ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

ISSN: 2636-8552
e-ISSN: 1307-2080,
Volume: 63, No: 3, 2025
Formerly: Eczacılık Bülteni / Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal GÜVEN

ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences
is Published Quarterly
ISSN: 2636-8552
e-ISSN: 1307-2080,
Volume: 63, No: 3, 2025
Formerly: Eczacılık Bülteni/Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal Güven

Address
İstanbul Medipol Üniversitesi
Kavacık Güney Kampüsü
Göztepe Mah. Atatürk Cad.
No: 40 34810 Beykoz/İSTANBUL
Tel: 0216 681 51 00

E-mail
editor@actapharmsci.com
secretary@actapharmsci.com

Web site
http://www.actapharmsci.com

Contents

Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is a continuation of the former "Eczacılık Bülteni" which was first published in 1953 by Prof. Dr. Kasım Cemal GÜVEN's editorship. At that time, "Eczacılık Bülteni" hosted scientific papers from the School of Medicine-Pharmacy at İstanbul University, Türkiye.

In 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and it became a journal for national and international manuscripts, in all fields of pharmaceutical sciences in both English and Turkish. (1984-1995, edited by Prof. Dr. Kasım Cemal GÜVEN, 1995-2001, edited by Prof. Dr. Erden GÜLER, 2002-2011, edited by Prof. Dr. Kasım Cemal GÜVEN)

Since 2006, the journal has been published only in English with the name, "Acta Pharmaceutica Sciencia" which represents internationally accepted high-level scientific standards. The journal has been published quarterly except for an interval from 2002 to 2009 in which its issues were released at intervals of four months. The publication was also temporarily discontinued at the end of 2011 but since 2016, Acta Pharmaceutica Sciencia has continued publication with the reestablished Editorial Board and also with the support of you as precious scientists.

Yours Faithfully

Prof. Dr. Gülden Zehra OMURTAG
Editor

INSTRUCTIONS FOR AUTHORS

Manuscripts must be prepared using the manuscript template.

Manuscripts should contain the following elements in the following order:

Abstract

Keywords

Introduction (without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of Interest Statement

Author Contributions

Funding Sources

Acknowledgments

References

*Obligatory files are manuscript main document, title page, ethical approval, tables, figures for submission. If exist, supplementary files should also be added.

1. Scope and Editorial Policy

1.1 Scope of the Journal

Acta Pharmaceutica Sciencia (Acta Pharm. Sci.), formerly known as Bulletin of Pharmacy and Acta Pharmaceutica Turcica is a peer-reviewed scientific journal publishing current research and reviews covering all fields of pharmaceutical sciences since 1953.

The original articles accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part. The reviews must provide critical evaluation of the state of knowledge related with the subject.

All manuscripts have to be written in clear and concise English.

Including the October 2023 issue, the journal has started to be published online only. It will also publish special issues for national or international scientific meetings and activities in the interested field.

1.2 Manuscript Categories

Manuscripts can be submitted as Original Articles or Review Articles.

Original Articles are definitive accounts of significant, original studies. They are expected to present important new data or provide a fresh approach to an established subject.

Each issue comprises 14 original articles and 1 review article, or 15 original articles in the absence of a review article.

1.3 Prior Publication

Authors should submit only original work that has not been previously published and is not under consideration for publication elsewhere. Academic theses, including those on the Web or at a college Web site, are not considered to be prior publication.

1.4 Patents and Intellectual Property

Authors need to resolve all patent and intellectual property issues. Acceptance and publication will not be delayed for pending or unresolved issues of this type. Note that Accepted manuscripts and online manuscripts are considered published documents.

1.5 Professional Ethics

The editorial board pursue the best practice guidelines of the Committee on Publication Ethics (COPE). To guaranty the integrity of the published papers, *Acta. Pharm. Sci.* editors are guided for using COPE's flowcharts whenever they suspect an ethical issue about the paper they process.

Editors, reviewers, and authors are expected to adhere to internationally accepted criteria for scientific publishing. Helsinki declaration is applied and accepted for the ethical standards of the journal.

World Medical Association. (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bulletin of the World Health Organization, 79(4), 373-374.

1.5.1 Author Consent

Submitting authors are reminded that the consent of all coauthors must be obtained prior to the submission of manuscripts. If an author is removed after submission, the submitting author must obtain the removed author's consent for the change by e-mail to the assigned editor.

1.5.2 Plagiarism

Manuscripts must be original in concept, content, and writing. It is not appropriate for an author to reuse wording from other publications, including their own previous work, whether or not that publication is cited. Suspected plagiarism should be reported immediately to the editorial office, and the report should specifically indicate the plagiarized material within the manuscript. *Acta Pharmaceutica Sciencia* uses iThenticate or Turnitin software to screen submitted manuscripts for similarity to published material. The maximum allowed plagiarism percentage is 20% or less. Please note that your manuscript may be screened during the submission process.

1.5.3 Use of Human or Animal Subjects

For research involving biological samples obtained from animals or human subjects, editors reserve the right to request additional information from authors. Studies submitted for publication must provide evidence that the described experimental activities have undergone local institutional review, ensuring the safety and humane usage of animal subjects. In the case of human subjects, authors must also provide a statement that study samples were obtained with the informed consent of the volunteers, or, in the absence of that, under the authority of the institutional board that approved the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section of 'Statement of Ethics'.

World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bulletin of the World Health Organization, 2001;79(4),373-374.

