallic acid (GAE)/mg extract) than in the methanol extract (75.70 ± 3.20 µg GAE/mg extract). On the contrary, the TFC was found to be higher in the methanol extract (46.93 ± 1.94 µg quercetin (QE)/mg extract) than in the water extract (41.22 ± 2.99 µg QE/mg extract). (Table 1). This result is higher than a prior investigation on *Stachys tmolea* and it was stated that methanol was found to be more appropriate for extraction of flavonoid compounds (Elfalleh, Kirkan, & Sarikurkcu, 2019). In this study, the methanol and water extracts exhibited substantial differences in each antioxidant activity assay, such as DPPH, ABTS, and iron chelating, as seen in Table 1. Utilizing the DPPH, and ABTS methods, it was found that the methanol extract possessed the strongest radical scavenging capabilities, with $IC_{50}$ values of 605.7 ± 1.04, and 19.40 ± 0.37 µg/mL, respectively. This could be because of certain flavonoid components in the methanol extract with high radical scavenging abilities. Otherwise, the water extract

**Table 1. The phenolic contents of the methanol and water extracts of *S. bombycina* (µg/mg, n=3).**

| Analyte | Retention time (min) | Methanol extract | Water extract |
|---|---|---|---|
| Gallic acid | 4.69 | 0.008 | - |
| 3,4-dihydroxy benzoic acid | 6.98 | 0.065 | 0.022 |
| Catechin | 7.97 | - | **0.411** |
| Chlorogenic acid | 8.79 | **11.817** | 0.113 |
| 4-hydroxy benzoic acid | 10.65 | 0.047 | 0.227 |
| 1,2-dihydroxy benzene | 11.09 | 0.066 | - |
| Epicatechin | 11.40 | 0.294 | 0.378 |
| Vanillic acid | 11.80 | - | 0.169 |
| Caffeic acid | 12.18 | **1.875** | **3.306** |
| Vallinin | 17.63 | 0.029 | 0.007 |
| *p*-Coumaric acid | 18.27 | - | 0.272 |
| Sinapic acid | 19.17 | 0.510 | 0.293 |
| *Trans*-Ferulic acid | 20.07 | 0.262 | 0.091 |
| Ellagic acid | 21.17 | **92.807** | 0.294 |
| Rutin | 22.40 | 0.207 | 0.091 |
| Salicylic acid | 32.88 | **3.182** | 0.201 |
| Quercetin | 36.26 | 0.241 | **0.596** |
| Kaempferol | 39.97 | 0.327 | 0.129 |

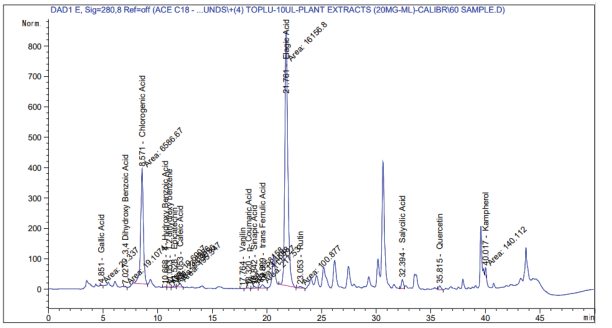


**Figure 1.** HPLC chromatogram of *S. bombycina* methanol extract.

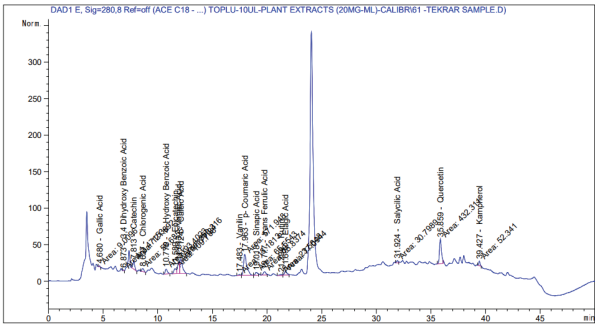


**Figure 2.** HPLC chromatogram of *S. bombycina* water extract.

exhibited stronger iron chelating activity with $IC_{50}$ value of 917.9 ± 3.55 µg/mL than the methanol extract with $IC_{50}$ value of 3098 ± 1.91 µg/mL. These findings were also consistent with previous research (Elfalleh et al., 2019).

**Enzyme inhibitory effects**

Table 2 shows the results of testing the enzyme inhibitory effects of the methanol and water extracts prepared from *S. bombycina* aerial parts. The inhibitions of the extracts were also comparable to that of positive control medicines at the same doses. To investigate the ability of cholinesterase enzyme inhibition by *S. bombycina*, the enzymes AChE, and BChE were used. According to our results, the methanol extract showed low inhibitions against AChE and BChE. The water extract demonstrated higher BChE inhibition ($IC_{50}$: 58.09 ± 1.18µg/mL) than the methanol extract. The potential of tyrosinase inhibition by the extracts progresses in a linear dosage pattern. The methanol and water extracts displayed low tyrosinase inhibitions (Table 2). The antidiabetic activity of *S. bombycina* was investigated by its inhibitory effects on enzymes α-glucosidase and α-amylase. The $IC_{50}$ value of the water extracton α-glycosidase was calculated as 749.3 ± 0.98 µg/mL (with acarbose as the positive control with $IC_{50}$ value of 825.7±1.03µg/mL). Otherwise, the methanol extract showed no activity on α-glycosidase. The strongest α-amylase inhibitory effect was found on the methanol extract with $IC_{50}$ value of 605.7 ± 1.04 µg/mL (with acarbose as the positive control with $IC_{50}$ value of 259.4 ±2.02 µg/mL). The present study showed that the water extract exhibited a higher selectivity against the α-glycosidase enzyme, while the methanol extract showed a stronger sensitivity against the α-amylase enzyme.

## DISCUSSION

In our research, TPC and TFC were detected to be more in the methanol extract of *S. bombycina* with 75.70 µg GAEs/mg, and 46.93 µg QEs/mg, respectively, compared to *S. tmolea* from Turkey previously tested by Elfalleh et al. (2019). Therefore, we proposed that variances in polyphenols and antioxidant properties could be caused by different extraction procedures and solvents.

Previous research has found that terpene-rich essential oils and extracts have a substantial inhibitory effect on AChE and BChE. Trans-caryophyllene and β-phellandrene were reported for their prospective cholinesterase inhibition properties (Bonesi et al., 2010). Similar to this, nonacosane, E-9-octadecenoic acid, hexadecanoic acid, *β*-caryophyllene, germacrene D, caryophyllene oxide, and phytol were identified as important components in *S. bombycina* essential oil (Kucukbay et al., 2011). In another investigation on *S. lavandulifolia*, the hexane

**Table 2. Extract yield, total phenol and flavonoid content, and antioxidant activities of *S. bombycina* methanol and water extracts.**

| Extract/ Reference | Extract yield (% g/g) | Total phenolic (*µ*g GAEs/mg)[b] | Total flavonoids (*µ*g QEs/mg)[c] | Antioxidant activity (µg/mL) | | |
|---|---|---|---|---|---|---|
| | | | | DPPH ($IC_{50}$) | ABTS ($IC_{50}$) | Iron chelating ($IC_{50}$) |
| Methanol | 17.93 | 75.70 ± 3.20 | 46.93 ± 1.94 | 605.7 ± 1.04 | 19.40 ± 0.37 | 3098 ± 1.91 |
| Water | 8.51 | 81.07 ± 4.71 | 41.22 ± 2.99 | 1960 ± 0.69 | 109.2 ± 1.03 | 917.9 ± 3.55 |
| Quercetin | | - | - | 9.62 ± 0.09 | - | - |
| BHT | | - | - | - | 0.7 ± 0.22 | - |
| EDTA | | - | - | - | - | 437.3 ± 2.31 |

a: Values expressed are means ± S.D. of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p < 0.05$)
b: GAEs. Gallic acid equivalents ($y = 0.003x + 0.0578$ gallic acid (µg) ($r^2 = 0.999$))
c:QEs. Quercetin equivalents ($y = 0.0068x + 0.0928$ quercetin (µg) ($r^2 = 0.9982$)).

**Table 3. Enzyme inhibitory activity of methanol and water extracts of *S. bombycina* ($IC_{50}$ µg/mL)[a]**

| Samples | Extract | AChE | BChE | Tyrosinase | α-glucosidase | α-amylase |
|---|---|---|---|---|---|---|
| *S. bombycina* | methanol | 5668 ± 0.83 | 3028 ± 0.54 | 3129 ± 0.21 | N.E. | 605.7 ± 1.04 |
| | water | 1418 ± 1.05 | 58.09 ± 1.18 | 1182 ± 0.67 | 749.3 ± 0.98 | 3686 ± 0.97 |
| Galanthamine | | 28.16 ± 2.01[b] | 27.34 ± 1.86[b] | - | - | - |
| Kojic acid | | - | - | 107.3 ± 0.66[b] | - | - |
| Acarbose | | - | - | - | 825.7±1.03[b] | 259.4 ±2.02[b] |

a: $IC_{50}$ values are given as the mean and standard deviation (Mean ± SD) of three parallel measurements
b: Reference compound
N.E.: not active

and dichloromethane extracts had the strongest AChE and BChE inhibition effects with $IC_{50}$ values of 13.7 and 143.9 µg/mL, respectively (Tundis et al., 2015). In another study, the most anticholinesterase activity with $IC_{50}$ values of *S. annua* against different enzymes was as follows: the methanol extract was 119.8 µg/mL on AChE, while the water extract was 186.7 µg/mL on BChE (Bursal et al., 2020).

As for antidiabetic activity, the water extract of *S. annua* was reported as the most active on *α*-glycosidase, and *α*-amylase with $IC_{50}$ values of 18.7 and 11.4 µg/mL, respectively (Bursal et al., 2020). In a previous work, ethyl acetate extract of *S. germanica* subsp. *heldreichii* showed higher α-amylase inhibition activity ($IC_{50}$: 2.24 mg/mL) than the hexane and methanol extracts. Moreover, it was stated that apigenin in this extract may be contributed to the activity, according to correlation analysis, of chemical composition and activity data (Sarikurkcu, Ceylan, Benabdallah, & Tepe, 2020).

Antityrosinase activity of the methanol extract of *S. germanica* subsp.*heldreichii* was found as important with an$IC_{50}$ value of 2.90 mg/mL (Sarikurkcu et al., 2020). In our findings, the water extract exhibited higher antityrosinase activity ($IC_{50}$: 1182 ± 0.67 µg/mL), than the result of the above-mentioned study. Otherwise, the methanol extract was found to have lower antityrosinase activity ($IC_{50}$: 3129 ± 0.21 µg/mL) than the other study.

## CONCLUSION

The findings showed that *S. bombycina* has significant antioxidant potentials, such as DPPH, ABTS, and iron chelating, and moderate enzyme inhibitory properties, with the water extract having particularly great data against BChE and α-glucosidase. There is no information on the phenolic content and biological activity of this plant that we are aware of. More chemical screening investigations using various solvents and phytochemical analyses are required to uncover novel antioxidant and key enzyme inhibitors in nature.

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- N.E., F.A.; Data Acquisition- N.E., F.A.; Data Analysis/Interpretation- N.E.; Drafting Manuscript- N.E.; Critical Revision of Manuscript- F.A.; Final Approval and Accountability- N.E., F.A.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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Original Article

# A comparative cross-sectional study investigating prevalence and patterns of sexual dysfunction among hypertensive and non-hypertensive men

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**ABSTRACT**

**Background and Aims:** Erectile dysfunction is prevalent among men with hypertension, but there is paucity of data on prevalence of other domains of sexual dysfunction (SD) in hypertensives and the general population. The study compared the prevalence and patterns of SD among treated hypertensive male patients with normotensive men. The effect of antihypertensive drugs on different domains of sexual function was also investigated in our study.
**Methods:** A total of 195 participants (95 hypertensive and 100 non-hypertensive men) were recruited from the medical outpatient department of a secondary health care facility in Lagos, Nigeria. Sexual function was assessed using International Index of Erectile Function (IIEF) which measures erectile function, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction.
**Results:** Sexual dysfunction affecting at least one domain was present in 82.1% of the hypertensive subjects and 52% of non-hypertensive controls (P<0.001). The hypertensive patient had more severe dysfunction in the multiple domains (p< 0.001). The use of methyldopa, furosemide and ß-blockers were associated with significantly lower scores while there was no significant difference in scores with the use calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, thiazide and potassium sparing diuretics. SD was higher in the older age group and with longer duration of hypertension and treatment.
**Conclusion:** SD is common in the adult male population with hypertension significantly increasing the risk. Hypertension is associated with involvement of multiple domains for sexual dysfunction. Use of methyldopa, furosemide and ß-blockers were associated with higher rates of SD in the hypertensive population.
**Keywords:** Sexual dysfunction, Hypertension, Erectile dysfunction, antihypertensive drugs

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## INTRODUCTION

Sexual dysfunction (SD) is a disorder of sexual sensation and activity which affects one or more components of sexual response cycle. In men, SD manifests as loss of libido, erectile dysfunction (ED) and reduced sexual activity. SD is common, and has been shown to be a contributor to poor quality of life and marital disharmony (Kessler, Sollie, Challacombe, Briggs, & Van Hemelrijck, 2019).Prevalence of SD varies across populations, but it has been reported that about 50% of men between the ages of 40-70 year have SD (Chen et al., 2019). ED is one of the most commonly reported manifestations of SD, and global prevalence varies from 3-76.5% (Kessler et al., 2019). A community based study in South-Western Nigeria reported a prevalence rate of 58% among participants (Oyelade, Jemilohun, &Aderibigbe, 2016).

Risk factors for the development of SD include increasing age, the presence of diseases affecting the cardiovascular and neuroendocrine systems, previous urological surgery, psychological disorders, toxins and drugs (Chen et al., 2019). SD has been associated with specific drugs such as selective serotonin reuptake inhibitors (SSRI), alpha-5-reductase inhibitors and antihypertensive medications (Healy, Le Noury, &Mangin, 2018; Imprialos et al., 2018). Hypertension is one of the most common cardiovascular diseases affecting the adult population, therefore men with hypertension are expected to have a significantly higher risk of developing SD from complications of hypertension and as an adverse effect of therapy. A study reported that more than half of men using antihypertensive drugs experienced SD (Oshodi, Adeyemi, Oke, & Seedat, 2010). SD often leads to poor drug adherence and ultimately poor clinical outcome in the management of hypertension, this emphasizes the need to address this disorder (Oyelade et al., 2016; Chen et al., 2019; Kretchy, Boima, Agyabeng, Koduah, & Appiah, 2020).

It has been shown that nearly all classes of antihypertensive drugs can cause SD although different classes of drugs have varying degree of severity (Ekman, Hägg, Sundström, &Werkström, 2010; Akinyede et al., 2020). We have also previously reported the occurrence of SD with the use of different classes of antihypertensive drugs (Akinyede et al., 2020). A recent publication by the Working Group on Sexual Dysfunction and Arterial Hypertension of the European Society of Hypertension which reviewed antihypertensive drugs and ED, reported that thiazide diuretics, centrally acting sympatholytics and beta-blockers have the worst profile, whereas angiotensin receptor blockers (ARB) and nebivolol (a beta-blocker which is a nitric oxide donor) have the best profile on ED (Imprialos et al., 2018; Viigimaa et al., 2020). Calcium channel blockers and angiotensin converting enzyme inhibitors (ACEI) have however been shown to have neutral effects on sexual function (Burnett, 2019; Fogari & Zoppi, 2002).

Hypertension and antihypertensive drugs use are both associated with SD, the real magnitude of sexual dysfunction associated with hypertension and antihypertensive drug use may be overestimated because SD is also commonly seen among non-hypertensive men population Several studies have investigated prevalence of ED among the general population with only a few addressing SD. This may lead to under-recognition of the prevalence of SD especially in men who have other forms of SD without ED. There is also paucity of data on the effect of antihypertensive drugs on different domains of sexual function. This may mask the true pattern of antihypertensive drug induced SD which has implications for its pathophysiology and management.

This study investigated the prevalence, patterns, and determinants of sexual dysfunction in a cohort of hypertensive men on treatment and compared to non-hypertensive controls. It also determined the effect of antihypertensive drugs on the different domains of sexual function.

## MATERIALS AND METHODS

### Study design

This is an analytical cross-sectional study carried out at the General Hospital Ikorodu, Ikorodu, Lagos State. The hospital provides secondary level of healthcare to people living in Ikorodu and its environs.

### Population

A total of 195 (95 patientsand 100 control group) consecutively consenting male adults were recruited for this study. The subjects were sexually active male adults aged 25 to 80 years who were attending the medical outpatient's clinic and had been on treatment for hypertension for > 3 months. Patients with co-morbid disorders that could affect sexual function like diabetes mellitus, renal disease, stroke, heart failure, history of previous urological surgery and penile injury were excluded from the study.

Controls were men aged 25-80 years, sexually active, consenting males who were normotensive with no previous history of hypertension. Those with disorders that could affect sexual function and who were non-consenting were excluded from the study.

### Ethical approval

Ethical clearance was obtained from the Lagos University Teaching Hospital Health Research Ethics Committee (CMUL HREC). Permission for use of the Ikorodu General Hospital was obtained from the Lagos State Health Service Commission. Informed consent was also obtained from each participant before inclusion in the study.

### Methodology

All interviews were conducted privately, and anonymity of the participants was maintained. Information was obtained using a semi structured interviewer administered questionnaire to document their socio-demographic and clinical data. The International Index of Erectile Function (IIEF) questionnaire was used to assess sexual function over the previous four weeks prior to the clinic days. Antihypertensive agents used by each subject in the last 3 months prior to presentation were also documented. The blood pressure of participants was measured using a mercury sphygmomanometer.