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1 General Considerations

Manuscripts should be kept to a minimum length. Authors should write in clear, concise English, employing an editing service if necessary. For professional assistance with improving English and/or the figures, or formatting in the manuscript before submission please contact to editorial office by e-mail.

The responsibility for all aspects of manuscript preparation rests with the authors. Applying extensive changes or rewriting of the manuscript will not be undertaken by the editors.

It is best to use the font "Times New Roman", 11 font size, and all kinds of articles must be 1.5 spaced including text, references, tables, and legends.

Ensure that all special characters (e.g., Greek characters, math symbols) are present in the body of the text as characters and not as graphic representations. Be sure that all characters are correctly represented throughout the manuscript—e.g., 1 (one) and l (letter l), 0 (zero) and O (letter o).

All text (including the abstract, all sections of the body of the paper, figure captions, scheme or chart titles, and footnotes and references) and tables should be in one file. Graphics may be included with the text or uploaded as separate files. Manuscripts that do not adhere to the guidelines may be returned to authors for correction.

Use A4 page size with all pages oriented vertically. Set a 2.5 cm margin on each side. There are no page limitations; however, the length should be kept to a minimum. The experimental procedures for all experimental steps must be clearly and fully included in the experimental section of the manuscripts.

2.1.1 Nomenclature

It is the responsibility of the authors to provide correct nomenclature. It is acceptable to use semisynthetic or generic names for certain specialized classes of compounds, such as steroids, peptides, carbohydrates, etc. In such a case, the name should conform to the generally accepted nomenclature conventions for the compound class. Chemical names for drugs are preferred. If these are not practical, generic names, or names approved by the World Health Organization, may be used.

Authors may find the following sources useful for recommended nomenclature:

- The ACS Style Guide; Coghill, A. M., Garson, L. R., Eds.; American Chemical Society: Washington DC, 2006.
- Enzyme Nomenclature; Webb, E. C., Ed.; Academic Press: Orlando, 1992.
- IUPHAR database of receptors and ion channels (http://www.guidetopharmacology.org/).

2.1.2 Compound Code Numbers

Code numbers (including peptides) assigned to a compound may be used as follows:

- Once in the manuscript title, when placed in parentheses AFTER the chemical or descriptive name.
- Once in the abstract.

- Once in the text (includes legends) and once to label a structure. Code numbers in the text must correspond to structures or, if used only once, the chemical name must be provided before the parenthesized code number, e.g., "chemical name (JEM-398)." If appearing a second time in the text, a bold Arabic number must be assigned on first usage, followed by the parenthesized code number, e.g., "1 (JEM-398)." Subsequently, only the bold Arabic number may be used. All code numbers in the text must have a citation to a publication or a patent on first appearance.

Compounds widely employed as research tools and recognized primarily by code numbers may be designated in the manuscript by code numbers without the above restrictions. Their chemical name or structure should be provided as above. Editors have the discretion of determining which code numbers are considered widely employed.

2.1.3 Trademark Names

Trademark names for reagents or drugs must be used only in the experimental section. Do not use trademark or service mark symbols.

2.1.4 Interference Compounds

Active compounds from any source must be examined for known classes of assay interference compounds and this analysis must be provided in the General Experimental section. Many of these compounds have been classified as Pan Assay Interference Compounds (PAINS; see Baell & Holloway, J. Med. Chem. 2010, 53, 2719-2740). These compounds shown to display misleading assay readouts by a variety of mechanisms by forming reactive compounds. Provide firm experimental evidence in at least two different assays that reported compounds with potential PAINS liability are specifically active and their apparent activity is not an artifact.

2.2 Manuscript Organization

Sections: (Capital letters should be used in) Abstract, Introduction, Methodology, Results and Discussion, Statement of Ethics, Conflict of Interest Statement, Author Contributions, Funding Sources, Acknowledgments.

2.2.1 Title Page

The title of the manuscript should reflect the purposes and findings of the work in order to provide maximum information in a computerized title search. The title should be concise and informative. Minimal use of nonfunctional words is encouraged. The title should be concise and informative. Only commonly employed abbreviations (e.g., DNA, RNA, ATP) are acceptable. Code numbers

for compounds may be used in a manuscript title when placed in parentheses AFTER the chemical or descriptive name. *Only the first letter of the first word of the title must be capitalized.*

Authors' Names and Affiliations: The authors' full first names, middle initials, last names (with capital letters for only last names), and affiliations with addresses at time of work completion should be listed below the title. The name of the corresponding author should be marked with an asterisk (*). E-mail belonging to the corresponding author is mandatory. The affiliations must be written in this order: Name of the University, Faculty, Department, City, Country. The ORCID numbers must be hyperlinked in title page for each author.

2.2.2 Abstract and Keywords

Articles of all types must have an abstract following the title. The maximum length of the Abstract should be 200 words, organized in a findings-oriented format in which the most important results and conclusions are summarized. Abstracts should not be separated into categories; it should be written in a paragraph format. After the abstract, a section of Keywords not more than five has to be given. Be aware that the keywords, chosen according to the general concept, are very significant during the searching and indexing of the manuscripts. The 'keywords' must be given as below.

Keywords: instructions for authors, template, journal

2.2.3 Introduction

The Introduction should argue the case for the study, outlining only essential background, and should not include the findings or the conclusions. It should not be a review of the subject area but should finish with a clear statement of the question being addressed. Authors should use this template when preparing a manuscript for submission to *Acta Pharmaceutica Sciencia*.

2.2.4 Methodology

Materials, synthetic, biological, demographic, statistical or experimental methods of the research should be given detailed in this section. The authors are free to subdivide this section in the logical flow of the study. For the experimental sections, authors should be as concise as possible in experimental descriptions. General reaction, isolation, preparation conditions should be given only once. The title of an experiment should include the chemical name and a bold Arabic identifier number; subsequently, only the bold Arabic number should be used. Experiments should be listed in numerical order. Molar equivalents of all reactants and percentage yields of products should be included. A general intro-

ductory section should include general procedures, standard techniques, and instruments employed (e.g., determination of purity, chromatography, NMR spectra, mass spectra, names of equipment) in the synthesis and characterization of compounds, isolates and preparations described subsequently in this section. Special attention should be called to hazardous reactions or toxic compounds. Provide analysis for known classes of assay interference compounds.

Latin names of the plants and names of the microorganisms must be written in italic form.

The preferred forms for some of the more commonly used abbrevations are mp, bp, ^{o}C, K, min, h, mL, μL, g, mg, μg, cm, mm, nm, mol, mmol, μmol, ppm, TLC, GC, NMR, UV, and IR. Units are abbreviated in table column heads and when used with numbers, not otherwise.

2.2.5 Results and Discussion

This section could include synthetic schemes and tables of biological, demographic, and statistical data. The discussions should be descriptive. Authors should discuss the analysis of the data together with the significance of results and conclusions.

2.2.6 Ancillary Information

Include pertinent information in the order listed immediately before the references.

PDB ID Codes: Include the PDB ID codes with assigned compound Arabic number. Include the statement "Authors will release the atomic coordinates and experimental data upon article publication."

Homology Models: Include the PDB ID codes with assigned compound Arabic number. Include the statement "Authors will release the atomic coordinates upon article publication."

Corresponding Author Information: Provide telephone numbers and e-mail addresses for each of the designated corresponding authors.

Present/Current Author Addresses: Provide information for authors whose affiliations or addresses have changed.

2.2.7 Statement of Ethics: Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section of 'Statement of Ethics'. If the study does not require any ethical approval, it could be stated as follows 'No need for ethical approval for this study.'.

2.2.8 Conflict of Interest Statement: Any conflict should be stated. If there is none, it could be stated as follows 'There is no conlict of interest.'.

2.2.9 Author Contributions: Include statement such as "These authors contributed equally."

2.2.10 Funding Sources: Provide the information of the financial support received for conducting the research, including grants, donations, or sponsorships from institutions, organizations, or agencies.

2.2.11 Acknowledgments: Authors may acknowledge people, organizations, and financial supporters in this section.

2.2.12 References and Notes

Vancouver style is used in the reference list and citations. Manuscripts available on Web with a DOI number are considered published. In-text citations should be given superscript numbers according to the order in the manuscript. Ensure that references that are included in the text in the correct numerical order, corresponding to the sequence of references list. Footnotes are not used. Begin your reference list on a new page and title it 'REFERENCES'. Use Arabic numerals (1, 2, 3, 4, 5, 6, 7, 8, 9) as superscripts in the text. Abbreviate journal titles in the style used in the NLM Catalog. Check the reference details against the actual source – you are indicating that you have read a source when you cite it. Years of the references should not be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication. *The use of a DOI URL at the end of the reference is strongly recommended and should be embedded as a hyperlink.*

2.2.12.1 For printed articles

• Article with 1-6 authors:

Author AA, Author BB, Author CC, Author DD. Title of the article. Abbreviated title of journal, Year;volume number(issue number):page numbers.

Sahin Z, Ertas M, Berk B, Biltekin SN, Yurttas L, Demirayak S. Studies on non-steroidal inhibitors of aromatase enzyme; 4-(aryl/heteroaryl)-2-(pyrimidin-2-yl)thiazole derivatives. Bioorg Med Chem, 2018;26(8):1986-1995. Doi:10.1016/j.bmc.2018.02.048

** It is recommended to hyperlink the DOI in the reference section by embedding the link in the DOI number provided.*

• Article with more than 6 authors:

Author AA, Author BB, Author CC, Author DD, Author EE, Author FF, et al. Title of the article. Abbreviated title of journal, Date of publication YYYY;volume number(issue number):page numbers.

2.2.12.2 For electronic journal articles

Author AA, Author BB, Author CC, Author DD, Author EE, Author FF. Title of article. Abbreviated title of Journal [Internet], Year of publication;volume number(issue number):page numbers. Available from: URL DOI

2.2.12.3 For books and book chapters

Books: a) Print book or b) Electronic book

a) Author AA. Title of book. Edition. Place of Publication: Publisher; Date.

b) Author AA, Author BB. Name of the book [Internet]. Edition number (if not the first edition). Publication city, publication country: Publisher; year [cited date]. Available from: URL.

Book chapters: a) Print book chapter or b) Electronic book chapter

a) Author AA. Title of the book chapter. In: Editor AA, Editor BB, editors. Title of the book. Publication city, publication country: Publisher; year. p. page numbers.

b) Author AA. Title of the book chapter. In: Editor AA, Editor BB, editors. Title of the book [Internet]. Publisher; year. p. page numbers. Available from: URL. Accessed date: [accessed date].

2.2.12.4 For theses and dissertations

Author AA. Title of the thesis. [Doctoral Thesis/Master's Thesis]. City: Name of the University; Year.

2.2.12.5 For conference or symposium reports

Name of the Organization. Title of the report. In: Name of the symposium or conference; Year Month Day(s); City, Country. Publisher; Year.