### International Index of Erectile Function

The 15-question International Index of Erectile Function (IIEF) Questionnaire is a validated, multi-dimensional, questionnaire

that has been validated for use in the clinical assessment of erectile dysfunction. A score of 0-5 is awarded to each of the 15 questions. The IIEF has five domains: erectile function (q1,2,3,4,5,15), orgasmic function (q9,10), sexual desire (q11,12), intercourse satisfaction (q6,7), and overall satisfaction (q13,14) (Rosen et al., 1997). Scores were rated as mild, moderate and severe with lower scores suggesting the presence of SD in the domain measured. For erectile function, a score of 26-30 was regarded as normal, 17-25 signified mild erectile dysfunction (ED), 11-16 showed moderate ED while a score of 1-10 signified severe ED. Intercourse satisfaction was graded as normal for a score of 12-15, mild dysfunction (9-11), moderate (6-8), while a score of 0-5signified severe dysfunction. Orgasmic function, sexual desire, and overall satisfaction were graded as normal for a score of 9-10, mild dysfunction (7-8), and moderate (5-6). A score of 0-4 suggested severe dysfunction affecting orgasmic function while a score of 2-4 suggested severe dysfunction in sexual desire and overall satisfaction.

### Data analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS VERSION 21). The data was assessed for normality. Variables were expressed as means ± standard deviation and percentages. Association between variables were determined using Chi-Square, Mann Whitney U test and Spearman correlation analysis.

## RESULTS

The study consisted of 95 patients and 100 non-hypertensive control group. The mean age for the hypertensive patients was 57.7 ± 12.6 years while that for the control subjects was 54.92± 8.73 years. The mean duration of hypertension was 75.26±63.07 months while the mean duration of treatment was 69.7161.12 months. Blood pressure control was poor among the hypertensive population. Sexual dysfunction was significantly higher among the hypertensive population with a prevalence of 82.1% and 52% among control. (Table 1).

The hypertensive patient had more severe dysfunction in the different domains ($p<0.001$) and also were more likely to have involvement of multiple domains of sexual dysfunction. (Results are shown in Tables 1 - 3).

The patterns of antihypertensive drug used is documented in Table 4. Calcium channel blockers (CCB), angiotensin receptor

**Table 1. Demographic profile and prevalence of sexual dysfunction among participants.**

| Variable | Patients n=95 | Control n=100 | P-value |
|---|---|---|---|
| **Age** | | | |
| Age range | 25-79 | 25-80 | <0.001* |
| Mean age | 57.7 12.6 | 47.23 12.12 | |
| **Marital status** | | | |
| Married | 89 (93.7) | 89 (89) | |
| Single | 6 (6.3) | 11(11) | |
| **Educational Status** | | | |
| Nil | 11(11.6) | 6(6) | |
| Primary | 23(24.2) | 14(14) | |
| Secondary | 26(27.4) | 35(35) | |
| Tertiary | 35(36.8) | 45(45) | |
| **Mean Systolic BP** | 147.55 19.61 | 120.15 15.93 | <0.001* |
| **Mean Diastolic BP** | 90.93 | 79.40 9.98 | <0.001* |
| **Mean duration of HTN (months)** | 75.26±63.07 | | |
| **Mean duration of treatment (months)** | 69.7161.12 | | |
| **Sexual dysfunction** | | | |
| Yes | 78 (82.1) | 52 (52) | <0.001# |
| No | 17 (17.9) | 48 (48) | |
| **Number of domains affected per patient** | | | |
| One domain | 2 (2.1) | 16 (16) | <0.001* |
| Two domains | 4 (4.2) | 6 (6) | |
| Three domains | 2 (2.1) | 2 (2) | |
| Four domains | 8 (8.4) | 5 (5) | |
| Five domains | 62 (65.3) | 23 (23) | |

Patients=Hypertensive males on treatment, Control= Normotensive males, BP= Blood pressure, HTN= Hypertension *p values were calculated using Mann-Whitney U test, # p values were calculated using Chi square

**Table 2.Table comparing IIEF scores among male patients on anti-hypertensive drugs and normotensive males.**

| | Subject | | | Control | | | P values |
|---|---|---|---|---|---|---|---|
| SD Domains | N | % | Mean score | n | % | Mean score | |
| Erectile dysfunction | 72 | 75.79 | 17.12 9.97 | 29 | 48.33 | 23.83 6.55 | <0.001* |
| Intercourse dissatisfaction | 63 | 66.31 | 7.68 5.11 | 28 | 46.66 | 11.15 3.16 | <0.001* |
| Orgasmic dysfunction | 74 | 77.89 | 5.863.11 | 30 | 50 | 8.18 1.85 | <0.001* |
| Sexual desire dysfunction | 75 | 78.94 | 6.0 2.8 | 30 | 50 | 8.12 1.98 | <0.001* |
| Overall dissatisfaction | 73 | 76.84 | 6.182.91 | 20 | 33.33 | 8.23 1.99 | <0.001* |

SD = Sexual dysfunction, *p values were calculated using Mann-Whitney U test

**Table 3.Table showing SD severity among hypertensive subjects and normotensive controls.**

| | Subjects n (%) | | | | Control n (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | NIL | Mild | Moderate | Severe | NIL | Mild | Moderate | Severe |
| Erectile dysfunction | 23(24.2) | 31(32.6) | 12(12.6) | 29(30.5) | 65(65) | 24(24) | 8(8) | 3(3) |
| Intercourse dissatisfaction | 32(33.7) | 13(13.7) | 20(21.1) | 31(32.6) | 69(69) | 20(20) | 8(8) | 3(3) |
| Orgasmic dysfunction | 21(22.1) | 27(28.4) | 17(17.9) | 31(32.6) | 65(65) | 26(26) | 7(7) | 2(2) |
| Sexual desire dysfunction | 20(21.1) | 21(22.1) | 20(21.1) | 33(34.7) | 65(65) | 19(19) | 14(14) | 2(2) |
| Overall dissatisfaction | 22(23.2) | 26(27.4) | 17(17.9) | 30(31.6) | 77(77) | 14(14) | 5(5) | 4(4) |

SD = Sexual dysfunction

**Table 4. Antihypertensive use pattern among the patients.**

| Number of drugs | N | % |
|---|---|---|
| One | 7 | 7.4 |
| Two | 52 | 54.7 |
| Three | 25 | 26.3 |
| Four | 9 | 9.6 |
| Five | 2 | 2.1 |
| **Drug class** | | |
| CCB | 62 | 65.3 |
| ARB | 40 | 42.1 |
| Thiazide | 38 | 40 |
| ACE | 30 | 31.6 |
| K sparing | 29 | 30.5 |
| Centrally acting sympatholytic | 13 | 13.7 |
| BB | 12 | 12.6 |
| Furosemide | 7 | 7.4 |
| Nitrate | 1 | 1.1 |

CCB- Calcium channel blockers,ACE Inhibitors- Angiotensin converting enzyme inhibitor, ARB- Angiotensin receptor blocker, BB- beta blockers

blockers (ARB) and thiazide diuretics were the most commonly used antihypertensive drugs in this population. Other classes of antihypertensive used include angiotensin converting enzyme inhibitor (ACEI), beta blockers (BB) and centrally acting sympatholytic drugs (Methyldopa). Majority (54.7%) of the patients were on two medications (Table 4). The most common two drugs combination were CCB + ACEI in 15.8% and CCB + ARB in another 15.8% of the patients. Other combinations include CCB and thiazide in 4 (4.2%) patients and CCB + BB in 3 (3.2%) . In patients on three drug combination the most frequent combination was CCB + ARB + thiazide which was seen in 4(4.2%) of the subjects.

The use of centrally acting sympatholytic (alpha methyl-dopa) significantly affected all domains, B-blockers affected all except sexual desire score (SDS). Furosemide, a loop diuretic significantly affected all domains of sexual function except erectile function score (EFS) (Table 5).

The age of the patient, duration of hypertension and duration of treatment in subjects were negatively correlated with sexual function among the hypertensive population in the study ($p<0.001$) (Table 6).

## DISCUSSION

The study showed that sexual dysfunction was prevalent among both hypertensive and normotensive men with more than half of both populations affected, the prevalence was however higher among the hypertensive population. About 82% of men in the hypertensive group had sexual dysfunction affecting at least one domain of SD, in comparison to about half of non-hypertensive males (Table 1). This is in consonance with previous studies, a study involving over 3000 community

**Table 5. Table Showing The relationship between classes of antihypertensive drug and mean score on the IIEF.**

| | | | | EFS | | ISS | | OFS | | SDS | | OSS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Drug** | **Use** | **N** | **%** | **Mean + SD** | **P** | **Mean+ SD** | **P** | **Mean+ SD** | **P** | **Mean+ SD** | **P** | **Mean + SD** | **P** |
| CCB | Yes | 62 | 65.3 | 17.29±10.45 | 0.677 | 7.81±5.43 | 0.712 | 5.85±3.28 | 0.865 | 6.05±2.99 | 0.791 | 6.16±3.07 | 0.968 |
| | No | 33 | 34.7 | 16.79 ±9.15 | | 7.45±4.54 | | 5.88±2.79 | | 5.91±2.47 | | 6.21±2.62 | |
| ACEI | Yes | 30 | 31.6 | 19.03±10.43 | 0.185 | 8.80±5.39 | 0.136 | 6.63±3.21 | 0.084 | 6.67±2.95 | 0.119 | 6.63±3.12 | 0.241 |
| | No | 65 | | 16.23 ± 9.69 | | 7.17±4.94 | | 5.51±3.02 | | 5.67±2.70 | | 5.97±2.80 | |
| ARB | Yes | 40 | 42.1 | 18.78±9.16 | 0.192 | 8.28±4.87 | 0.371 | 6.13±2.95 | 0.513 | 6.33±2.66 | 0.360 | 6.68±2.75 | 0.166 |
| | No | 55 | | 15.91±10.43 | | 7.25±5.29 | | 5.67±3.23 | | 5.76±2.91 | | 5.82±2.99 | |
| B-blockers | Yes | 12 | 12.6 | 11.58±8.20 | 0.032* | 5.17±3.61 | 0.046* | 4.08±2.19 | 0.016* | 4.75±2.18 | 0.089 | 4.67±2.39 | 0.046* |
| | No | 83 | | 17.92±9.99 | | 8.05±5.21 | | 6.12±3.14 | | 6.18±2.85 | | 6.40±4.67 | |
| Central acting | Yes | 13 | 13.7 | 9.31±7.79 | 0.003* | 4.00±4.06 | 0.006* | 3.77±2.49 | 0.007* | 4.31±2.32 | 0.018* | 4.46±2.90 | 0.025* |
| | No | 82 | | 18.35±9.75 | | 8.27±5.04 | | 6.20±3.08 | | 6.27±2.79 | | 6.45±4.46 | |
| Thiazide | Yes | 38 | 40 | 17.89±9.53 | 0.596 | 8.24±4.81 | 0.429 | 6.16±2.82 | 0.552 | 6.13±2.61 | 0.689 | 6.42±2.65 | 0.639 |
| | No | 57 | | 16.60±10.30 | | 7.32±5.32 | | 5.67±3.29 | | 5.91±2.95 | | 6.02±3.09 | |
| K- sparing | Yes | 29 | 30.5 | 17.86±8.41 | 0.820 | 7.69±4.28 | 0.887 | 5.93±2.51 | 0.838 | 5.86±2.31 | 0.692 | 6.14±2.39 | 0.743 |
| | No | 66 | | 16.79±10.62 | | 7.68±5.47 | | 5.83±3.35 | | 6.06±3.01 | | 6.20±3.13 | |
| Furosemide | Yes | 7 | 7.4 | 10.29±7.82 | 0.054 | 3.71±3.14 | 0.030* | 3.29±1.80 | 0.015* | 4.00±1.63 | 0.047* | 4.00±1.92 | 0.033* |
| | No | 88 | | 17.66±9.95 | | 8.00±5.12 | | 6.07±3.10 | | 6.16±2.82 | | 6.35±2.91 | |

EFS= erectile function score, ISS= intercourse satisfaction score, OFS=orgasmic function score, SDS= sexual desire score, OSS= overall satisfaction score, IIEF- International Index of Erectile Function Questionnaire, CCB- calcium channel blockers, ACEI-angiotensin converting enzyme inhibitors, ARB- angiotensin receptor blocker. B-Blocker- Beta blockers, central sympatholytic

*p values were calculated using independent sample Mann-Whitney U test.

**Table 6. Correlation between IEEF score and other variables affecting sexual functions among male hypertensive patients.**

| | EFS | | ISS | | OFS | | SDS | | OSS | |
|---|---|---|---|---|---|---|---|---|---|---|
| | rho | P- value | rho | P- value | rho | P- value | rho | P- value | rho | P- value |
| Age | -0.560 | <0.001* | -0.586 | <0.001* | -0.557 | <0.001* | -0.536 | <0.001* | -0.522 | <0.001* |
| No of antihypertensive | -0.021 | 0.838 | -0.039 | 0.71 | -0.074 | 0.477 | -0.054 | 0.605 | -0.05 | 0.632 |
| Diastolic blood pressure | -0.042 | 0.590 | -0.030 | 0.710 | -0.028 | 0.732 | -0.020 | 0.809 | -0.054 | 0.509 |
| Systolic blood pressure | -0.097 | 0.205 | -0.096 | 0.211 | -0.073 | 0.352 | -0.057 | 0.467 | -0.086 | 0.274 |
| Hypertension duration | -0.370 | <0.001* | -0.415 | <0.001* | -0.329 | <0.001* | -0.349 | <0.001* | -0.456 | <0.001* |
| Treatment duration | -0.315 | <0.001* | -0.317 | <0.001* | -0.357 | <0.001* | -0.275 | <0.001* | -0.294 | <0.001* |

EFS= erectile function score, ISS= intercourse satisfaction score, OFS=orgasmic function score, SDS= sexual desire score, OSS= overall satisfaction score, IIEF- International Index of Erectile Function Questionnaire, CCB- calcium channel blockers, ACEI-angiotensin converting enzyme inhibitors, ARB- angiotensin receptor blocker. B-Blocker- Beta blockers, central sympatholytic
rho -Spearman's rank correlation coefficient, *p values were calculated using Spearman's correlation

dwelling adults aged 57-85 in the USA showed that treated hypertensive male patients had significantly higher rates of SD (69.1%) compared to untreated hypertensives (57.7%), and non-hypertensives (54.3%) (Spatz, Canavan, Desai, Krumholz, & Lindau, 2013). Another study carried out in treated hypertensive male patients in France reported a prevalence of 49% (Hanon et al., 2002). A study at a tertiary centre in Lagos, Nigeria documented a prevalence of 56.7% in both male and female patients on antihypertensive drugs (Oshodi et al., 2010).

The study also showed that about half of the non-hypertensive men have sexual dysfunction affecting at least one domain. Prevalence of sexual dysfunction is highly variable among different populations, a study reported a range of 25- 61% with the higher rates found in older population (Derogatis & Burnett, 2008). Other studies have however reported lower prevalence. A multidisciplinary committee review reported that about 20-30% of adult men have SD affecting at least one domain (Lewis et al., 2010). The prevalence rates reported among non-hypertensive control in this study appears high but falls within the range found in previous studies. The age of study population and the instrument used to investigate sexual function are major determinants of prevalence rates of SD and may explain the variation in results across different studies (Derogatis & Burnett, 2008; Lewis et al., 2010).

In this study, hypertensive patients were more likely to have involvement of multiple domains compared to controls. In the hypertensive patients, about 73.3% of the 82% with SD had involvement of 4 or 5 domains pointing to an overlap of domains in the same patient. A total of 75.8 % reported ED which is comparable to other domains like orgasmic dysfunction (77.9%) and problems with sexual desire (78.9%) and is in consonance with a previous study (Akinyede et al., 2020). This suggests that any of these domains may be used singly as screening for SD in hypertension since there is involvement of multiple domains concurrently in same patient. The utility of a single domain for the assessment of SD in the normotensive population may however lead to underreporting of SD.

The differences in domains involved may be a pointer to the aetiopathogenesis of SD especially in the non-hypertensive populations. Problems with sexual desire and orgasm have been linked to neuroendocrine changes (Krüger et al., 2003; Motofei & Rowland, 2005). On the other hand, ED has been attributed to vascular, endocrine and neurological dysfunction (Bleustein, Arezzo, Eckholdt, &Melman, 2002; Santi et al., 2016; Burnett, 2019), although ED associated with hypertension is mainly related to vascular dysfunction (Nilsson, Viigimaa, Giwercman, &Cifkova, 2020).

Several studies have reported a high prevalence of ED among hypertensive patients. A study carried out in the same geographical region as ours showed a prevalence of 65.8% which is similar to our report (Fafiolu, Adebayo, Akande, & Akinbobooye, 2014). Another study in Italy among hypertensive patients attending clinics reported a prevalence of 50.6% (Artom et al., 2016). Although a much lower prevalence of 35.2% among hypertensive patients was reported in Greece, it was significantly higher compared to the normotensive population that had a

prevalence of 14.1% (Doumas et al., 2006).There is paucity of data comparing other domains of SD.