2.2.12.6 For guidelines

Guildelines: a) Print guidelines or b) Electronic guidelines

a) Organization or Author(s). Title of the guideline. Edition (if applicable). Place of publication: Publisher; Year of publication. Total number of pages (if applicable).

b) Organization or Author(s). Title of the guideline [Internet]. Edition (if applicable). Place of publication: Publisher; Year of publication [cited date]. Available from: URL.

2.2.13 Tables

Table titles should not be abbreviated exp. tab. is not acceptable. It should be written as; Table 1. Tables should be centered, while table captions should be justified. When writing the table title, "**Table 1.**" should be in bold, followed by the caption in regular font.

Tabulation of experimental results is encouraged when this leads to more effective presentation or to more economical use of space. Tables should be numbered consecutively in order of citation in the text with Arabic numerals. The font used in tables should be Times New Roman, size 10. Footnotes in tables should be given as lowercase size 9 letter designations and cited in the tables as superscripts. The sequence of letters should proceed by row rather than by column. If a reference is cited in both table and text, insert a lettered footnote in the table to refer to the numbered reference in the text. Each table must be provided with a descriptive title that, together with column headings, should make the table self-explanatory. Titles and footnotes should be on the same page as the table. Table captions should be written on the top. Tables may be created using a word processor's text mode or table format feature. The table format feature is preferred. Ensure each data entry is in its own table cell. If the text mode is used, separate columns with a single tab and use a return at the end of each row. Tables may be inserted in the text where first mentioned or may be grouped after the references.

2.2.14 Figures, Schemes/Structures, and Charts

Figure titles should not be abbreviated exp. fig. is not acceptable. It should be written as; Figure 1. Figures should be centered, while figure captions should be justified. When writing the figure title, "**Figure 1.**" should be in bold, followed by the caption in regular font.

Figure captions: A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used. Figure captions should be written on the bottom.

The use of illustrations to convey or clarify information is encouraged. Structures should be produced with the use of a drawing program such as ChemDraw. Authors using other drawing packages should, in as far as possible, modify their program's parameters so that they conform to ChemDraw pref-

erences. Remove all color from illustrations, except for those you would like published in color. Illustrations may be inserted into the text where mentioned or may be consolidated at the end of the manuscript. If consolidated, legends should be grouped on a separate page(s). Include as part of the manuscript file.

To facilitate the publication process, please submit manuscript graphics using the following guidelines:

1. The preferred submission procedure is to embed graphic files in a Word document. It may help to print the manuscript on a laser printer to ensure all artwork is clear and legible.

2. Additional acceptable file formats are: TIFF, PDF, EPS (vector artwork) or CDX (ChemDraw file). When submitting individual graphic files and embedding them in a Word document, make sure to name the files according to their graphic function (e.g., Scheme 1, Figure 2, Chart 3), not the scientific name.

EPS files: Ensure that all fonts are converted to outlines or embedded in the graphic file. The document settings should be in RGB mode. NOTE: While EPS files are accepted, the vector-based graphics will be rasterized for production. Please see below for TIFF file production resolutions.

3. TIFF files (either embedded in a Word doc or submitted as individual files) should have the following resolution requirements:

- Black & White line art: 1200 dpi

- Grayscale art (a monochromatic image containing shades of gray): 600 dpi

- Color art (RGB color mode): 300 dpi

- The RGB and resolution requirements are essential for producing high-quality graphics within the published manuscript. Graphics submitted in CMYK or at lower resolutions may be used; however, the colors may not be consistent and graphics of poor quality may not be able to be improved.

- Most graphic programs provide an option for changing the resolution when you are saving the image. Best practice is to save the graphic file at the final resolution and size using the program used to create the graphic.

4. Graphics should be sized at the final production size when possible. Single column graphics are preferred and can be sized up to 240 points wide (8.38 cm). Double column graphics must be sized between 300 and 504 points (10.584 and 17.78 cm's). All graphics have a maximum depth of 660 points (23.28 cm) including the caption (please allow 12 points for each line of caption text).

Consistently sizing letters and labels in graphics throughout your manuscript will help ensure consistent graphic presentation for publication.

2.2.15 Image Manipulation

Images should be free from misleading manipulation. Images included in an account of research performed or in the data collection as part of the research require an accurate description of how the images were generated and produced. Apply digital processing uniformly to images, with both samples and controls. Cropping must be reported in the figure legend. For gels and blots, use of positive and negative controls is highly recommended. Avoid high contrast settings to avoid overexposure of gels and blots. For microscopy, apply color adjustment to entire image and note in the legend. When necessary, authors should include a section on equipment and settings to describe all image acquisition tools, techniques and settings, and software used. All final images must have resolutions of 300 dpi or higher. Authors should retain unprocessed data in the event that the editors request them.

2.3 Specialized Data

2.3.1 Biological Data

Quantitative biological data are required for all tested compounds. Biological test methods must be referenced or described in sufficient detail to permit the experiments to be repeated by others. Detailed descriptions of biological methods should be placed in the experimental section. Standard compounds or established drugs should be tested in the same system for comparison. Data may be presented as numerical expressions or in graphical form; biological data for extensive series of compounds should be presented in tabular form.

Active compounds obtained from combinatorial syntheses should be resynthesized and retested to verify that the biology conforms to the initial observation. Statistical limits (statistical significance) for the biological data are usually required. If statistical limits cannot be provided, the number of determinations and some indication of the variability and reliability of the results should be given. References to statistical methods of calculation should be included.