Antihypertensive medications are known to cause SD, the study therefore compared IEEF scores in different domains in patients who used specific classes of hypertensive drugs compared to those who did not use these drugs. The study showed that patients who were on antihypertensive combinations containing furosemide, ß-blockers and methyldopa had a significantly higher risk of SD compared to other groups of antihypertensive in this study (Table 5). It has been reported that drugs like diuretics, centrally acting sympatholytic and beta–blockers are notorious for SD (Nicolai et al., 2014; Kretchy et al., 2020). This study follows same trend except for the profile of diuretics which appears to deviate from reported patterns. The only diuretic significantly associated with SD in this study was furosemide (a drug which is used infrequently in the management of hypertension). Potassium sparing diuretics has not been linked to high rates of SD, this was replicated in our study (Nicolai et al., 2014). Thiazide diuretic use was however not associated with higher rates of SD, which conflicts with previous data (Nicolai et al., 2014; Artom et al., 2016). The reason for this is unknown, but it appears the combination of drug may influence the adverse effect profile, majority of the patients were on a combination of ARB and thiazide. ARBs have been reported to be protective against SD and the combination with of thiazide and ARB may be protective against SD in this study (Ismail et al., 2019).

ß-blockers, methyldopa and furosemide are not recommended as first line drugs in blacks, as such were more likely to be given in combination with two or more drugs. It could be argued that the high rate of SD associated with these drugs was secondary to a higher number of pills co-medicated in our study. Higher drug loads have been associated with a higher prevalence of developing SD although a few studies have reported conflicting reports (Doumas et al., 2006; Hanon et al., 2002; Oshodi et al., 2010). Our study however found no association between number of antihypertensive drugs and scoring on IIEF index (Table 6).

Other variables significantly associated with higher prevalence of SD in this study include increasing age, and a longer duration of hypertension and treatment, this has been consistently replicated in several studies (Hanon et al., 2002; Doumas et al., 2006; Oshodi et al., 2010). Variables like systolic and diastolic BP on the other hand showed inconstant finding as regards the development of SD in both men and women (Hanon et al., 2002;Doumas et al., 2006; Oshodi et al., 2010; Foy et al., 2016). This study found no association between SD and blood pressure control.

The higher risk of SD with advancing age and longer duration of hypertension is a sequela of multiple processes which include a progressive in endothelial dysfunction. Normal endothelial function is required to maintain erectile function, secretion of gonadal hormones and spermatogenesis that is required for normal sexual function (Santi et al., 2016; Burnett, 2019). In the physiological state, endothelial cells release NO which enters the corpus cavernosum smooth muscle cells of the penis, to activate guanosine cyclase. Guanosine cyclase converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP) which further activates protein kinases downstream leading to the relaxation of the smooth muscle cells. This process favours increased blood flow into the penile shaft to maintain tumescence (Burnett, 2019). One or more of this pathway is disrupted in when there is a reduction in NO production.

One of the strength of this study is the use of IIEF which assessed different domains of sexual function and also included a control population which helped to give a more holistic assessment of the SD in the population studied (Rosen et al., 1997). Although this study investigated the effect of each class of antihypertensive drug on SD by comparing patients who were on specific drug class to those who were not, it is still difficult to distinguish between patterns of SD caused by hypertension alone or SD associated with antihypertensive drugs. The introduction of an untreated hypertensive population would help to address this. Another limitation to the study is that the control population were relatively younger than the hypertensive population. Since SD has been shown to be more severe with advancing age, the actual prevalence among the older population who are not hypertensive might be much higher. In addition, the patterns of SD may be different across age groups. Further studies involving age-matched controls are needed.

SD is common in both normotensive and hypertensive adult males although prevalence is higher in the hypertensive males. The use of antihypertensive drugs, like methyldopa, furosemide and ß-blockers was associated with higher degrees of sexual dysfunction among the hypertensive population.

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Original Article

# The drug burden index medication use in older people in north-east Nigeria

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**ABSTRACT**

**Background and Aims:** Evidence has shown that physical and cognitive impairment in older people is linked with the Drug Burden Index (DBI). The study aimed to describe the prescription pattern of DBI medications, estimate the frequency of contraindicated DBI medication use, determine the rate of exposure to high-risk DBI medications, and identify the potential predictors of exposure to high-risk DBI medications..

**Methods:** This one-year retrospective study was conducted in a secondary healthcare facility. It included patients over 65 years of age who were prescribed at least one anticholinergic and/or sedative medication. The study data were summarized using descriptive statistics, while multivariable logistic regression analysis was used to identify potential predictors of exposure to high-risk DBI medications. Statistically, a significant level was set at $p < 0.05$.

**Results:** Most patients were exposed to cardiovascular drugs (57.5%) followed by antihistamines (25.8%). A total of 23 (6.3%) contraindicated DBI medications were identified. Sixty (19.6%) older patients were prescribed high-risk DBI medications. Patients over 70 years were 3.08 times significantly more likely to be exposed to high-risk DBI medications. Also, patients with a low number of non-DBI co-medications (adjusted odds ratio [AOR] 3.40, 95% CI 1.03 - 11.23), polypharmacy (AOR 7.38, 95% CI 2.20 – 24.73), and those that had contraindicated DBI medications (AOR 3.93, 95% CI 1.14 – 13.53) were significantly more likely to be exposed to high-risk DBI medications.

**Conclusion:** The study demonstrated that most older people in the study were exposed to anticholinergic medications. A considerable proportion of these older people were exposed to contraindicated and high-risk DBI medications. Patients over 70 years of age, a low number of non-DBI co-medications, polypharmacy, and contraindicated DBI prescriptions were the significant predictors of exposure to high-risk DBI medications.

**Keywords:** Anticholinergic Medications; Sedative Medications; Drug Burden Index; Older People; North-East, Nigeria

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## INTRODUCTION

Medications with sedative and anticholinergic effects pose considerable risk of negative health outcomes in older people (Sumukadas, McMurdo, & Mangoni, 2014). These medications are used to treat a variety of conditions that commonly occur in later life, such as sleep disturbances, nausea, pain, and urinary incontinence (Kouladjian, Gnjidic, & Chen, 2014). It is worth noting that an altered response to medications, in terms of efficacy as well as adverse drug events (ADEs), is an important aspect of the human aging process (Le Couteur, McLachlan, & de Cabo, 2012). The observation that older people are at higher risk of drug-drug interactions, increased comorbidities, and polypharmacy due to age-related alterations in pharmacokinetics and pharmacodynamics, is of clinical importance (McLean, & Le Couteur, 2004; Tinetti, Bogardus, & Agostini, 2004; Hilmer, McLachlan & Le Couteur, 2007). Polypharmacy is the most significant medication-related risk factor for ADEs that require hospitalization (Mannesse, Derkx, & de Ridder, 2000; Onder, et al., 2002; Leendertse, Egberts, & Stoker, 2008; Olivier, et al, 2009). To reduce medication-related problems (MRPs) in the older population, Beers et al. developed criteria that can be used to assess potentially inappropriate prescribing in this population (The American Geriatrics Society, 2019).

Aside from other MRPs, medications with sedative and anticholinergic effects pose significant risks to older people. Older people's exposure to anticholinergic medications is linked to decreased cognitive performance, physical performance, and functional status (Mulsant, et al, 2003; Lechevallier-Michel, Molimard, & Dartigues, 2005; Landi, et al., 2007; Nebes, Pollock, & Halligan, 2007; Han, Agostini, & Allore, 2008). Sedative medications have also been linked to ADEs in older people, including lower levels of physical function, falls, fractures, and cognitive impairment (Cumming, & Le Couteur, 2003; Hilmer, et al., 2007; Hartikainen, Lonnroos, & Louhivuori, 2007; Gnjidic, et al., 2009; Hilmer, et al., 2009). As a result, high-risk prescribing, such as polypharmacy and exposure to medications that increase the drug burden index (DBI), contribute to the downward spiral in physical and cognitive function in older people (Gnjidic, et al., 2009; Hilmer, et al., 2009; Xue, 2011; Lowry, Woodman, & Soiza, 2011). However, in a bid to formulate measures to reduce the irrational use of sedative and anticholinergic medications in older people, a valid method of quantifying the issue and identifying areas for enhanced rational prescribing is required.

The DBI is a pharmacological measure of an individual's exposure to medications with anticholinergic and sedative effects (Hilmer, et al., 2007). This measure provides a model for the measurement of the effects of cumulative exposure to both anticholinergic and sedative medications on physical and cognitive function in older people. The DBI as a clinical risk assessment tool was developed to estimate the risk of physical and cognitive impairment from medications in older people. It is an easy-to-use, evidence-based prescribing tool that can enhance the quality of drug use in older people.

A review of the literature revealed some previous studies on older people's exposure to anticholinergic and sedative medications in some non-African countries (Gnjidic, et al., 2009; Hilmer, et al., 2009; Wouters, van der Meer, & Taxis, 2017; Jamieson, et al., 2018). The paucity of such data from Africa, including Nigeria, justified the need for this study. Therefore, the present study aimed to describe the prescription pattern of DBI medications, estimate the frequency of contraindicated DBI medication use, determine the rate of exposure to high-risk DBI medications, and identify the potential predictors of exposure to high-risk DBI medications.

## MATERIALS AND METHODS

### Study design and setting

This retrospective study was conducted at a public secondary hospital in Maiduguri, Nigeria. This hospital is currently a 460-bed healthcare facility. It is the primary service and referral hospital for the Maiduguri Metropolis and the entire Borno State in North-East Nigeria. The hospital's General Out-patients' Department (GOPD) served as the study site.

### Sample size and patients' selection

The study included all patients that were 65 years old or older who were prescribed at least a sedative or anticholinergic medication in the study hospital's GOPD between January 1, 2019, and December 31, 2019.

### Data collection and measurements

A master list of available medications with potential sedative and/or anticholinergic effects in Nigeria was developed (Appendix I) by reviewing previously published studies (Ness, Hoth, & Barnett, 2006; Landi, et al., 2007; Cao, et al., 2008; Duran, Azermai, & Vander Stichele, 2013; Gnjidic, et al., 2013; Salahudeen, Hilmer, & Nishtala, 2015; Byrne, et al., 2018; O'Connell, et al., 2018; Zhang, Zhou, & Li, 2019). Data were taken at a one-time point for each participant. For those who had repeated visits, only data on their last visit were collected. Data including age, sex, marital status, religion, comorbidities, prescribed medications, and dosages were extracted from the patient's medical records.

### Definition of variables and data processing

In the present study, medications without anticholinergic or sedative medications were considered as co-medications. Polypharmacy was defined as the presence of five or more medications in a prescription, including anticholinergic and/or sedative medications following previous studies (Bosboom, Alfonso, & Almeida, 2012; Best, Gnjidic, & Hilmer, 2013; Saka, Oosthuizen, & Nlooto, 2018; Akande-Sholabi, Adebusoye, & Olowookere, 2018; Alhawassi, Alatawi, & Alwhaibi, 2019; Assefa, Kedir, & Kahaliw, 2020; Seixas, & Freitas, 2021). The 2019 updated American Geriatrics Society Beers Criteria were used to assess for contraindicated DBI medications (The American Geriatric Society, 2019). An individual's DBI was calculated by adding the burdens from all sedative and anticholinergic medications they take regularly, using the equation below (Hilmer, et al., 2007):

$$\sum D/(\delta+D)$$

Where D is the dose taken in a day, and δ is the lowest licensed dose/day, which is used as an estimate of the dose/

day needed to elicit half of the highest effect at a steady state. In this study, the lowest effective dose for each of the sedative or anticholinergic medications was determined using a Nigerian drug formulary (EMDEX, 2019). For medications administered intravenously, the lowest effective dose/day for the oral route was used. Also, for medications available as combined products, the lowest effective dose/day for the sedative or anticholinergic medication only was used to define the lowest effective dose/day.

The DBI score for a patient is the sum of scores for his or her number of DBI medications in a prescription. In other words, for each patient, the DBI for each medication with anticholinergic and sedative effects in a prescription was calculated and added together. In this study, the DBI score was categorized dichotomously as low-risk (0 > 1), and high-risk (≥ 1). The DBI score 0 > 1 received zero points for logistic regression analysis, while the DBI score ≥ 1 received one point. The variables that were taken into account were classified as follows: age (categorized into 65 - 70 years [reference], and >70 years), gender (female [reference], and male), polypharmacy (no [reference], and yes), anticholinergic exposure (no [reference], and yes), sedative exposure (no [reference], and yes), and Beers criteria (not contraindicated [reference], and contraindicated).

**Statistical analysis**

Descriptive statistics for continuous data, such as age and DBI scores, were reported as mean ± standard deviation. The frequencies and percentages were used to express categorical data. The association between categorical variables was investigated using Chi-square or Fisher's exact test, as appropriate. The association between patient's variables (sex, age group, and the number of non-DBI co-medications, polypharmacy, anticholinergic prescriptions, sedative prescriptions, and contraindicated DBI prescriptions) and exposure to high-risk DBI medications was investigated using logistic regression. The statistical analysis was done using SPSS (IBM) Statistics for Windows, Version 21.0 (Armonk, NY: IBM Corp). A statistically significant level was set at $p < 0.05$.

## RESULTS

The study included 306 patients that were 65 years old or older and were prescribed at least one anticholinergic and/or sedative medication in 2019. A high proportion (64.4%, n=197/306) of the patients were between the ages of 65 and 70 years. Males made up the majority (53.9%, n=165/306), 58.8% (180/306) were married and 92.2% (282/306) practice Islam. The most common chronic disease in the study population was hypertension (52.3%, n=160/306), followed by arthritis (10.5%, n=32/306) (Table 1).

The majority of patients were exposed to cardiovascular drugs (57.5%) and antihistamines (25.8%) (Figure 1).

Out of the 194 cardiovascular medications prescribed, 33.3% were for furosemide and 15.5% were for nifedipine. Out of the 82 antihistamines prescriptions, loratadine constituted 17.4%, promethazine 3.0%, and meclizine 0.8% (Table 2).

**Table 1. Background Characteristics of the Study Population (n=306).**

| Variable | n (%) |
|---|---|
| **Age Group (years)** | |
| 65 – 70 | 197 (64.4) |
| ≥ 70 | 109 (35.6) |
| **Sex** | |
| Female | 137 (44.8) |
| Male | 165 (53.9) |
| Unreported | 4 (1.3) |
| **Marital Status** | |
| Single/Widowed | 40 (13.1) |
| Married | 180 (58.8) |
| Unreported | 86 (28.1) |
| **Religion** | |
| Christianity | 21 (6.8) |
| Islam | 282 (92.2) |
| Unreported | 3 (1.0) |
| [+]**Chronic Diseases** | |
| None | 110 (36.0) |
| Hypertension | 160 (52.3) |
| Arthritis | 32 (10.5) |
| Chronic kidney disease | 20 (6.5) |
| Diabetes mellitus | 19 (6.2) |
| Heart failure | 15 (4.9) |
| Being prostate hyperplasia | 10 (3.3) |
| Asthma | 5 (1.6) |
| [#]Others | 8 (2.6) |
| **Number of Co-medications (those without anticholinergic or sedative effects)** | |
| 0 – 2 | 142 (46.4) |
| > 2 | 164 (53.6) |
| **Polypharmacy (all medications including those with anticholinergic or sedative effects )** | |
| Yes | 122 (39.9) |
| No | 184 (60.1) |

[+]Some patients had multiple chronic diseases;
[#]Others=Osteoporosis, Chronic liver disease, Angina, Stroke, Pyelonephritis, and Cancer

Most of the patients (80.1%) were prescribed one DBI medication per prescription, followed by two (19.6%) as shown in Figure 2.

Furosemide was the highest (29.7%) mono DBI medication prescribed followed by loratadine (15.7%), while furosemide and loratadine were the highest (3.3%) among the dual DBI therapy followed by loratadine and codeine (2.9%). The only

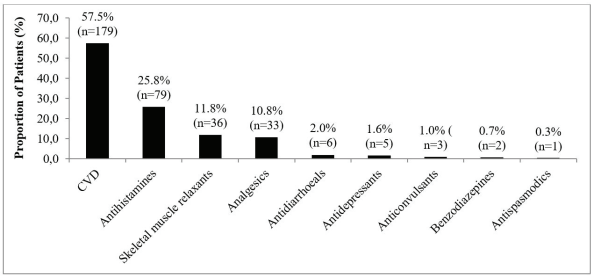


**Figure 1.** The Distribution of Patients Exposure to DBI Medication Classes (N=306)

CVD: Cardiovascular Drugs

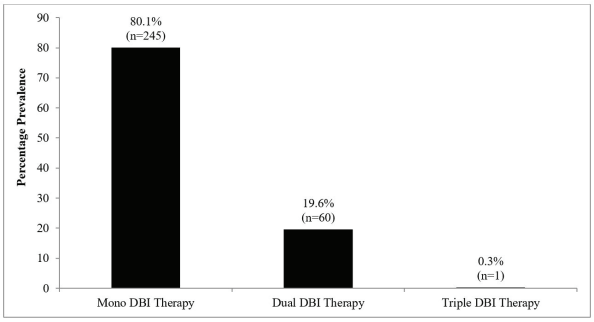


**Figure 2.** The Distribution of Patients Based on the Number of DBI Medications/ Prescription (n=306).

three DBI medications prescribed concomitantly were nifedipine, furosemide, and codeine (0.3%) as presented in Table 3.

The study identified a total of 23/368 (6.3%) contraindicated DBI medications according to the 2019 updated Beers criteria. The most frequently potentially inappropriate DBI medication in the study population was promethazine (47.8%, n=11/23), followed by amitriptyline (17.4%, n=4/23) (Figure 3).

The average DBI score in the cohort was 0.72 ± 0.28. Out of the 306 individuals prescribed DBI medications in 2019, 296 (96.7%) were exposed to anticholinergic medications, and 41 (13.4%) were exposed to sedative medications alone. Thirty-one (10.1%) were exposed to both anticholinergic and sedative medications. Two hundred and forty-six (80.4%) of the patients were prescribed low-risk DBI medications, while 60 (19.6%) were prescribed high-risk DBI medications. The analysis of prescriptions of both sexes for DBI score medications did not show any significant difference. In contrast, patients aged 70 years or older or with polypharmacy were more often prescribed high-risk DBI drugs ($p < 0.05$) (Table 4).

After adjusting for confounders, the multivariate logistic analysis revealed that patients over 70 years of age, with a low number of co-medications, polypharmacy, and contraindicated DBI medication prescribing were significantly associated with exposure to high-risk DBI medications ($p < 0.05$). Patients over 70 years of age were 3.08 times significantly more likely to be exposed to high-risk DBI medications compared to those 70 years and younger ($p = 0.006$). Patients that had a low number of co-medications were significantly more likely (adjusted odds ratio [AOR] 3.40, 95% CI=1.03 - 11.23, $p = 0.045$) to be exposed to high-risk DBI medications. Similarly, patients with

**Table 3. Prescriptions Patterns Based on the Number of Individual DBI Medications Prescribed Per Encounter in 2019 (n=306).**

| Medication | n (%) |
|---|---|
| **Mono DBI Therapy (n=245)** | |
| Furosemide | 91 (30.0) |
| Loratadine | 48 (15.7) |
| Nifedipine | 38 (12.4) |
| Tizanidine | 30 (9.8) |
| Codeine | 8 (2.6) |
| Hydrochlorothiazide | 8 (2.6) |
| Promethazine | 7 (2.3) |
| Loperamide | 4 (1.3) |
| Amitriptyline | 4 (1.3) |
| Lorazepam | 1 (0.3) |
| Pregabalin | 1 (0.3) |
| Orphenadrine | 1 (0.3) |
| Diazepam | 1 (0.3) |
| Hyoscine | 1 (0.3) |
| Cimetidine | 1 (0.3) |
| Cetrizine | 1 (0.3) |
| **Dual DBI Therapy (n = 60)** | |
| Furosemide + Nifedipine | 10 (3.3) |
| Loratadine + Codeine | 9 (2.9) |
| Furosemide + Codeine | 7 (2.3) |
| Furosemide + Loratadine | 6 (2.0) |
| Nifedipine + Codeine | 5 (1.6) |
| Codeine + Tizanidine | 3 (1.0) |
| Furosemide + Hydrochlorothiazide | 2 (0.7) |
| Nifedipine + Hydrochlorothiazide | 2 (0.7) |
| Furosemide + Meclizine | 2 (0.7) |
| Codeine + Hydrochlorothiazide | 2 (0.7) |
| Promethazine + Codeine | 2 (0.7) |
| Furosemide + Pregabaline | 1 (0.3) |
| Nifedipine + Meclizine | 1 (0.3) |
| Furosemide + Tizanidine | 1 (0.3) |
| Furosemide + Cetrizine | 1 (0.3) |
| Furosemide + Baclofen | 1 (0.3) |
| Nifedipine + Amitriptyline | 1 (0.3) |
| Promethazine + Loperamide | 1 (0.3) |
| Promethazine + Gabapentin | 1 (0.3) |
| Tizanidine + Methocarbamol | 1 (0.3) |
| Loratadine + Loperamide | 1 (0.3) |
| **Triple DBI Therapy (n=1)** | |
| Nifedipine + Furosemide + Codeine | 1 (0.3) |

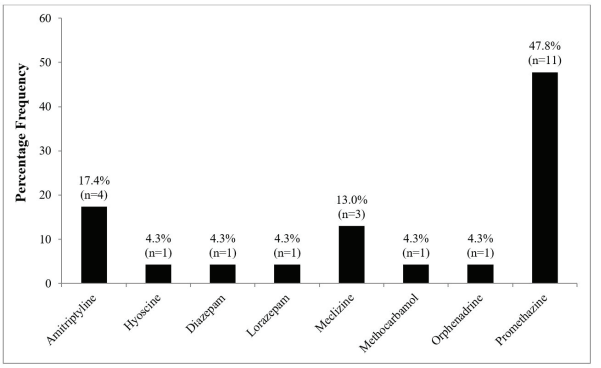


**Figure 3.** The Distribution of Contraindicated DBI Medications in the Study Population.

polypharmacy were significantly more likely (AOR 7.38, 95% CI = 2.20 - 24.73, p = 0.001) to be exposed to high-risk DBI medications than those with no polypharmacy. Also, patients prescribed contraindicated DBI medications were more likely (AOR 3.93, 95% CI = 1.14 - 13.53, p = 0.030) to be exposed to high-risk DBI medications as compared to those that received recommended DBI medications (Table 5).

## DISCUSSION

The study found that most of the patients were exposed to anticholinergic medications with cardiovascular drugs being the most frequently prescribed class followed by antihistamines. More than two-thirds of the study population was prescribed one DBI medication per encounter. In the study population, some contraindicated DBI medications were found, while an appreciable proportion of older people were exposed to high-risk DBI medications, particularly those over 70 years, and those with polypharmacy. Furthermore, after adjusting for confounders, over 70 years of age, low number of co-medications, polypharmacy, and contraindicated DBI medication prescriptions were all found to be significant predictors of exposure to high-risk DBI medications.

In our study, patients' exposure to cardiovascular medications (57.5%, n = 179/306) was significantly higher compared to other DBI medications. This was most likely due to the high prevalence of cardiovascular diseases in the study population. This finding emphasized the importance of prescribing this medication class with caution to older people to avoid adverse effects. A systematic review of hospitalization due to various MRPs identified cardiovascular disease medications as one of the main therapeutic categories linked with MRPs (Al Hamid, Ghaleb, & Aljadhey, 2013). In contrast, the highest exposure to antipsychotics (56.0%, n = 298/532) was observed in the older population in Ireland (O'Connell, et al., 2018), antidepressants other than selective serotonin reuptake inhibitors and sympathomimetics (15.3%, n = 90/589, respectively) in Australia (Gnjidic, et al., 2009), and analgesics in France (Dauphinot, et al., 2014). This variation could be due to varying disease burdens in the study populations. The high exposure to antihistamines (25.8%, n = 79/306) is also noteworthy. Consistent with our result, high use of antiallergic medications (23.2%, n = 184/792) was reported previously in the study area (Okoro, & Shekari, 2013). This may be due to the incessant air pollution of our semi-arid study area with allergens (dust). This finding also highlights the need for rational prescribing of this medication class to older people to prevent negative health outcomes.

In the present study, a considerable proportion of patients (19.9%, n = 61/306) concomitantly used two or more DBI medi-

**Table 4. The Distribution of Patients Based on DBI Categories (n=306).**

| Variables | All Patients n (%) | DBI Category | | p-value |
|---|---|---|---|---|
| | | Low (0 > 1) n (%) | High (≥ 1) n (%) | |
| **Sex** | | | | |
| Female | 137 (45.4) | 111 (45.5) | 26 (44.8) | 0.927 |
| Male | 165 (54.6) | 133 (54.5) | 32 (55.2) | |
| **Age Group (Years)** | | | | |
| 65-70 | 197 (64.4) | 168 (68.3) | 29 (48.3) | 0.004* |
| >70 | 109 (35.6) | 78 (31.7) | 31 (51.7) | |
| **Number of Co-medications (those without anticholinergic or sedative effects)** | | | | |
| 0 – 2 | 142 (46.4) | 112 (45.5) | 30 (50.0) | 0.553 |
| > 2 | 164 (53.6) | 134 (54.5) | 30 (50.0) | |
| **Polypharmacy (all medications including those with anticholinergic or sedative effects)** | | | | |
| Yes | 122 (39.9) | 91 (37.0) | 31 (51.7) | 0.037* |
| No | 184 (60.1) | 155 (63.0) | 29 (48.3) | |
| **Exposure to Anticholinergics** | | | | |
| Yes | 296 (96.7) | 236 (95.9) | 60 (100.0) | 0.219 |
| No | 10 (3.3) | 10 (4.1) | 0 (0.0) | |
| **Exposure to Sedatives** | | | | |
| Yes | 41 (13.4) | 11 (4.5) | 30 (50.0) | < 0.001* |
| No | 265 (86.6) | 235 (95.5) | 30 (50.0) | |
| **Beer's Criteria** | | | | |
| Contra-indicated DBI medication | 23 (7.5) | 15 (6.1) | 8 (13.3) | 0.057 |
| Indicated DBI medication | 283 (92.5) | 231 (93.9) | 52 (86.7) | |

*Chi-square or Fisher's exact test is significant at p < 0.05

**Table 5. Independent Predictors of Older Peoples' Exposure to High-Risk DBI Medications (n=306).**

| Independent Variables | AOR (95% CI) | p-value |
|---|---|---|
| **Sex** | | |
| Female | 1.19 (0.53 to 2.66) | 0.674 |
| Male | 1.00 | |
| **Age Group (Years)** | | |
| 65 – 70 | 1.00 | |
| > 70 | 3.08 (1.38 to 6.89) | 0.006* |
| **Number of Co-medications (those without anticholinergic or sedative effects)** | | |
| 0 – 2 | 3.40 (1.03 to 11.23) | 0.045* |
| > 2 | 1.00 | |
| **Polypharmacy (all medications including those with anticholinergic or sedative effects)** | | |
| Yes | 7.38 (2.20 to 24.73) | 0.001* |
| No | 1.00 | |
| **Exposure to Anticholinergics** | | |
| Yes | - | - |
| No | - | |
| **Exposure to Sedatives** | | |
| Yes | 326.94 (39.30 to 272.00) | < 0.001* |
| No | 1.00 | |
| **Beer's criteria** | | |
| Contraindicated DBI medication | 3.93 (1.14 to 13.53) | 0.030* |
| Indicated DBI medication | 1.00 | |

*Significant at p < 0.05; AOR; Adjusted Odds Ratio; CI: Confidence Interval

cations comparable with a higher rate of 45.6% (n = 169/371) in a previous Australian study (Wilson, et al., 2011). Concomitant use of DBI medications is of particular concern when the additive effects that result in a high DBI score. It is already known that the older population is more susceptible to the negative outcomes of most medications (Sumukadas, et al., 2014). As a result, the cumulative effect of taking two or more DBI medications together could result in negative outcomes in older patients. Available evidence has demonstrated that increasing DBI is linked to decline in activities of daily living (DBI > 0.8 - 1.65: OR 0.17, 95% CI = 0.08 - 0.25, DBI > 1.65: OR 0.19, 95% CI = 0.09 - 0.29) (Wouters, et al., 2020), greater number of falls (incidence rate ratio [IRR] 1.56, 95% CI = 1.48 - 1.65) (Nishtala, Narayan, & Wang, 2014) and 2.11, 95% CI = 1.47 - 3.04) (Wilson, et al., 2011), greater number of general practitioner visits (IRR 1.13, 95% CI = 1.12 - 1.13) (Nishtala, et al., Narayan & Wang, 2014), increased risk of hospitalization (relative risk [RR] 1.26, 95% CI = 1.18 - 1.35) (Lo¨nnroos, et al., 2012), and mortality (hazard ratio [HR] 1.29, 95% CI = 1.25 - 1.33) (Nishtala, et al., 2014). These findings suggest that in older patients, deprescribing DBI medications might be an option. This is because older people's exposure to these medications may have a significant impact on their health. The rising number of chronic conditions and the aging population necessitates a greater number of medication regimens (or polypharmacy), which may contribute to MRPs in older people. However, the efficacy of some DBI medications is frequently insufficient to overlook the attendant consequences in older people (Glass, Lanctôt, & Herrmann, 2005; Nishtala, et al., 2014). Based on the available evidence, deprescribing some DBI medications may have a positive impact on an older patient's health (Cumming, & Le Couteur, 2003; Garfinkel, & Mangin, 2010). As a result, decreasing the irrational use of these medications in the older population is a critical public health concern.

The prevalence (7.5%) of contraindicated DBI medication noted in the present study population is considerable, although, our study did not assess patients' home medications which may have underestimated this prevalence. The most commonly prescribed contraindicated DBI medications in the study population were promethazine (47.8%, n = 11/23) and amitriptyline (17.4%, n = 4/23). These medications should be avoided in older people due to their highly anticholinergic effects, reduced clearance with advanced age, and higher risk of side effects such as confusion, dizziness, dry mouth, constipation, blurred vision, and other anticholinergic effects or toxicity (The American Geriatric Society, 2019). Therefore, evidence-based alternative medications, along with non-pharmacological approaches when appropriate are recommended to avoid these problems (The American Geriatric Society, 2019).

Our study found that a considerable proportion (19.6%, n = 60) of the study population was exposed to high-risk DBI medications comparable with 33.3% (n = 22/66) in a Finnish study (Lo¨nnroos et al., 2012). In contrast, another study in Finland found a higher prevalence of 35.4% among 257 community-dwelling older people exposed to DBI medications (Gnjidic, Le Couteur, & Abernethy, 2012). Also, a study of 532 older adults with intellectual disabilities exposed to DBI medications in Ireland found a much higher proportion of 69.0% (O'Connell et al., 2018). The observed variations could be due to differences in disease burden, medications prescribed, and methods of prevalence calculation.

When the study data were analyzed for potential independent predictors of high-risk DBI medication exposure, it was discovered that patients over 70 years of age (OR 3.08, 95% CI = 1.38 - 6.89), those with low non-DBI co-medications (OR 3.40, 95% CI=1.03 - 11.23), polypharmacy (OR 7.38, 95% CI = 2.20 - 24.73), and contraindicated DBI medication (OR 3.93, 95% CI = 1.14 - 13.53) had significantly higher odds of exposure to high-risk DBI medications (p < 0.05). In agreement with the finding of the present study, a similar study linked increased DBI medication exposure with increasing age (OR 1.02, 95% CI = 1.02 - 1.02) (Nishtala, et al., 2014). The significantly increased likelihood of being exposed to high-risk DBI medication with a low number

of non-DBI co-medications confirmed that an increasing number of non-DBI co-medications do not translate to increased DBI. In addition, consistent with the finding of the present study, polypharmacy is significantly associated with increased DBI (OR 4.92, 95% CI = 4.86 - 4.98) (Nishtala, et al., 2014).

Furthermore, several studies demonstrated that inappropriate medication use, which included the use of sedative and anticholinergic medications, was linked to increased inpatient visits (OR 1.99, 95% CI = 1.76 - 2.26); increased outpatient visits (OR 1.53, 95% CI = 1.43 - 1.63); increased physician office visits (OR 1.89, 95% CI = 1.55 - 2.30); and increased emergency hospital room visits (OR 1.98; CI = 1.77 - 2.20) (Fick, Mion, & Beers, 2008), a shorter time to hospitalization (HR 1.20; 95% CI = 1.04 - 1.39) (Fillenbaum, et al., 2004), and a higher risk of at least one acute hospitalization (RR 2.03, 95% CI = 1.49 - 2.77) (Klarin, Wimo, & Fastbom 2005). Also, a study showed that a higher DBI was associated with an impairment in cognitive performance which corresponded to lower Mini-Mental State Exam scores (coefficient: –0.161, 95% CI = –0.250 - –0.071) (Kris, et al., 2017). These findings emphasized the potential benefit of clinical medication reviews to stop or reduce the prescribing of unnecessary medications, particularly DBI medications to this high-risk population. Targeted efforts involving a multidisciplinary approach are thus required to reduce irrational use of sedative and/or anticholinergic medications in older people.

The implications of the study findings for practice are: (i) the high use of cardiovascular disease medications and antihistamines, combined with an appreciable concomitant prescribing of two DBI medications, suggest that interventions to educate patients and physicians about the risks associated with sedative and anticholinergic medications in older people should be prioritized. (ii) Regular medication reviews by a clinical pharmacist should also be considered, with a particular emphasis on those over 70 years of age and those with polypharmacy. Also, the DBI can be deployed as a screening tool to identify older patients with high exposure to sedative and/or anticholinergic medications who may have compromised bodily or psychological functions (Wouters, et al., 2017).

To the best of our knowledge, this is the first study in Nigeria to investigate older peoples' exposure to sedative and anticholinergic medications. Secondly, a list of DBI medications formulated in this study may be useful for further DBI studies in Nigeria. The study had some limitations, which includes the inability to review patients' home medications due to a lack of information in the medical records and the retrospective study design. Secondly, a single-center study with a small sample size may have an impact on the generalizability of the findings. Thirdly, while all DBI medications prescribed were included in the analyses, it was impossible to ascertain whether they were dispensed and ingested by the patients, or how long they were consumed. As a result, the DBI calculations may not accurately reflect true exposure. Concerning intention to prescribe, the results, however, reflect prescribing practice. Finally, the DBI medication main list provided in this study was based on medications and dosages applicable to Nigeria.

## CONCLUSION

This study demonstrates that most older people were exposed to anticholinergic medications, especially cardiovascular disease medications, and antihistamines. A considerable proportion of these older people had contraindicated DBI and high-risk DBI medications. The significant independent predictors of exposure to high-risk DBI medications identified in our study were over 70 years of age, low number of non-DBI co-medications, polypharmacy, and contraindicated DBI prescriptions. These findings suggest that there are opportunities for interventions targeting these identified significant predictors to ensure rational prescribing in this high-risk population. Future studies on the effects of DBI medications on health outcomes in older people in Nigeria are recommended. Interventional studies to reduce the irrational use of DBI medications in older people are also warranted.