Doses and concentrations should be expressed as molar quantities (e.g., mol/kg, μmol/kg, M, mM). The routes of administration of test compounds and vehicles used should be indicated, and any salt forms used (hydrochlorides, sulfates, etc.) should be noted. The physical state of the compound dosed (crystalline, amorphous; solution, suspension) and the formulation for dosing (micronized, jet-milled, nanoparticles) should be indicated. For those compounds found to be inactive, the highest concentration (*in vitro*) or dose level (*in vivo*) tested should be indicated.

If human cell lines are used, authors are strongly encouraged to include the following information in their manuscript:

· the cell line source, including when and from where it was obtained;

· whether the cell line has recently been authenticated and by what method;

· whether the cell line has recently been tested for mycoplasma contamination.

2.3.2 Purity of Tested Compounds

Methods: All scientifically established methods of establishing purity are acceptable. If the target compounds are solvated, the quantity of solvent should be included in the compound formulas. No documentation is required unless asked by the editors.

Purity Percentage: All tested compounds, whether synthesized or purchased, should possess a purity of at least 95%. Target compounds must have a purity of at least 95%. In exceptional cases, authors can request a waiver when compounds are less than 95% pure. For solids, the melting point or melting point range should be reported as an indicator of purity.

Elemental Analysis: Found values for carbon, hydrogen, and nitrogen (if present) should be within 0.4% of the calculated values for the proposed formula.

2.3.3 Confirmation of Structure

Adequate evidence to establish structural identity must accompany all new compounds that appear in the experimental section. Sufficient spectral data should be presented in the experimental section to allow for the identification of the same compound by comparison. Generally, a listing of 1H or 13C NMR peaks is sufficient. However, when the NMR data are used as a basis of structural identification, the peaks must be assigned.

List only infrared absorptions that are diagnostic for key functional groups. If a series contains very closely related compounds, it may be appropriate merely to list the spectral data for a single representative member when they share a common major structural component that has identical or very similar spectral features.

3. Submitting the Manuscript

3.1 Communication and Log in to Author's Module

All submissions to Acta Pharmaceutica Sciencia should be made by using e-Collittera (Online Article Acceptance and Evaluation) system on the journal main page (www. actapharmsci.com).

3.2 Registration to System

It is required to register into the e-Collittera system for the first time while entering by clicking the "Create Account" button on the registration screen and the filling out the opening form with real information. Completing all the required fields in the form is essential for a successful registration. Without providing the necessary information, the registration process will not be able to proceed.

After the registration, a "Welcome" email is sent to the user by the system, reminding your user name and password. Authors are expected to return to the entry screen and log in with their user name and password for the submission. Please use only English characters while determining your username and password.

If you already registered into the e-Collittera system and forget your password, you should click on "Forgot My Password" button and your user name and password will be e-mailed to you in a short while.

3.3 Submitting a New Article

The author module's main page is divided into various sections that display the status of your manuscripts in progress. Authors can initiate a new submission by clicking the "New Manuscript" button, which involves a series of nine consecutive levels. In the first 7 levels, information such as the article's kind, institutions, authors, title, summary, keywords etc. are asked respectively as entered. Authors can move back and forth while the information is saved automatically. If the ~~is~~ transaction is discontinued, the system will move the new submission to the "Partially Submitted Manuscripts" part and the transaction can be continued from there.

3.3.1 Sort of Article Authors should first select the type of article from the drop-down menu.

Warning. If "Return to Main Page" button is clicked after this level, the article will be automatically assigned as "Partially Submitted Manuscripts".

3.3.2 Authors The authors' surnames, names, institutional information appear as entered order in the previous page. Filling all e-mail addresses are required. Institutional information is available in Manuscript Details table at the top of the screen. After filling all required fields, you may click the Continue button.

3.3.3 Title should be English, explaining the significance of the study. If the title includes some special characters such as alpha, beta, pi or gamma, they

can easily be added by using the Title window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text.

Warning. No additions to cornered parenthesis are allowed. Otherwise, the system will not be able to show the special characters.

3.3.4 Abstract The summary of the article should be entered to the Abstract window. There must be an English summary for all articles and the quantity of words must be not more than 200. If special characters such as alpha, beta, pi or gamma are used in summary, they can be added from the Abstract window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text. The abstract of the articles is accessible for the reviewers; so, you should not add any information related to the institutions and authors in this summary part. Otherwise, the article will be returned without evaluation. Authors will be required to comply with the rules.

Warning. No additions to cornered parenthesis are allowed. Otherwise, the system will not be able to show the special characters.

3.3.5 Keywords There must be five words to define the article at the keywords window, which will be diverged with commas. Authors should pay attention to use words, which are appropriate for "Medical Subjects Headings" list by National Library of Medicine (NLM).

3.3.6 Cover Letter If the submitting article was published as a thesis and/or presented in a congress (or elsewhere); all information about the thesis, presented congress (or elsewhere) should be delivered to the editor and must be mentioned in the "Cover Letter" section.

3.3.7 Adding Article This process consists of four different steps, beginning with the uploading of the article into the system. The "Browse" button under the "Choose a file to upload" tab is used to reach the article file. After finding and selecting the article file, you may click "Choose File" and the file will be uploaded.

Second step is to select a file category. Options are: Main Document, Black and White Figure, Color Figure and Video.

The explanation of the files (e.g., Figure 1, Full Text Word File, supplements etc.) should be added on the third step. The last step is submitting the prepared article into the system. Then, click the "Download" button.