### Abbreviations

ADEs = Adverse Drug Events

AOR = Adjusted Odds Ratio

CI = Confidence Interval

CVD = Cardiovascular Drug

DBI= Drug Burden Index

GOPD = General Outpatients' Department

HR = Hazard Ratio

IRR = Incidence Rate Ratio

MRPs = Medication Related Problems

OR = Odds Ratio

RR = Relative Risk

**Peer-review:** Externally peer-reviewed.

**Informed Consent:** Written consent was obtained from the participants.

**Author Contributions:** Conception/Design of Study- R.N.O.; Data Acquisition- A.I.I.; Data Analysis/Interpretation- R.N.O., A.I.I.; Drafting Manuscript- R.N.O.; Critical Revision of Manuscript- R.N.O.; Final Approval and Accountability- R.N.O., A.I.I.

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Ethics Committee Approval:** This study was approved by the State Specialist Hospital Research and Ethics Committee (No: SSH/GEN/641).

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**Appendix I**

| Drug class | Anticholinergics | Sedatives |
|---|---|---|
| **Cardiovascular drugs** | | |
| | | Methyldopa |
| | Nifedipine | |
| | | Prazosin |
| | Furosemide | |
| | Digoxin | |
| **Antihistamines** | | |
| | Imipramine | Imipramine |
| | | Cetirizine |
| | Chlorpheniramine | Chlorpheniramine |
| | | Loratadine |
| | Meclizine | Meclizine |
| | Cimetidine | Cimetidine |
| | Clemastine | Clemastine |
| | Clomipramine | Clomipramine |
| | Prochlorperazine | Prochlorperazine |
| | Promethazine | Promethazine |
| | Ranitidine | |
| | Diphenhydramine | Diphenhydramine |
| | Hydroxyzine | Hydroxyzine |
| | Triprolidine | Triprolidine |
| | Cyproheptadine | Cyproheptadine |
| **Skeletal muscle relaxants** | | |
| | | Baclofen |
| | Methocarbamol | Methocarbamol |
| | Orphenadrine | |
| | Tizanidine | Tizanidine |
| **Analgesics** | | |
| | | Codeine |
| | | Tramadol |
| | | Pentazocine |
| **Antidiarrhoeals** | | |
| | Loperamide | |
| **Antidepressants** | | |
| | Amitriptyline | Amitriptyline |
| | Paroxetine | Paroxetine |
| | | Citalopram |
| | | Paroxetine |
| | | Escitalopram |
| | | Fluoxetine |
| | | Sertraline |
| | | Venlafaxine |

**Appendix I. Continue**

| Drug class | Anticholinergics | Sedatives |
|---|---|---|
| **Anticonvulsants** | | |
| | | Amobarbital |
| | | Aprobarbital |
| | | Benzylbutylbarbiturate |
| | | Butabarbital |
| | | Butalbital |
| | | Gabapentin |
| | | Phenobarbital |
| | | Pregabalin |
| | | Secobarbital |
| | | Thiopental |
| | Carbamazepine | Carbamazepine |
| | | Phenytoin |
| **Benzodiazepines** | | |
| | | Diazepam |
| | | Bromazepam |
| | | Clonazepam |
| | | Flunitrazepam |
| | | Lorazepam |
| | | Nitrazepam |
| | | Oxazepam |
| | | Temazepam |
| | | Estazolam |
| | | Flurazepam |
| **Antispasmodics** | | |
| | Propantheline | |
| | Oxybutynin | Oxybutynin |
| | Hyoscine Butylbromide | |
| | Trihexyphenidyl | |
| | Benztropine | |
| **Antipsychotics** | | |
| | Chlorpromazine | Chlorpromazine |
| | Olanzapine | Olanzapine |
| | Fluphenazine | Fluphenazine |
| | | Resperidone |
| | Trifluoperazine | Trifluoperazine |
| | Clozapine | Clozapine |
| | Thioridazine | Thioridazine |
| | | Haloperidol |

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**Original Article**

# Evaluation of summary of product characteristics and patient information leaflet of the best-selling drugs in Turkey in terms of readability

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**ABSTRACT**

**Background and Aims:** Readability can be defined as the easiness or difficulty of texts to be understood by readers. In our study, it was aimed to evaluate the patient information leaflet and the summary of product characteristics in terms of readability in Turkish.
**Methods:** Our study is a cross-sectional study. For our study, the best-selling drugs included in the "Turkish Pharmaceutical Market Monitoring Report-8, 2020 Market Status in Terms of Sales Volume and Value" prepared by the Turkish Medicines and Medical Devices Agency in 2021, were evaluated by using Turkish readability formulas (Ateşman and Bezirci-Yılmaz).
**Results:** 138 patient information leaflet and summary of product characteristics of a total of 69 products were evaluated. It has been determined that an average of at least undergraduate education is required for the readability of the texts. The patient information leaflets are significantly shorter than the summary of product characteristics (p=0.000). However, in terms of readability, it was easier in Ateşman calculation and more difficult in Bezirci-Yılmaz calculation (p=0.007 and p=0.000, respectively).
**Conclusion:** It has been seen that patient information leaflets are not easy to read texts prepared for patients. While preparing the texts to be read by the patients, the texts should be easily understandable and should be read and understood by people of all education levels.
**Keywords:** Health literacy, comprehension, Pharmaceutical Preparations, Prospectus

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## INTRODUCTION

In today's health system, the expectation of service providers from service users is increasing(Nielsen-Bohlman, Panzer, & Kindig, 2004). The patriarchal doctor-patient relationship has been replaced by a relationship in which individuals understand, decide, and apply the information given in writing or verbally, and as a result, they take their own health responsibilities. An effective health literacy is needed for all these roles to be fulfilled effectively (Ilbars & Özkan, 2020; Nielsen-Bohlman et al., 2004).

Health literacy is a new concept in which health and literacy come together. The World Health Organization examines health literacy in three dimensions: functional, interactive, and critical literacy(Kanj & Mitic, 2009). Functional health literacy refers to the ability of individuals to understand and act in accordance with written texts such as drug prospectuses, informed consent, and informational texts given by health personnel (Erdoğan & Araman, 2017; Williams, Baker, Parker, & Nurss, 1998). While low functional literacy directly affects the health of the individual, it also increases the unnecessary use of health facilities (Baker, Parker, Williams, & Clark, 1998).

With the Regulation on Licensing of Medicinal Products for Human Use published in our country in 2005, summary of product characteristics (SmPC) and patient information leaflet (PIL) were introduced for newly licensed products (Sağlık Bakanlığı, 2005). Accordingly, SmPC will be prepared only to inform health professionals and to use the medicinal product effectively, which is not included in the product box. There will also be PIL in the product box prepared in accordance with the SmPC to inform the patients (Sağlık Bakanlığı, 2005). Making this distinction, in a way, shows that patients are expected to read the relevant product information and take responsibility for their own health.

Readability can be defined as the texts' being easy or difficult to be understood by the reader and it can be measured objectively. In the readability calculation, parameters such as the number of words in the sentence, the average number of syllables of words, and the number of multi-syllable words are included. Although there are more than 40 readability formulas that come from the past and are still used, most of these formulas have been prepared in accordance with the English language structure (Philipson, Doyle, Gabram, Nightingale, & Philipson, 1995). In Turkey, certain readability formulas such as Ateşman and Bezirci-Yılmaz formulas, which are suitable for Turkish language structure, are used (Ateşman, 1997; Bezirci & Yilmaz, 2010).

In our study, it was aimed to evaluate PIL prepared for patients and SmPC prepared for health professionals in terms of readability in Turkish using mathematical formulas and to determine which education level in patients it appeals to.

## MATERIAL AND METHODS

For our study, the "Turkish Pharmaceutical Market Monitoring Report-8, 2020 Market Status in terms of Sales Volume and Value" report prepared by the Turkish Medicines and Medical Devices Agency (Türkiye Ilaç ve Tibbi Cihaz Kurumu; TITCK) in 2021 was taken as a basis. According to the sales volumes of 2020 in this report, the top 20 drugs, which are the most sold in total, the most sold without a prescription, covered by Social Security Institution (Sosyal Güvenlik Kurumu, SGK) and private insurance, were evaluated (a total of 80 drugs) (Table 1). (Sağlık Bakanlığı, 2021).

The current SmPC and PIL information of the drugs in this list was obtained from the official website of TITCK (https://www.titck.gov.tr/kubkt). If there is more than one SmPC or PIL information for a drug, the most up-to-date one was included in the evaluation. Although it is included in the report, "fortini multifibre strawberry flavored, 200 ml", which does not have SmPC- PIL information on TITCK's website, could not be evaluated.

With 20 drugs in each group, a total of 80 drugs from 4 groups were evaluated, but in the case of a drug that has the same active ingredients and the product names and is included twice in the list due to the number of tablets in it, only one of these drugs has been evaluated (such as Parol 500 mg tablet, 20 tablets and Parol 500 mg tablet, 30 tablets). Therefore, 10 drugs listed twice and 1 drug without SmPC-PIL information were excluded, and a total of 69 drugs were evaluated.

Ateşman and Bezirci-Yılmaz readability formulas were used in the readability calculation. Ateşman readability formula was developed in 1997 as an adaptation of Flesch readability formula into Turkish (Ateşman, 1997). It is calculated as: Readability score = 198.825 – 40.175 x word length (total syllables / total words) – 2.610 x sentence length (total words / total sentences). An increase in the score indicates an increase in readability. The difficulty levels and the level of education required by the scores are shown in Table 2.

The Bezirci-Yılmaz readability formula was developed in 2010 in accordance with the Turkish language structure, without being an adaptation of a foreign formula (Bezirci & Yilmaz, 2010). According to the results obtained, the level of education required by the text is determined (Table 3). The formula is calculated as follows: Readability score= √OKS x ((H3 x 0.84) + (H4x1.5) + (H5x3.5) + (H6x26.25)) OKS: average word count; H3: mean number of 3-syllable words; H4: mean number of 4-syllable words; H5: mean number of 5-syllable words; H6: the average number of words with 6 or more syllables.

In order not to affect the calculation, the product names and registration information in the SmPC-PIL were not taken into consideration. A software developed by Bezirci-Yılmaz (BET-okunabilirlik.exe) was used to evaluate the remaining parts (Bezirci & Yilmaz, 2010). The fractional results obtained for the education level were rounded to the nearest integer.

The "Word Frequency Dictionary of Written Turkish" published by the Turkish Language Association in 2018 was used to look at the number of difficult words in theSmPC and PILs, and the words that are not among the basic 3000 words here are defined as "difficult words".

SPSS 18 package program was used in the analysis of the data. Whether the data were normally distributed or not was evalu-

**Table 1. The top 20 drugs, which are the most sold in total, the most sold without a prescription, covered by Social Security Institution (Sosyal Güvenlik Kurumu, SGK) and private insurance (Sağlık Bakanlığı, 2021).**

| TOP 20 DRUGS SOLD TOTAL | TOP 20 DRUGS SOLD WITHOUT PRESCRIPTION* |
|---|---|
| PAROL 500 MG TABLET, 20 TB | PAROL 500 MG TABLET, 20 TB |
| CORASPIN 100 MG ENTERIC COATED TABLET, 30 TB | DOLOREX DRAJE, 20 DRAJE |
| ARVELES 25 MG FILM TABLET, 20 TB | ARVELES 25 MG FILM TABLET, 20 TB |
| DOLOREX DRAJE, 20 DRAJE | CORASPIN 100 MG ENTERIC COATED TABLET, 30 TB |
| BELOC ZOK CONTROLLED RELEASE FILM TABLET 50 MG 20 TB | DEVIT-3 IM/ORAL AMP. |
| NEXIUM ENTERIC COATED PELLET TABLET 40 MG 28 TABLET | MAJEZIK 100 MG 15 FILM TABLET |
| PAROL 500 MG TABLET (30 TABLET) | PAROL 500 MG TABLET (30 TABLET) |
| ECOPIRIN 100 MG ENTERIC COATED TABLET, 30 TABLET | ASPIRIN TABLET 20X0.5G (20 TABLET) |
| LANSOR MICROPELLET CAPSULE 30 MG 28 CAP | VERMIDONE TABLET (30 TABLET) |
| MAJEZIK 100 MG 15 FILM TABLET | NOVALGIN 500 MG TABLET, 20 TB |
| TRAVAZOL LEATHER CREAM (15 G) | DEVIT-3 ORAL DROPS 50.000 IU (15 ML) |
| DEVIT-3 IM/ORAL AMP. | NEXIUM ENTERIC COATED PELLET TABLET 40 MG 28 TB |
| FORTINI MULTIFIBER STRAWBERRY FLAVORED, 200 ML | CALPOL SUSPENSION |
| DEVIT-3 ORAL DROPS 50.000 IU (15 ML) | A-FERIN FORT FILM TABLET 30 TB |
| INFATRINI 200 ML. | NUROFEN COLD & FLU 200MG/30MG FILM |
| GLIFOR 1000 MG FILM TABLET (100 TABLET) | COATED TABLET (24 TABLET) |
| PLAVIX 75 MG 28 FILM TABLET | TRAVAZOL LEATHER CREAM (15 G) |
| AUGMENTIN BID 1000 MG FILM TABLET, 14 TABLET | ECOPIREN 100 MG ENTERIC COATED TABLET, 30 TB |
| NOOTROPIL FILM TABLET 800 MG 30 TB | VENTOLIN INHALER 200 DOSES |
| VENTOLIN INHALER 200 DOSES | APRANAX FORT FILM COATED TABLET, 20 TABLET |
| | THERAFLU FORTE FILM TABLET (20 TB) |

| TOP 20 DRUGS PAID BY SGK | TOP 20 DRUGS PAID BY SPECIAL INSURANCES |
|---|---|
| CORASPIN 100 MG ENTERIC COATED TABLET, 30 TB | CORASPIN 100 MG ENTERIC COATED TABLET, 30 TB |
| PAROL 500 MG TABLET, 20 TB | AUGMENTIN BID 1000 MG FILM TABLET, 10 FILM TB |
| ARVELES 25 MG FILM TABLET, 20 TB | PAROL 500 MG TABLET, 20 TB |
| BELOC ZOK CONTROLLED RELEASE FILM TABLET 50 MG 20 TB | TRANKO-BUSKAS 10 + 10 MG COATED TABLET (20) |
| DOLOREX DRAJE, 20 DRAJE | ARVELES 25 MG FILM TABLET, 20 TB |
| NEXIUM ENTERIC COATED PELLET TABLET 40 MG 28 TB | RITALIN 10 MG TABLET (30 TB) |
| ECOPIREN 100 MG ENTERIC COATED TABLET, 30 TABLET | BELOC ZOK CONTROLLED RELEASE FILM TABLET 50 MG 20 TB |
| LANSOR MICROPELLET CAPSULE 30 MG 28 CAP | AUGMENTIN BID 1000 MG FILM TABLET, 14 TABLET |
| PAROL 500 MG TABLET (30 TABLET) | DEVIT-3 ORAL DROPS 50.000 IU (15 ML) |
| FORTINI MULTIFIBER STRAWBERRY FLAVORED, 200 ML | DEVIT-3 IM/ORAL AMP. |
| INFATRINI 200 ML. | GERALGINE-K TABLET 20 TB |
| TRAVAZOL LEATHER CREAM (15 G) | DOLOREX DRAJE, 20 DRAJE |
| GLIFOR 1000 MG FILM TABLET (100 TABLET) | NEXIUM ENTERIC COATED PELLET TABLET 40 MG 28 TB |
| PEDIASURE PLUS FIBER STRAWBERRY FLAVORED 220 ML | LYRICA 300 MG CAPSULES ( 56 CAPSULES ) |
| NOOTROPIL FILM TABLET 800 MG 30 TB | NEURONTIN 800 MG NOTCHED FILM COATED TABLET (50 TB) |
| PLAVIX 75 MG 28 FILM TABLET | MAJEZIK 100 MG 15 FILM TABLET |
| PEDIASURE PLUS FIBER BANANA FLAVORED 220 ML | XANAX 1MG 50 TABLET |
| VASOXEN 5 MG TABLET, 28 TB | BELOC ZOK CONTROLLED RELEASE FILM TABLET 25 MG 20 TB |
| FORTINI MULTI FIBER BANANA FLAVORED 200 ML | LANSOR MICROPELLET CAPSULE 30 MG 28 CAP |
| FORTINI MULTI FIBER CHOCOLATE FLAVORED 200 ML | PLAVIX 75 MG 28 FILM TABLET |

* It refers to the first 20 drugs obtained from pharmacies by patients without SGK payment and prescription.

ated with the Kolmogrov - Smirnov test. Student's t test, Mann Whitney U test, two-way ANOVA and descriptive statistics were performed. Normally distributed data are given mean. ± Std Deviation, non-normally distributed data are given as mean (min, max). The statistical significance value was taken as $p<0.05$.

Ethics committee approval was obtained for the study from Erzincan Binali Yıldırım University Clinical Research Ethics Committee with the date 28.04.2022 and decision number 15.

## RESULTS

138 SmPC-PIL of 69 products in total were evaluated. The mean readability score was calculated as 43.8±6.2 for Ateşman and 15±2.4 for Bezirci-Yılmaz, respectively; It corresponds to 13th-15th grade level education requirement for Ateşman and undergraduate level education requirement for Bezirci-Yılmaz.