Reminder: If the prepared article includes more than one file (such as main document, black and white figure, video), the transaction will be continued by starting from the first step. The image files must be in the previously defined format. After all required files are added, "Continue" button should be clicked. All details and features of the article can be reached from the Article Information page.

This page is the last step of the transaction which ensures that the entered information is controlled.

3.3.8 Your Files After submitting the article you may find all information related to the article under "Your Files" window.

File Information: This window includes file names, sizes, forming dates, categories, order numbers and explanations of files. The details about the files can be reached by clicking on "Information" button.

If you click on "Name of File", the file download window will be opened to reach the copy of the file in system.

File Download: This window submits two alternatives; one of them is to ensure that the file can be opened on a valid site and the second is to ensure that the submitted file can be downloaded to the computer.

Opening the Category part on the fourth column can change the category of the file.

Opening the Order column on the fifth column can change the order of the file.

The file can be deleted by clicking on Delete button on the last column. Before deleting, system will ask the user again if it is appropriate or not.

3.3.9 Completing the Submission Last level is submitting the article and the files into the system. Before continuing the transaction; it is important to control the Article Information window so that you can easily go back and make any necessary corrections using the "Previous" button. Otherwise, by clicking the Send the Article button, you will successfully finalize the transaction.

3.3.10 Page to Follow the Article The Main Page tab of the author ensures possibility to follow the article. This page consists of three different parts; some information and bridges related to the sent articles, revision required articles and the articles that are not completed to be sent.

3.3.11 Articles Not Yet Completed for Submission After the sending transaction was started, if article is not able to continue until the ninth step or could not be sent due to technical problems shown at this part, you may find

the information such as the article's number which is assigned by system, title and formation date. You may delete the articles by using Delete button on the right column.

3.3.12 Articles that Require Revision Articles, which were evaluated by the reviewer and accepted by the editor with revision, passes to Waiting for Revision tab.

The required revisions can be seen in "Notes" part by clicking the articles title.

In order to send any revision, Submit Revision button on the last column should be clicked. This connection will take the author to the first level of Adding Article and the author can complete the revision transaction by carrying out the steps. All changes must be made in the registered file, and this changed file must be resent. In addition to the revised manuscript, author's most efficacious replies relating to the changes must be submitted in "Cover Letter" part.

If the is transaction is discontinued, the system moves the revised article to Submitted Manuscripts tab and the transaction can be continued from there.

After the transaction was completed, the system moves the revised article to "Submitted Manuscripts" part.

3.3.13 Submitted Manuscripts Information related to articles can be followed through the Submitted Manuscripts tab. Here you can find the information such as the article's number assigned by system, title, sending date and transaction situation. The Manuscript Details and summary files can be reached by clicking the title of the article and the Processing Status part makes it possible to follow the evaluation process of the article.

Article Review Process

Articles uploaded to the Manuscript submission system are checked by the journal administration for format consistency and similarity rate which is required to be less than 20%. Then sent to the chief editor if found appropriate.

Articles that are not suitable are sent back to the author for correction and re-submission (sent back to the author). Studies that have not been prepared using the draft for submitting to Acta Pharmaceutica Sciencia "acta_msc_tmp" and that have not been adapted in terms of format, will be directed to the editor-in-chief, after the 3rd time, by giving the information that "the consistency requirements have not been met".

The manuscripts sent to the chief editor will be evaluated and sent to the "language and statistics editor" if deemed necessary.

Studies found appropriate after language and statistics editor will be sent to field editors. If the field editor does not deem it appropriate after evaluating the article scientifically, he/she will inform the editor-in-chief of its negative comments, otherwise, at least two independent reviewers' comments will be asked.

Authors should consider that this process may take time because of the reviewer assignments and acceptance for review may take time for some cases.

Our review system is double-blind. The editor, who evaluates according to the comments of the reviewers, submits his/her comment and suggestion to the editor-in-chief. In this way, the article receives a decision of either acceptance, rejection, or revision. After all the revision process, the editor submits his/her final opinion to the editor-in-chief. The editor-in-chief conveys his or her final decision to the author. After the accepted articles are subjected to the final control by the journal and the corresponding author, the article starts to be included in the "accepted papers" section by giving the inactive DOI number. When the article is placed in one of the following issues, the DOI number will be activated and displayed in the "current issue" section on the homepage of the journal.

EDITORIAL

Do current pharmacovigilance systems enable early public protection?

Editorial Article

Semra ŞARDAŞ*

Istinye University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Istanbul, Türkiye

Regional Pharmacovigilance (PV) systems, coordinated by regulatory authorities, collect and analyze safety data, primarily through spontaneous reporting (SRS) remains a cornerstone of PV. However, underreporting, delays, and limited real-time analysis restrict their public health impact. Adverse drug reactions (ADRs) significantly impact healthcare systems, leading to increased hospitalization rates and costs.

Drug toxicity determination is a main step in drug design and involves identifying the adverse events (AEs) of chemicals on humans, plants, animals, and the environment. Pre-clinical evaluations are a necessity for preventing toxic drugs from reaching clinical trials. Despite this, high toxicity is still a major contributor to drug failure accounting for two-thirds of post-market drug withdrawals and for one-fifth of failures during clinical trials. Thus, accurate toxicity estimates are necessary for ensuring drug safety, and can help reduce the cost and development time of bringing new drugs to market.