The number of words, sentences, words, difficult words, syllables and polysyllabic words of SPC-IFUs are given in the t Table

**Table 2. Difficulty and education levels corresponding to the score obtained with the Ateşman readability formula(Ateşman, 1997).**

| Score | Difficulty level | Education level |
|---|---|---|
| **90-100** | Very easy | Can be read by anyone with a 4th grade and below. |
| **80-89** | Easy | Can be read by anyone with a 5th or 6th grade education |
| **70-79** | | Can be read by anyone with a 7th or 8th grade education |
| **60-69** | Medium difficulty | Can be read by anyone with a 9th or 10th grade education |
| **50-59** | | Can be read by anyone with an 11th or 12th grade education |
| **40-49** | Hard | Can be read by anyone with a 13th or 15th grade education. |
| **30-39** | | Can be read by anyone with a bachelor's degree. |
| **1-29** | Very hard | Can be read by anyone with a postgraduate degree. |

**Table 3. Education level corresponding to the score obtained with the Bezirci–Yılmaz readability formula (Bezirci & Yilmaz, 2010).**

| Grade | Education level |
|---|---|
| **1st - 8th** | Primary education |
| **9th – 12th** | Secondary education |
| **12th – 16th** | Undergraduate |
| **16th+** | Academic level education |

SmPc and PIL scores are given in the Table 6 according to the most sold in total, the most sold without a prescription, covered by SGK and private insurance ,and no significant difference was found between the groups in terms of both Ateşman scores and Bezirci-Yılmaz scores (respectively p=0.815, p=0.760).

## DISCUSSION

The World Health Organization (WHO) defines health literacy as "an individual's ability to access, understand and use health information for the protection and maintenance of health"(Kanj & Mitic, 2009). The concept of "readability", which can be measured objectively and indicates the level of easy readability of the read text by the reader, is directly related to health literacy. In our study, it was aimed to investigate the readability level of SmPC and PILs .

Studies have shown that most of the patients forgot or misunderstood the information they received from the physician or other health personnel(Calkins et al., 1997; Makaryus & Friedman, 2005). In a study, it was found that patients forgot at least half of what the physician said about 5 minutes after leaving the

**Table 4. Comparison of SmPC* and PILs** in terms of number of sentences, words, syllables and polysyllabic words.**

| | SmPC / PIL | Mean | Min | max | p |
|---|---|---|---|---|---|
| Number of sentences | SmPC | 339.03 | 164 | 571 | 0.000 |
| | PIL | 178.07 | 98 | 284 | |
| Word count | SmPC | 3363.61 | 1813 | 6822 | 0.000 |
| | PIL | 2260.54 | 1389 | 3358 | |
| Difficult word count | SmPC | 3526.46 | 1775 | 6558 | 0.000 |
| | PIL | 2205.29 | 1363 | 3273 | |
| Number of syllables | SmPC | 1074.,62 | 5194 | 18860 | 0.000 |
| | PIL | 6347.48 | 3485 | 9312 | |
| Number of polysyllabic words | SmPC | 1291.83 | 607 | 2240 | 0.000 |
| | PIL | 721.99 | 332 | 1049 | |

*SmPC: Summary of Product Characteristics; ** PIL: Patient Information Leaflet

4, and in all groups,PILs are statistically shorter than SmPCs (p=0.000) (Table 4).

When we look at the difficult word ratio in SmPC-PIL it was seen that 97.09 ±1.12 of SmPC and 97.50±1.65 of PIL consisted of difficult words. Although this rate was higher in PILs the difference was not found to be significant (p=0.083).

Considering the readability scores of the SmPC and PIL a significant difference was found between both Ateşman and Bezirci-Yılmaz scores (p=0.007, p=0.000, respectively) (Table 5).

**Table 5. Comparison of the readability scores of SmPC* - PILs.**

| | | n | Mean | Std. Deviation | Corresponding education level | p |
|---|---|---|---|---|---|---|
| *Ateşman* | SmPC | 69 | 42.43 | 5.00 | 13-15th grade | 0.007 |
| | PIL | 69 | 45.25 | 6.95 | 13-15th grade | |
| *Bezirci-Yılmaz* | SmPC | 69 | 14.57 | 1.67 | Undergraduate | 0.000 |
| | PIL | 69 | 15.51 | 2.93 | Undergraduate | |

* SmPC: Summary of Product Characteristics; **PIL: Patient Information Leaflet

**Table 6. Comparison of readability scores of best selling drug groups.**

| | | Ateşman | | | Bezirci-Yılmaz | |
|---|---|---|---|---|---|---|
| | | n | Mean | Std. Error | Mean | Std. Error |
| *Most sold in total* | SmPC | 17 | 43.61 | 5.23 | 14.30 | 1.76 |
| | PIL | 17 | 45.37 | 6.52 | 15.26 | 3.00 |
| | Total | 34 | 44.49 | 5.89 | 14.78 | 2.47 |
| *Most sold without a prescription** | SmPC | 20 | 41.41 | 5.62 | 14.50 | 1.81 |
| | PIL | 20 | 45.96 | 7.45 | 15.36 | 2.84 |
| | Total | 40 | 43.68 | 6.90 | 14.93 | 2.39 |
| *Covered by SGK* | SmPC | 13 | 41.61 | 3.90 | 15.10 | 1.15 |
| | PIL | 13 | 44.34 | 7.62 | 15.75 | 3.39 |
| | Total | 26 | 42.98 | 5.88 | 15.42 | 2.50 |
| *Covered by private insurance* | SmPC | 19 | 43.00 | 4.82 | 14.53 | 1.78 |
| | PIL | 19 | 45.02 | 7.08 | 15.74 | 2.82 |
| | Total | 38 | 44.01 | 6.06 | 15,13 | 2.40 |

* It refers to the first 20 drugs obtained from pharmacies by patients without SGK payment and prescription.

exam room(Kitching, 1990). In another study conducted with 623 patients, only 31% of the patients stated that they were adequately informed by the physician about the side effects of the medication (Enlund, Vainio, Wallenius, & Poston, 1991).

Today, due to the increasing need for health care and increasing workload, especially with the Covid-19 pandemic, physicians cannot spare enough time for their patients and provide the necessary information (Auwal, Tanimu, Samira, & Hadiza, 2022; Desideri et al., 2021). For this reason, the importance of the instructions for use in medicine boxes and especially prepared for the patients to read is increasing. Patients are expected to take more responsibility for their own health problems.

In our study, it was seen that an average of 13$^{th}$-15$^{th}$ grade is required for Ateşman, and undergraduate education is required for Bezirci-Yılmaz in order to understand the texts. According to the data of the Turkish Statistical Institute for the year 2020, 63% of the citizens in Turkey have received secondary education and below, and are considered within the scope of the population with low education (Türkiye İstatistik Kurumu, 2020). The rate of getting education at the level of 13$^{th}$ grade or higher is only 16% (Türkiye İstatistik Kurumu, 2020). According to these statistics, SmPC and PILs have been prepared at a level that cannot be understood by a large part of the society.

Although there were close values in the calculations, it was seen that the readability was slightly higher in the Ateşman calculation. As mentioned before, the Ateşman readability formula is not a formula prepared entirely in accordance with the Turkish language structure, but is an adaptation of the Flesch readability formula to Turkish (Ateşman, 1997). Although Turkish and English are two completely different languages in terms of structure,the quantities considered in the readability formulas are almost the same. Since Turkish has an additive language structure, it can be said that it is a more difficult language than English in terms of language learning (Solak & Bayar, 2015). For this reason, the Bezirci-Yılmaz readability formula, which is more suitable for the Turkish language structure, was developed by Bezirci and Yılmaz in 2010 (Bezirci & Yilmaz, 2010). Although this formula seems to be more suitable for the Turkish language structure, both formulas are frequently used in the literature. Therefore, both formulas were used in our study.

Considering the average number of sentences, words and syllables, PILs are significantly shorter than SmPCs. However, this brevity was not reflected in the texts at the same level as readability. Although PILs were found to be more readable in terms of Ateşman score, in the Bezirci-Yılmaz calculation prepared in accordance with the Turkish language structure, PILs were

found to be less readable. In addition, the words that are considered as "difficult words" because they are not among the basic 3000 words in Turkish were used at a higher rate in PILs, but at a rate of over 97% in both SmPC and PILs. From this point of view, it has been seen that PILs are short forms of SmPCs rather than texts that are easier to read and prepared for patients.

There was no difference in readability between the drug groups that are most sold, most sold without a prescription, and most sold covered by SGK and private insurance. Here, it would be appropriate to regulate the PILs of the most sold without prescription drugs and to prepare more legible texts.

SmPC and PILs should have certain standarts.The current standards in Turkey were published in the period of 2007-2008 and were prepared according to the 2005 European Union guidelines (Türkiye İlaç ve Tıbbi Cihaz Kurumu, 2007, 2008). In these guides, the order of the subtitles to be used in SmPC and PIL, the font and size to be used, even the paper type etc. are clearly stated. On the other hand, suggestions were made as "short sentences should be used", but features such as what is meant by shortness, number of syllables, number of words were not specified. We think that it would be appropriate to evaluate SmPC and PILs with readability formulas accepted by the literature before they are used, and to set certain standards in this respect by taking into account the education level of the society.

It can be said that readability is a new concept in the medical literatüre (Ay & Duranoğlu, 2022). No other study that has previously evaluated the readability of SmPC and PILs has been found in the literature. However, one of the biggest limitation in our study is to evaluate only the readability, not the intelligibility of the text. Therefore, there is a need for further studies, such as the Patient Education Materials Evaluation Tool, in which the understanding levels of patients are also evaluated (Vishnevetsky, Walters, & Tan, 2018). Nevertheless, our study is one of the first studies in this field and is a valuable study in this respect.

Using a plain and simple language, preparing texts consisting of words with few syllables and short sentences are essential in improving readability. While preparing the texts that the patients will read, the texts should be prepared at a level that everyone can read and understand. Those who prepared these texts should never make the mistake of only shortening the texts prepared for healthcare professionals. Such an approach would be more appropriate in the form of today's changing health service delivery.

## CONCLUSION

The patient information leaflets (PILs) are at the same level as the texts prepared for health professionals in terms of readability.

While preparing PILs instead of using a simple and more understandable language, the original texts were shortened.

In today's health system, where the patient is expected to take more responsibility for their own health, the texts prepared for patients (PILs) should be prepared at a level that can be read by all segments.

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Original Article

# Evaluation of the role of the pharmacists in the rational use of inhaler devices in the treatment of asthma and COPD

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**ABSTRACT**

**Background and Aims:** Inhaled drug-delivery is the cornerstone for the treatment of asthma and chronic obstructive pulmonary disease (COPD). However, these diseases cannot be adequately controlled in most patients due to the misuse of inhaler devices and poor patient compliance. Patient education is a critical component of treatment and requires the cooperative effort of physicians and pharmacists. This study aimed to evaluate a pharmacists' ability to use inhaler devices and their practical knowledge, and to assess the extent to which pharmacists have consulted on this issue for the rational drug use of asthma or COPD patients.

**Methods:** A questionnaire containing demographic information, steps for the correct use of inhaler devices, information source for inhaler use, and patient counseling was applied to fifty community pharmacists in Istanbul and the data was evaluated.

**Results:** The number of pharmacists who have adequate knowledge about the use of a Metered-Dose Inhaler was 59%. It was 50% for Turbuhaler®, 54% for Diskus®, and 56% for a Capsule Inhaler. Pharmacists operating in community pharmacies for up to 20 years were more knowledgeable about the correct use of inhaler devices, but there was no statistically significant difference according to the pharmacists years in practice ($p<0.05$ for each device).

**Conclusion:** About half of the pharmacists did not have enough knowledge for the correct use of each inhaler device. This suggests that there is a clear need for special and continuing educational programs for pharmacists to increase their knowledge of inhaler use and attitudes towards inhaled therapy to provide better patient counseling and training in the management of asthma and COPD.

**Keywords:** Asthma, Chronic Obstructive Pulmonary Disease, Inhaler Devices, Community Pharmacist, Inhaler Technique

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## INTRODUCTION

Chronic respiratory diseases are the third leading cause of mortality worldwide, behind cardiovascular diseases and neoplasms. Of all chronic respiratory diseases, asthma and chronic obstructive pulmonary disease (COPD) are the most common. This prevalence has been increasing over the years in the world due to higher smoking and increased air pollution (GINA, 2020; GOLD, 2020). In most patients, COPD and asthma are associated with significant comorbidities such as impaired mobility, insomnia, depression, sinusitis, migraine, high blood pressure, stomach ulcers and cancer (Van Manen et al., 2001). The economic impact of asthma and COPD on a patient's spending is substantial, including both direct healthcare costs such as medication, routine and emergency care, and also indirect healthcare costs, including reduced quality of life and productivity, missed school and workdays (GINA, 2020; GOLD, 2020).

Inhaled drug delivery is the cornerstone for the treatment of pharmaceutical management of asthma and COPD, since it allows drugs to reach the target site at effective concentrations with the advantages of faster onset of action, low systemic bioavailability, and consequently less side effects compared to systemic delivery routes (Broeders, Sanchis, Levy, Crompton, & Dekhuijzen, 2009; Al-Jahdali et al., 2013; Mortensen & Hickey, 2014). In both diseases an adequate treatment with inhaled drugs can reduce symptoms and the number of exacerbations, provide clinical control, and improve patients' quality of life. A wide variety of inhaler devices are currently available on the drug market. However, this wide range also represents a disadvantage as each requires different inhalation techniques and the correct completion of multiple steps to ensure optimal and effective drug delivery to the lungs (Basheti, Bosnic-Anticevich, Armour, & Reddel, 2014; Mortensen & Hickey, 2014; Chrystyn et al., 2017). Although effective corticosteroid and bronchodilator treatments are available with inhaled devices, the benefits of inhalation therapy may be limited by inadequate inhalation maneuvers and inhaler use (Broeders et al., 2009; Sanchis, Corrigan, Levy, & Viejo, 2013; Molimard et al., 2017). Many published studies have shown that the majority of patients do not use inhaler devices correctly, and they need to be educated and trained about inhaler techniques (Melani et al., 2011; Chrystyn et al., 2017; Ruud, Rønningen, Faksvåg, Ariansen, & Hovland, 2018). Misuse of inhaler devices and poor compliance of patients are the most common causes of treatment failure. Errors in the use of inhaler devices have been associated with uncontrolled asthma and increased rates of severe COPD exacerbations in patients due to reduced drug delivery and decreased efficacy of the inhaled drugs (Maricoto et al., 2015; Molimard et al., 2017; Price et al., 2017).

In the successful management of asthma and COPD, patient education is a critical component of treatment and requires the cooperative effort of physicians and pharmacists. Since community pharmacists are the healthcare providers that patients often encounter just before taking obtaining their medication, they play an important role in educating and counselling patients on the correct use of different inhaled medicines and devices and also, they have a great responsibility to ensure that patients are using their prescribed drugs correctly. Training and follow-up of the patients by community pharmacists is considered as an important step in the management of asthma and COPD (Ballantyne, 2007; Benavides, Rodriguez, & Maniscalco-Feichtl, 2009; Hesso, Gebara, & Kayyali, 2016). Therefore, pharmacists must be confident and competent in the correct use of different inhaler devices. Several studies have attempted to evaluate the knowledge and ability of pharmacists to properly educate patients for the correct use of inhaler devices, and the impact on the management of chronic respiratory diseases such as asthma and COPD. It was evident in these studies that pharmacists lack the knowledge of the use of inhaler devices and inhaled therapy (Cain, Cable, & Oppenheimer, 2001; Dizdar, Civelek, & Sekerel, 2007; Benavides et al., 2009; Plaza, Giner, Rodrigo, Dolovich, & Sanchis, 2018). A literature search showed that there was only one published survey study done in Turkey in 2014 that evaluated pharmacists' knowledge of using inhaler devices (Gemicioglu, Borekci, & Can, 2014). Therefore, in order to currently address this important issue, this study aimed to evaluate the skills of inhaler device use and practical knowledge of community pharmacists, and to assess the extent to which pharmacists have consulted on this issue for rational drug use of asthma and COPD patients.

## MATERIAL AND METHODS

### Design and study population

This is a survey study, the objective of which is to assess the level of inhaler device usage skills, practical knowledge, and attitudes of community pharmacists. After the coordination and legal permissions were obtained, pharmacies were randomly visited and a specifically designed questionnaire was distributed to community pharmacists in Istanbul city between 1st of February 2019 and 1st of May 2019. After the research goals were announced and the satisfaction of the community pharmacists, questionnaires were distributed and then collected when the survey was completed the same day. Informed consent was obtained before starting data collection.

### Questionnaire

A questionnaire was created based on those used in the relevant survey studies previously published, and piloted for use as a tool in this study (Basheti et al., 2014; Gemicioglu et al., 2014; Giner et al., 2016). Seventy-five community pharmacists in Istanbul city were asked to fill out the questionnaire. However, only fifty of them voluntarily agreed to participate in the study. Data was evaluated within the framework of the principles of rational drug use. The questionnaire included questions about demographics, the most frequently prescribed inhaler devices for patients, the source of information on inhaler use among community pharmacists, steps for the correct use of Metered Dose Inhaler (MDI) and Dry Powder Inhalers (DPIs) including Diskus®, Turbuhaler® and Capsule Inhaler (Aerolizer®, HandiHaler®), and patient counseling. The questionnaire also included questions about the pharmacists' knowledge on storage and cleaning of inhaler devices. A checklist of steps for using each inhaler device was used to assess pharmacists' knowledge on inhalation techniques (Table 1). These checklists were designed according to the pharmaceutical company

| Table 1: Checklist Items of Demonstrating the Use of Inhaler Devices |
|---|
| **Steps for the Use of Metered Dose Inhaler (MDI)** |
| **1.** Remove the cap of the inhaler. |
| **2.** Hold inhaler upright. |
| **3.** Shake the inhaler vigorously. |
| **4.** Tilt your head back slightly and breathe out gently. |
| **5.** Put your lips around the mouthpiece. |
| **6.** Start breathing slowly and then operate the inhaler once during inspiration. |
| **7.** Keep inhaling through mouth while pressing the canister down. |
| **8.** Hold your breath for 10 seconds, then exhale slowly through the nose. |
| **9.** If a second dose is needed, wait half a minute before repeating. |
| **10.** Replace the cap. |
| **Steps for the Use of Turbuhaler®** |
| **1.** Unscrew the cover and lift it up. |
| **2.** Hold the Turbuhaler® upright. |
| **3.** Turn the colored base in one direction as possible. |
| **4.** To load the drug, turn it in the opposite direction until it clicks. |
| **5.** Tilt your head back slightly. |
| **6.** Breathe out gently away from the Turbuhaler®. |
| **7.** Put your lips around the mouthpiece. |
| **8.** Breathe in strongly and deeply through your mouth. |
| **9.** Hold your breath for 10 seconds. |
| **10.** Remove the Turbuhaler® from your mouth and exhale slowly through the nose. |
| **11.** Replace the cap and screw it shut. |
| **12.** After using the Turbuhaler®, rinse your mouth with water. |
| **Steps for the Use of Diskus®** |
| **1.** Put your thumb on the thumb handle, push your thumb away from you. |
| **2.** Slide the tab away from you until you hear a click sound. |
| **3.** Tilt your head back slightly. |
| **4.** Breathe out gently away from the device. |
| **5.** Put your lips around the mouthpiece. |
| **6.** Breathe in strongly and deeply through your mouth. |
| **7.** Hold your breath for 10 seconds. |
| **8.** Remove the Diskus from your mouth and breathe out through the nose. |
| **9.** Replace the cap and screw it shut. |
| **10.** Rinse your mouth and gargle with water, then spit it out. |
| **Steps for the Use of Capsule Inhaler** |
| **1.** Open the cap and lift the mouthpiece. |
| **2.** Remove capsule from foil, place in the internal chamber. |
| **3.** Close the mouthpiece firmly until you hear a click sound. |
| **4.** Hold the inhaler upright and press the button firmly only once to pierce the capsule. |
| **5.** Tilt your head back slightly. |
| **6.** Breathe out gently away from the inhaler. |
| **7.** Put your lips around the mouthpiece. |

**Table 1: Continue**

| **Steps for the Use of Capsule Inhaler** |
|---|
| **8.** Inhale slowly and deeply at a rate sufficient to hear whirring sound or feel the capsule vibrate. |
| **9.** Hold your breath for 10 seconds. |
| **10.** Breathe out normally through nose away from the inhaler. |
| **11.** Open the mouthpiece, tip out the used capsule and discard. |
| **12.** Close the mouthpiece and cap. |

instructions for each inhaled medication device, standardized, and validated according to the relevant survey studies (Basheti et al., 2014; Gemicioglu et al., 2014; Giner et al., 2016).

**Inhalation technique assessment**

Participants were individually classified according to the correct response rates they marked in the checklist for the correct use of each inhaler device. When a respondent marked 30% or less of the correct steps, it was considered as "Poor or No Knowledge Level", if between 30-70%, it was considered as " Inadequate Knowledge Level", and if 70% or more than, this was considered as "Adequate Knowledge Level."

**Statistical analysis**

GraphPad Prism 8.00 for Windows (GraphPad Software, San Diego, California, USA) was used to conduct the statistical analyses. Data was expressed as percentage (%). Scores were converted into the percentage of correct steps for each pharmacist. The relationship between years of working as a community pharmacist and their practical knowledge of the correct use of inhaler devices was examined by using the chi-squared test. The value of $p<0.05$ was considered statistically significant.

## RESULTS

Seventy-five community pharmacists in Istanbul were asked to fill out the questionnaire but only 50 voluntarily agreed to participate in the study (response rate= 66.6%). As demographic data, the gender rates, and the years of practicing as a community pharmacist are given in **Table 2.** There was no statistically significant difference in knowledge scores of pharmacists regarding the adequate use of inhaler devices in terms of gender. The most common inhaler devices prescribed for asthma and COPD patients were the MDI/spacer, followed by Inhaler Capsule (Handihaler® and Aerolizer®), Turbuhaler®, and Diskus® **(Figure 1).** The rates of the knowledge levels of community pharmacists on the correct use of each inhaler device are given in **Figure 2.**

Regarding the critical steps in using MDI, 44% of the pharmacists did not know that MDI should be held upright with the mouthpiece at the bottom. Thirty-four percent were unaware that MDIs had to be shaken before use. Only 62% had the knowledge to start inhalation at the same time as pressing down the canister as the most critical step in MDI use. Seventy-eight percent knew that they had to hold their breath for 10 seconds after inhalation. Only 48% marked the correct statement exhaling through the nose after inhalation. As the most critical step in the use of Turbuhaler®, only 42% of pharmacists had the knowledge that the colored base must be twisted on both sides to load the drug and make it ready for use. When we ask if the Turbuhaler® was accidentally loaded multiple times before use, only 44% of them marked the correct statement that the dose counter drops off by the loaded dose, but still only single dose is inhaled **(Table 2).** Seventy-three percent of the pharmacists knew that to turn on the Diskus®, the thumb had to be placed on the thumb handle and pushed until hearing a click sound, but the rest (29%) did not know this. Only 67% of them knew that to make the drug ready, the thumb had to be placed on the tab and slid away until it clicked in place. The rest (33%) did not know how to load the drug into the device before using it. With regard to capsule inhaler use, 70% had the knowledge that after inserting the capsule into the capsule-chamber, the spike buttons had to be pressed only once to make the drug ready for inhalation, which was a critical step. Only 53% of them knew that while breathing rapidly and deeply, a whirring sound had to be heard, otherwise, the capsule could get stuck. Eighty-five percent of the pharmacists knew how to empty the used capsule and discard after use.

The response rates of pharmacists to general questions regarding inhaler use and patient counseling, storage, and cleaning of devices are given in **Table 2.** The relationship between years practicing as a community pharmacist and knowledge of the adequate use of each inhaler device is shown in **Figure 3.** There were no statistically significant differences between community pharmacists by years of practice (n=50, p=0.12 for MDI, p=0.66 for Diskus®, p=0.88 for Turbuhaler®, and p=0.64 for Capsule Inhaler). The source of the knowledge on the use of inhaler devices among community pharmacists is given in **Figure 4.**

Within the scope of pharmaceutical care, pharmacists were asked what recommendations they made to ensure the rational use of inhaler drugs in the treatment of asthma and COPD and to increase the effectiveness of the treatment. Fifty-seven percent stated that special training and education programs should be organized so that they can educate their patients about the correct use of inhaler devices. Fifteen percent suggested that patient follow-up should be done to properly keep asthma and COPD under control. Seven percent recommended that brochures should be prepared for patients on the correct use technique of inhaler devices. Seven percent recommended that patients should have their pulmonary function tests done regularly. Seven percent stated that only

**Table 2: Questions about inhaler use and patient counseling, and response rates of pharmacists**

| **Gender** | **Pharmacist (%) (n=50)** |
|---|---|
| Male | 44 |
| Female | 56 |
| **Years practicing as a community pharmacist** | **Pharmacist (%)** |
| >30 years | 14 |
| 21-30 years | 56 |
| 10-20 years | 20 |
| >10 years | 10 |
| **After inhalation, if the inhaler device contains a corticosteroid drug, should the mouth be rinsed with water?** | **Pharmacist (%)** |
| Yes | 68 |
| No | 19 |
| I don't know | 13 |
| **What should be considered if no taste is sensed during inhalation with the inhaler device?** | **Pharmacist (%)** |
| I don't know | 32 |
| It is normal, the drug in the inhaler has no taste | 58 |
| The drug is not inhaled | 10 |
| **What happens if Turbuhaler® is accidentally loaded several times before using it?** | **Pharmacist (%)** |
| The dose counter drops off as much as the loaded dose but still only one dose is inhaled | 44 |
| All loaded dose is inhaled | 20 |
| I don't know | 36 |
| **How should inhaler devices be stored?** | **Pharmacist (%)** |
| At room temperature, in a dry place | 75 |
| In refrigerator, at +4°C | 9 |
| I don't know | 16 |
| **How should inhaler devices be cleaned?** | **Pharmacist (%)** |
| By using dry clothes | 64 |
| By using detergents and water | 9 |
| I don't know | 27 |
| **Do you think that you are providing adequate healthcare services in your pharmacy to ensure the rational use of drugs in the treatment of asthma and COPD?** | **Pharmacist (%)** |
| Yes, I think I am providing an adequate service | 48 |
| No, I do not think I am providing an adequate service | 52 |
| **In your pharmacy, do you teach the patients the inhalation technique of the prescribed devices?** | **Pharmacist (%)** |
| Always | 23 |
| Usually | 32 |
| Sometimes | 25 |
| Rarely | 12 |
| Never | 8 |
| **Which suggestions do you make to ensure the rational use of inhaler drugs and to increase the effectiveness of therapy in the treatment of asthma and COPD?** | **Pharmacist (%)** |

pharmacists should inform patients about inhaler drugs and devices, not pharmacy technicians, and 7% declared that it is the responsibility of physicians to inform patients about inhaler devices.

## DISCUSSION

The correct use of inhaler devices and adherence to prescribed therapy are critical components in the management of asthma and COPD. However, most patients do not use their inhaler de-

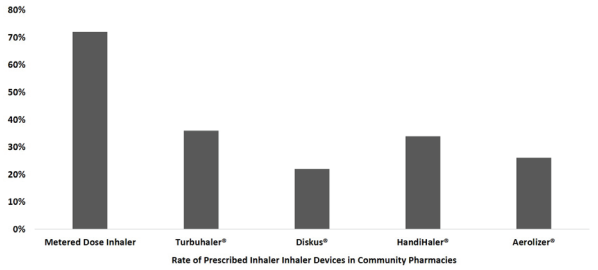


**Figure 1.** Most common inhaler devices prescribed for asthma and COPD patients.

The total percentage is higher than 100%, since respondents were able to select more than one device.

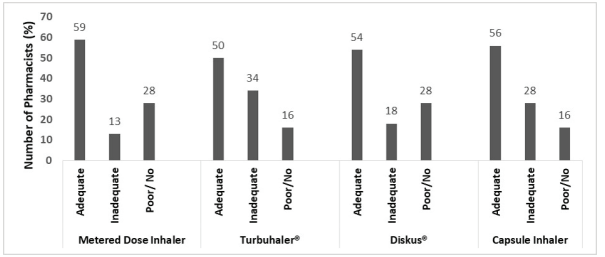


**Figure 2.** Rates of the knowledge levels of community pharmacists on the correct use of inhaler devices.

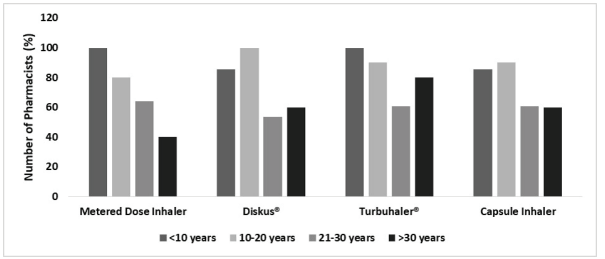


**Figure 3.** Relationship between years practicing as a community pharmacist and their knowledge on adequate use of inhaler devices.

There were no statistically significant differences between community pharmacists according to the practicing years (n=50). p=0.12 for Metered Dose Inhaler, p=0.66 for Diskus®, p=0.88 for Turbuhaler®, and p=0.64 for Capsule Inhaler.

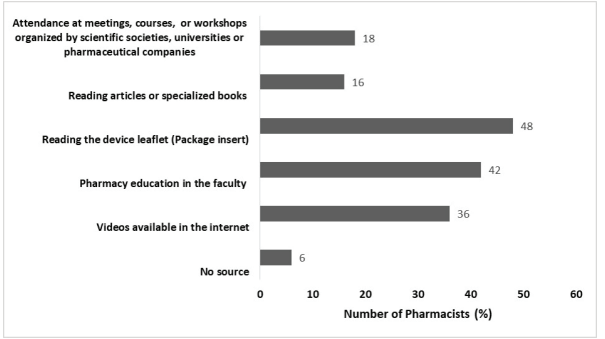


**Figure 4.** Source of knowledge on the inhaler device use among community pharmacists.

The total percentage is higher than 100%, because respondents were able to select more than one device.

vices correctly, and should be trained in terms of inhalation techniques (Melani et al., 2011; Chrystyn et al., 2017; Ruud et al., 2018). Community pharmacists, as being healthcare providers, are responsible for the correct use of medications, as is the prescribing physicians, to ensure that patients use inhaled drugs correctly. Pharmacists have important roles in education, training, and follow-up of the patients in terms of correct inhaler techniques, rational use of medications and in the management of asthma and COPD (Ballantyne, 2007; Benavides et al., 2009; Usmani et al., 2018). Therefore, this survey study aimed to examine inhaler device usage skills and practical knowledge of community pharmacists in Istanbul city and to assess the extent to which pharmacists have consulted this issue in terms of the rational drug use of in asthma and COPD patients.

In this study, more than half of the pharmacists who participated were aware of the most important steps in the correct inhalation techniques with MDI and DPIs and had adequate knowledge about the use of commonly used inhaler devices. However, about half of the respondents were lack of complete knowledge on their proper use. Overall, pharmacists demonstrated the same level of knowledge with all inhaler devices. Regarding the proper inhaler techniques among pharmacists, the MDI technique was slightly better than other inhaler devices. This may be because MDIs have been on the drug market for a long time. Pharmacists operating in community pharmacies for up to 20 years were more knowledgeable on the correct use of inhaler devices, however there was no significant difference according to the years in practice.

Numerous studies have been conducted around the world to elucidate the factors associated with inhaler technique errors and why mistakes are made by patients (Sanchis, Gich, & Pedersen, 2016; Chrystyn et al., 2017; Ruud et al., 2018; Makhinova, Walker, Gukert, Kalvi, & Guirguis, 2020). A high proportion of pharmacists from different countries also made mistakes in the usage steps of inhaler devices (Cain, et al., 2001; Basheti et al., 2011; Gemicioglu et al., 2014; Plaza et al., 2018). Common mistakes and problems in inhaler devices include the deficiencies in preparing the devices for use, lack of knowledge on how to turn on the device, how to load the dose and how to keep the device, inability to coordinate operation, not breathing out before inhalation, not breathing deeply and strongly, not holding the breath long enough when a second dose is required, not waiting for a half minute before repeating the dose, not breathing out through the nose after inhalation, not rinsing the mouth with water after inhaling a corticosteroid drug (Cain et al., 2001; Basheti et al., 2011; Gemicioglu et al., 2014; Plaza et al., 2018; Usmani et al., 2018; Makhinova et al., 2020).

This study's findings also revealed an alarming lack of knowledge among community pharmacists regarding the steps in the correct use of inhaler devices, as nearly half of them had inadequate, poor, or no knowledge on the correct use of inhaler devices. This study addressed some critical errors made by pharmacists in the use of various type of inhalers. The most critical and common lack of knowledge in pharmacists when using MDI were not shaking the device before use (34%) and not starting inhalation at the same time when pressing the canister to get the proper dose (38%), which are the most critical steps for adequate use of the device. More than half of the

pharmacists (58%) did not know how to load the drug into the Turbuhaler® and prepare it for inhalation. The most common and critical lack of knowledge in using the Turbuhaler® was not turning the colored base in two directions to load the dose into the device. Ten percent of the pharmacists thought that the drug was not inhaled when no taste was sensed during inhalation with the device, which may lead to the risk of overdose if the patient is misinformed. Twenty percent thought that if the Turbuhaler® was accidentally loaded multiple times before it was used, that the loaded dose was completely inhaled, and 36% had no knowledge, which may lead to noncompliance with treatment if the patient is misinformed. The most critical lack of knowledge among pharmacists in the use of Diskus® included not knowing how to turn on the device (27%) and load the drug dose for inhalation (33%). The most common mistakes with Capsule Inhaler use were not pressing the button to pierce the capsule (30%), not breathing deeply enough to hear the whirring sound or feel the vibration of the capsule (47%) and forgetting to open the mouthpiece to tip out the used capsule and discard (22%). Makhinova et al. showed that patients swallowed the capsules, did not know to load the capsule into the device, did not puncture the capsule, and did not release the puncture needle prior to inspiration while using the Capsule Inhaler (Makhinova et al., 2020). The pharmacists in our study also did not have the adequate knowledge of cleaning (24%) and storage (36%) of the devices. The study questionnaire focused on identifying the most problematic steps and common mistakes in the use of inhaler devices among community pharmacists. Gemicioglu et al. (2014) showed that in general the most common patient errors with inhaler devices were forgetting to hold their breath for 10 seconds after breathing in and not waiting 30-60 seconds before the second use of the device. In this study we found that the pharmacists lack of knowledge in how to load a drug dose and remind patients to breathe deeply and strongly.

In this study, the most common source of knowledge for inhaler device use among community pharmacists were to read the patient information leaflet (PIL) in the product package insert (48%), followed by faculty pharmacy education (42%), and videos for inhaler use available on the internet (36%). Only a few acknowledged attendance at meetings, courses, or workshops organized by scientific societies, universities, or pharmaceutical companies (18%), and reading articles or specialized books (16%). Our study shows that the instructions given by the pharmaceutical companies are not sufficient. It is highly recommended that attendance to appropriate and continuing education programs will enhance pharmacists' ability and knowledge on the correct use of different inhaler devices and improve the quality of counselling that patients receive.