Animal studies have historically been the most conventional approach taken to assess toxicity. However, these studies are constrained by cost, time, and ethical considerations. Numerous computational, in silico, approaches have demonstrated utility in estimating the toxicity of drug candidates. These approaches predict toxicity by evaluating various features of the drug and include target-based predictions and Quantitative Structure- Activity Relationships (QSAR).

With the growing adoption of Artificial Intelligence (AI) in healthcare, machine learning models offer promising solutions for ADR prediction. While drug ap-

* Corresponding author: Sema ŞARDAŞ
E-mail: semra.sardas@istinye.edu.tr
ORCID: 0000-0001-5456-8636

proval requires rigorous clinical testing, many adverse reactions only become apparent after widespread public use.

When we look at the global PV practices that emphasizes mandatory reporting by manufacturers and healthcare institutions in Europe, the European Medicines Agency (EMA) and EudraVigilance which is the EU's central database for adverse event reporting facilitates early detection of safety signals across EU member states. The PV Risk Assessment Committee (PRAC) plays a key role in assessing and managing risks. In the United States Food and Drug Administration (FDA) collects ADRs through MedWatch and the FDA Adverse Event Reporting System (FAERS). Japan's Pharmaceuticals and Medical Devices Agency (PMDA) operates a robust PV framework, which includes reexamination and reevaluation of drugs based on post-marketing data.

WHO and the Uppsala Monitoring Centre (UMC) manages VigiBase, the global database of individual case safety reports (ICSRs). It promotes harmonization of safety data and collaborates with more than 130 countries to enhance global PV practices.

The question is; Do Current Systems Enable Early Public Protection?

While existing systems provide a vital safety net, they often fail to ensure early intervention for several reasons;(i) Underreporting: It is estimated that less than 10% of all ADRs are reported. (ii) Latency: Signal detection and regulatory action can take months or even years. (iii) Bias and Incompleteness: Many reports lack crucial information, and rare or long-term side effects are easily missed. (iv) Public Communication Gaps: Safety updates may not effectively reach or influence public behavior. Consequently, while global PV systems are essential, they do not consistently enable the public to take early preventive measures before facing adverse effects.

AI can significantly improve PV. For example, by incorporating the present large datasets rapidly to AI and detect complex tools for active surveillance. Such contributions would not only reduce health risks but also place AI-driven PV innovation for early signal detection and patient risk profiling. By the help of this Predictive Modeling, AI systems can forecast which patient populations are at higher risk of specific ADRs for early signal detection and patient risk profiling. Privacy and Consent in using real-world data for PV must respect patient confidentiality

In conclusion PV systems around the world have made remarkable progress in identifying and managing drug-related risks. However, to empower the public and minimize harm proactively, these systems must evolve beyond traditional

spontaneous reporting. AI offers promising tools to enhance real-time monitoring, predictive risk assessment, and effective communication by developing ethical and transparent AI solutions that protect public health and align with global PV regulations. Since, PV is essential to post-marketing surveillance of pharmaceuticals, ensuring that the benefits of medicines outweigh their risks (AI) will improve signal detection, enhance real-time surveillance, and support risk minimization by presenting opportunities and challenges for public health which is yet unclear. Therefore, AI can have an emerging role of in PV for adverse drug reaction prediction.

REFERENCES

Raies AB, Bajic VB. *In silico* toxicology: computational methods for the prediction of chemical toxicity. WIREs Comput Mol Sci, 2016;6(2):147-172. Doi: 10.1002/wcms.1240

Onakpoya IJ, Heneghan CJ, Aronson JK. Worldwide withdrawal of medicinal products because of adverse drug reactions: a systematic review and analysis. Crit Rev Toxicol, 2016;46(6):477-489. Doi: 10.3109/10408444.2016.1149452

Segall MD, Barber C. Addressing toxicity risk when designing and selecting compounds in early drug discovery. Drug Discov Today, 2014;19(5):688-693. Doi: 10.1016/j.drudis.2014.01.006

Anna OB, Alexandre Y, Nicholas PT. Artificial intelligence for drug toxicity and safety. Trends Pharmacol Sci, 2019;40(9):624-635. Doi: 10.1016/j.tips.2019.07.005

REVIEW ARTICLE

Acta Pharm. Sci. Vol 63:(3), 2025
Review article
DOI: 10.23893/1307-2080.APS6331

Neuropsychiatry of psychological resilience: An overview

Ipek EROGLU[1,2*], Caglar MACIT[3], Ulas Basar GEZGIN[4], Gulden Zehra OMURTAG[5], Asena HIMMETOGLU[6], Esra ATASOY SUBASI[4]

1 Istanbul Medipol University, School of Pharmacy, Pharmacy Business Administration Department, Istanbul, Türkiye
2 Istanbul Esenyurt University, Institute of Graduate Studies, Hospital and Health Institutions Management Master Program, Istanbul, Türkiye
3 Istanbul Medipol University, School of Pharmacy, Pharmacology Department, Istanbul, Türkiye
4 Istanbul Galata University, Faculty of Arts and Social Sciences, Psychology Department, Istanbul, Türkiye
5 Istanbul Medipol University, School of Pharmacy, Toxicology Department, Istanbul, Türkiye
6 Bahçeşehir University, Graduate Education Institute, Neuroscience Doctorate Program, Istanbul, Türkiye