Only half of the pharmacists thought that they provided adequate healthcare services to their patients in their pharmacies to ensure rational use of drugs in the treatment of asthma and COPD. About half of them did not find the service they provided sufficient. They believed that they could not educate patients adequately in the correct use of inhaler devices, and were not confident with their knowledge. While most of the participating pharmacists considered it their responsibility to teach patients how to use inhaled devices correctly, while a minority believed that it was the physicians' role. The time a physician spends with a patient counseling is very limited, resulting in fewer opportunities to educate patients in the correct use of their medication and inhaler devices. As community pharmacists are the most accessible healthcare providers, they have the opportunity to evaluate, train, and counsel patients on their medications and inhaler techniques. There are many studies in the literature showing the pivotal role of community pharmacists in the management of asthma and COPD, especially in inhaler technique training and adherence to medication. In these studies community pharmacists' educational interventions improved the adherence to the proper use and technique of inhalers, reduced the number of patients who made errors in the use of inhaler devices, decreased the need for medication and drug waste, and significantly reduced the frequency of exacerbations and hospitalizations (Hämmerlein, Müller, & Schulz, 2011; Hesso et al., 2016; Takemura et al., 2013; Ottenbros et al., 2014; Tommelein et al., 2014) According to this study's findings, only half of the pharmacists demonstrated the inhalation technique of the prescribed device to their patients at the pharmacy. For this reason, we suggest pharmacists should take a more active role in patient care and counseling as well as paying more attention to evaluating and educating their patients on the correct use of inhaler devices.

In conclusion, community pharmacists who participated in this survey may have limited knowledge regarding the use of inhaler devices and are not sufficiently qualified in the inhalation technique to effectively educate their patients about the correct use of different devices. In order to increase patient participation in pharmaceutical care for the management of asthma and COPD, community pharmacists' knowledge of inhaler uses and their attitudes towards inhaled therapy needs to be improved by further education, so that they can provide better patient counseling and training. This will help to minimize poor disease control and frequent emergency department visits, and thereby, alleviating the economic burden of the disease. Besides, the findings of consistent problems with certain steps among both patients and pharmacists may encourage the manufacturers to perform field testing early in the development of new inhaler devices, thus, making future inhalers easier to use. On the other hand, this study was performed in only one city with a small sample size; therefore, the results cannot be generalized. Studies in other locations with larger sample sizes are necessary. However, the results of the study can give pharmacists and other healthcare professionals an insight into ideal strategies for managing persistent asthma and COPD.

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**Review**

# A smarter, tactical approach for combating Covid-19

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**ABSTRACT**

As human beings, we communicate with each other just like other creatures. In the same way we need to communicate, COVID-19 has to communicate with other viruses. Following the latest Pandemic, combating COVID-19 has become a major need today. Several theories are being formulated and tested for the efficient prevention and treatment of the virus. Vaccination is the ultimate solution but access to the vaccine and getting vaccinated is limited. The purpose of this review paper is to present a new approach. This approach is based on the Quorum sensing of viruses like bacteria. Bacteria use this for communication and it has recently been proven for viruses too. It can be used as a new way or strategy to stop viral communication, therefore restricting the viral spread will possibly help people around the world or reduce the disease's side effects. This new tactic involves the use of functionalized Quantum dots nanoparticles, and when they are coupled with carbon atoms and put to use in different delivery forms, these will be useful for maximum efficacy. The use of carbon quantum dots can be useful to minimize certain possible toxic effects. This may be greatly enhanced by doping boron atoms to the structure to trigger their synergistic effects. We suggest here that the inhaler form of this proposed drug delivery system should simultaneously provide a fairly high efficiency and a less toxic solution.

**Keywords:** Quorum sensing, Viral communication, Quantum dots, Carbon quantum dots, Pulmonary drug delivery

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## INTRODUCTION

Human beings appeared in the world more than 130.000 years ago and so society began . Building social networks needs many interactions between humans and the other social groups. Since their first appearance, they have started to share their experiences and warned others about danger by talking and using other kinds of communication skills. Even today, people like to get as much information as possible from others. Today, the internet and related networks play an important role. According to the Smithsonian National Museum of Natural History reports, a three-year old female baby named *Australopithecus afarensis,* may have been socially active 3.3 million years ago and she might have been able to interact with others. Another two-year-old Neanderthal baby, who was found lying on her back in a deep burial pit inside a cave with a slab of limestone on top of her head, may be telling the unknown story about her death 70,000-50,000 years ago. Scientists found this skeleton in that position and understood that its burial was prepared by others. As we interact with others to enhance our experiences aiming to live longer and better, other creatures also do the same. The theology of "Quorum sensing" (QS) explains how bacteria learn from others (Jiang, Chen, Yang, Yin & Yao, 2019). Bacterial quorum sensing can be activated by the self-produced extracellular signals in the milieu. These signals are mainly produced by certain chemicals which may play key roles in the regulation of bacterial pathogenesis. Some study results showed that these signals participate in the synthesis of virulence factors in microorganisms during bacterial growth and infection (Pearson, Pesci, & Iglewski, 1997; Dietrich, Price-Whelan, Petersen, Whiteley, & Newman, 2006). The synthesis and secretion of these signal-creating chemicals are regulated by these microorganisms (Yarwood, Bartels, Volper & Greenberg, 2004; Carnes et al., 2010). These virulence factors regulated by QS help bacteria to obtain nutrition from the host and evade its immune system. Some scientists believe that if these signal producing chemicals are blocked, this may be an alternative to antibiotics due to its capacity to reduce bacterial virulence and promote clearance of pathogens (Yarwood, Bartels, Volper & Greenberg, 2004; Jiang, Chen, Yang, Yin & Yao, 2019). Some recent evidence (Carnes et al., 2010; Jiang, Chen, Yang, Yin & Yao, 2019) indicates that antibiotic treatments tend to be less effective for patients because of drug-resistance from overuse (Li & Knetsch, 2018; Xu, Dong, Han, Li & Liu, 2018). These days we are faced with a major viral infection, namely COVID-19. The question here is "Can viruses communicate like bacteria?". Some scientists believe that viruses are rather less developed organisms, and they may not have an ability to communicate but, one very interesting and short review published in *Nature* last year explains the secret social lives of viruses (Dolgin, 2019). In this review, Dolgin discussed the possibility of viral communication with evidence to prove it. According to this report, viruses can actually interact with other viruses and are able to make their own decisions. Viruses can control their own destiny according to Wei Cheng, who was described in the report as a microbiologist from Sichuan University in Chengdu, China. Other reports also indicate that the communication skills of viruses work much like the system used in bacteria quorum sensing to share information about cell density and they adjust the population accordingly. It was the first demonstration of molecular messaging between viruses (Erez et al., 2017). This contradictory finding explains that viruses are much more sophisticated and social - they may also communicate using their own kind of language. In fact, this was not clear or useful for anyone until 1999 and maybe after the last COVID-19 pandemic. It was shown that viruses talk and behave like a *Prisoner's Dilemma* strategy game, working in partnership under certain circumstances and acting in their own self-interests in others (Turner & Chao, 1999). It is certain that, to learn this communication pattern and language will help us to learn more about viruses and this will give us more opportunities to combat them appropriately. This may also be useful for eliminating resistances. The shape of COVID-19 is approximately circular with spikes on it. The overall diameter is about 0.3 microns or slightly less, but the spherical core is about 100 nm (Kirtley, 2020). That is roughly 1/100th the diameter of the average human hair. The space between spikes can be reckoned as around 1-20 nm. This can lead us to think that the size of the particles should be in the size range of 1-20 nm if the virus is actually targeted. The particle in this range can be quantum dots (QDs). However, many known QDs are small enough (1-10 nm) but they are highly toxic. They are useful for developing new generation diagnostic kits and may be used for diagnosis only rather than as a carrier for antiviral drugs (Ghaderi, Ramesh & Seifalian, 2011). However, the introduction of new generation non-toxic composite QDs and their potential to be used as drug delivery systems are becoming possible because they are easy to produce with a very cheap technique (Lim, Shen, & Gao, 2014). In addition, some of them can have some other doping atoms such as nitrogen or boron. Known and previously developed QDs have been produced from CdSe, InAs, CdS, GaN, InGeAS, CdTe, PbS, PbSe, ZnS, and ZnO so far. The reason they are called QDs is because the band gap can be changed by changing its dimensions. In other words, size is a controllable parameter in QDs, and when this feature is combined with the effect of 'quantum confinement', QDs can gain some extraordinary optical and electrical properties (Degim & Kadioglu, 2013). With the change of the dimensions of the QDs, the color of the emitted light changes with the effect of quantum restriction (Figure).

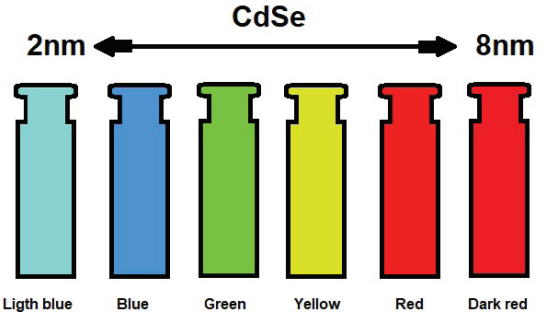


**Figure 1.** The color of Quantum Dots depending on their sizes.

While the smallest size QDs look blue, large QDs emit red light (Girma, Fahmi, Permadi, Abate, & Chang, 2017). QDs generally have very toxic effects since they are made up of metal atoms. Even though the quantum point, which has very superior properties, is generally made, their toxicity could not be

reduced causing a major problem. The ultimate effect and the real biodistributions or changes of biodistributions of QDs in the body have not been fully understood or determined yet. At this stage carbon quantum dots (CQDs) appear to be the safest nanoparticles to be used for medical purposes. In recent years, the production and preparation methods of CQDs have been proposed. The very futuristic usage of CQDs has been shown in the literature. The use of carbon materials including CQDs is generally accepted as safe. Carbon materials are also known to be adsorptive materials, being quite useful to deliver the drug molecule by simple adsorption and subsequent desorption. If the carbon material is good enough to carry, the drug molecule can be released at the site of action by desorption. It is a rather easy procedure, and the material is known to be safe. The only problem is the size of the carbon material because while in many cases the material is safe when it is in a bigger form, it can be very toxic if it is in a nanometer size. Moreover, in many other particulate products, nanoparticles (NPs) have been widely used in diverse food fields, including food processing, safety assessment, packaging, and nutrition delivery (Duncan, 2011; Bi et al., 2017). These NPs may potentially enter the body *via* several different routes such as inhalation, ingestion, or uptake through the skin (Martin et al., 2011). All these show that some nanoparticles may be safe for use. It is very interesting that when NPs are exposed to biological fluids or when they enter the body, they will probably be covered immediately with the protein or be covered to form a kind of protein corona on the nanoparticles (Yan et al., 2013; Huang, Carney, Ikuma, Stellacci & Lau, 2014). This indicates the affinity of nanoparticles to protein. If NPs are smaller, this version of nanoparticles can be QDs. The attraction of protein to the QDs may be increased. The interaction between nanoparticle and protein also affects the toxicity (Chen, Ganesh, Wang & Amiji, 2017; Chen, Ganesh, Wang & Amiji, 2019). The formation of the protein nanoparticle complex depends on size, chemical composition, and surface characteristics (Nayak et al., 2019). W. Hu et al. reported that carbon material (graphene oxide) can form a complex with 10% fetal bovine serum, thus mitigating the cytotoxicity (Hu et al., 2011). Coating graphene oxide with bovine serum albumin significantly attenuated its toxicity.

Food-borne CQDs have been found in roast salmon after the flesh of the fish was heated at about 200°C for 50 minutes (Song et al., 2019). When the roast salmon is consumed, the CQDs are inevitably transferred into the circulatory system. It is possible to get CQDs when we eat roasted salmon. These CQDs may not be very dangerous. These food-borne CQDs might encounter various kinds of serum proteins and be absorbed by these proteins via interactions of the functional groups (Zhu, Wang, Sun, Liu & Wang, 2010). Among the serum proteins, human serum albumin (HSA) is the major soluble protein constituent (40 mg/mL) in human blood plasma with many physiological functions (Arumugam & Malaichamy, 2015) and it has been shown that CQDs can interact with proteins in the body (Song et al., 2020). The formation of the human serum albumin (HSA) and CQDs complex, like a corona from roast salmon, as well as its biological effects, including acute toxicity in mice, have been investigated. The HSA-CQD complex has been introduced because of its static binding mechanism (Yi et al., 2004). The HSA-CQD complex mentioned enters the cytoplasm and has been found to be present in lysosomes or autolysosomes. The HSA coronas have been reported to mitigate the cytotoxicity of CQDs from 18.65% to 9.26%, and the energy metabolism was rectified from glycolytic to aerobic metabolism (Song et al., 2020). This shows the detoxification mechanism and the affinity of proteins to the CQDs. The COVID virus has proteins (S1 and S2) to bind to the receptor to enter the cell. It has been reported that if the S protein is blocked with a molecule, it can be used for preventing the host cells from COVID entering (Yi et al., 2004). If CQDs are not very toxic and we consume them without noticing without having toxicity problems, they may be useful for preventing COVID infections. Because CQDs are capable of interacting with proteins and the corona virus has a protein to enter cells, we may be able to stop the virus using CQDs. A very interesting study result showed that QDs made from tea leaves destroyed lung cancer cells (Shivaji et al., 2018). Their research confirmed previous evidence that tea leaf extract can be a non-toxic alternative to making QDs using chemicals. The cadmium sulfur (CdS) QDs derived from tea leaf extract is reported to show exceptional fluorescence emission in cancer cell bioimaging compared to conventional CdS nanoparticles but Cd is still not very good to use being a quite toxic or even carcinogenic element. However, all these show us that QDs can be a good alternative for the therapy of lung diseases including COVID-19.

Alongside this, another very interesting paper appeared in the literature highlighting the positive effect of functional CQDs as medical countermeasures to human coronavirus (Loczechin et al., 2019). Researchers produced a series of functionalized CQDs. They tested their functionalized CQDs in terms of antiviral activity. It was very interesting to note that all boron functionalized CQDs were found to be antiviral. Moreover, they found that the antiviral effect was maximum (EC50=5.7 mcg/mL) when they functionalized their CQDs with amino boronic acid. Authors claim that the underlying mechanism of the action of these CQDs can due to the inhibition of the virus entry receptors by the interaction of functional groups of the CQDs; the activity was reported to be observed at the viral replication step (Loczechin et al., 2019). If the boron containing CDQs are effective for the therapy of COVID-19, boron compounds or boron doped CQDs may be a better alternative.

**Strategy**

The strategy should be logical when viruses are subjected to drug treatment. The delivery system should be in a proper size range to interact with the virus body and be able to pass through the spikes and should reach to the virus' spherical body. More than that, the drug delivering nanoparticle should destroy the communication ability of the virus or destroy the viral message. This can be a smarter way to combat COVID-19 - one of the century's biggest problems. The strategy should target the virus directly. To administer a drug and wait for it to reach enough concentration in the lungs or other target tissues requires more time and higher doses because of the elimination, metabolism and hepatic first pass effect. Therefore, the drug should be administered directly in a proper formulation.

**Administration rationale**

COVID-19's target is lung epithelial cells therefore our target for stopping viral entry should be the epithelial surface of the lung. If this is the case, we can deliver the drug formulation using an inhaler; drug formulation can be sprayed and delivered by inhalation. To be delivered by inhalation, the drug formulation should be in solution or dry powder form. A solution form can be more useful because if a powder form of CCQDs is going to be used, the actual particle size will be quite small. Particles can reach the deeper sites of the lungs and alveoli but they can be exhaled as well. If the spray form is applied to the lung by inhalation the droplet size can be controlled better using a proper spray head and proper pressure. Also, it can be sent to the site of action much better. When CCQDs reach the surface of the epithelial cells in alveoli they can interact with COVID-19 viruses and block the adhesion or viral replication.

CQDs are a new class of fluorescent carbon nanomaterials having an approximate size in the range of 2–10 nm, as mentioned earlier. The majority of the reported review articles have discussed the development of the CQDs, especially their use in bio-imaging and chemical-/biological-sensing. However, there is still a severe lack of consolidated knowledge on the recently developed CQDs (especially doped/co-doped) and their therapeutic effects. Still, there are number of works in the literature indicating a number of recent developments in doped and co-doped CQDs using boron (B), fluorine (F), nitrogen (N), sulphur (S), and phosphorous (P) (Kandasamy, 2019). The green synthesis methods of this boron doped CQDs has also been introduced (Bourlinos et al., 2015) but the many extraordinary properties of these CQDs still need to be discovered.

## CONCLUSION

Looking at all these theoretical and aspects, and compiling the results, it appears that CQDs can be considered as a better delivery system for effective and rational therapy for life-threatening diseases including COVID-19 infections. Other atom doped CQDs in particular appeared to be the most effective. To deliver the drug to the lungs in a spray form may be another alternative because the final target is the epithelial surface of the lungs. This system can simply be transferred by inhalation of the sprayed solution of CQDs. It may be a better strategy to stop the virus at the entry site. These can be a starting point for the development of as effective solution. We strongly believe that these results and points of view will help readers to think through new pathways which will open a new window for new and better strategies to fight diseases.

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