ABSTRACT

Psychological resilience is the operational software of competencies that enables us to cope positively with life's drawbacks. As with all competencies and associated skills, the level of psychological resilience may vary among individuals. This research addresses the neuropsychiatric dimension of psychological resilience and discusses its potential applications in improving public health and pharmacy practices. To conduct a modeling study to identify, diagnose and disseminate to the society the individual characteristics that constitute the building blocks of a resilient society that will cope with pandemics, climate change, wars, waves of migration, inability to meet the basic needs of the increasing population and infrastructure problems, global economic crisis, technological challenges, digital transformation pressure, disruptive changes, and the VUCA (volatility, uncertainty, complexity, ambiguity) world environment, led us to a detailed literature review. Neuropsychiatry of psychological resilience is exemplified by recent global developments, biological underpin-

* Corresponding author: İpek EROĞLU
E-mail: ipek.eroglu@medipol.edu.tr
ORCIDs:
Ipek EROGLU: 0000-0002-6980-4192
Caglar MACIT: 0000-0002-5532-2395
Ulas Basar GEZGIN: 0000-0002-6075-3501
Gulden Zehra OMURTAG: 0000-0002-2018-9619
Asena HIMMETOGLU: 0000-0002-9367-798X
Esra ATASOY SUBASI: 0000-0003-3528-0850
(Received 12 Feb 2025, Accepted 6 May 2025)

nings, genetic variations, clinical perspectives, and developmental aspects.

Keywords: psychological resilience, neuropsychiatry, biological basis of resilience, genetics of resilience, pharmacist-led resilience

INTRODUCTION

Psychological resilience, often described as the "operational software" of human competencies, enables individuals to adapt positively and maintain well-being amidst adversity. This capacity has drawn significant attention in neuropsychiatry and mental health, especially given global stressors like the Corona Virus Disease-19 (COVID-19) pandemic, climate crises, and socio-political upheavals[1]. These challenges underscore the need to understand resilience both individually and as a societal asset.

Resilience research has evolved from focusing on innate traits to encompassing dynamic, multifaceted constructs shaped by biological, psychological, and social factors. Definitions range from "positive adaptation despite adversity" to "dynamic processes enabling individuals to thrive," reflecting the integration of neurobiological, genetic, and environmental perspectives. Foundational theories include Michael Rutter's (1987) protective factors framework, Ann Masten's (2001) "ordinary magic," and Michael Ungar's (2011) socio-ecological model emphasizing context and culture[2-4].

The neuropsychiatric dimension of resilience reveals roles for neural networks, the HPA axis, genome-wide association studies (GWAS), and genetic variations like 5-HTTLPR polymorphisms[1]. However, gaps persist, including limited longitudinal and cultural research, particularly in low- and middle-income countries. Promising digital interventions and strategies like mindfulness require robust validation across diverse populations. Additionally, there is growing interest in how resilience research can inform pharmacy practices, particularly in patient care, medication adherence, and mental health support. This review synthesizes global studies conducted from 1980 to 2024, identifying gaps and proposing future directions for resilience research.

METHODOLOGY

Search strategy

A systematic review was conducted to assess the neuropsychiatric dimensions of psychological resilience. PubMed, PsycINFO, Scopus, and Web of Science were searched for peer-reviewed articles published from January 1980 to December 2024. Keywords included "psychological resilience,"

"neuropsychiatry," "biological basis of resilience," "genetics of resilience" and "pharmacist-led resilience." Boolean operators (AND, OR) refined the search strategy, ensuring diverse perspectives were included. Filters for language (English) and article type (empirical studies, systematic reviews, meta-analyses) were applied to enhance relevance.

Inclusion and exclusion criteria

Studies on resilience's neuropsychiatric aspects, including biological, genetic, or clinical dimensions, feature empirical research, systematic reviews, and meta-analyses were included and grey literature, non-peer-reviewed studies, and conference abstracts, articles not available in English and studies unrelated to psychological resilience were excluded.

Screening process

Following the PRISMA 2020 flow diagram, a total of 1,200 articles were identified. After duplicate removal (n=300), 900 articles underwent title and abstract screening, excluding 500 irrelevant studies. Full-text reviews were conducted for 400 articles, resulting in 85 studies meeting all criteria.

Quality assessment

Methodological rigor was evaluated using the Critical Appraisal Skills Program (CASP) checklists. Studies with robust designs, adequate sample sizes, and appropriate statistical analyses were included. Weak studies were excluded to maintain review integrity.

Data extraction and analysis

Data were systematically extracted using a template to capture objectives, methodologies, findings, and limitations, with thematic synthesis categorizing biological mechanisms, genetics, clinical implications, and trends.

Ethical considerations

This review adhered to ethical guidelines for systematic research. All studies included were previously published and publicly available, ensuring compliance with ethical standards. No direct data collection involving human, or animal subjects was undertaken.

Limitations

Non-English articles and grey literature were excluded. This exclusion may have resulted in the omission of findings from diverse cultural or socio-economic contexts. Reliance on published studies introduces potential publication bias, warranting cautious interpretation.

RESULTS and DISCUSSION

Studies on psychological resilience

Resilience research lacks a definitive consensus on its conceptualization. Studies in this field are categorized into three models: the stress-resilience model, the biopsychosocial model, and the dynamic systems theory[5]. Research on resilience dates to the 1970s, with significant contributions shaping its development. Michael Rutter pioneered resilience research, emphasizing protective factors in reducing the impact of risk factors[2]. Norman Garmezy identified factors that enable individuals to thrive amidst adversity[6]. Ann Masten introduced the concept of "ordinary magic," highlighting resilience as part of normal life processes[3]. Emmy Werner's longitudinal study demonstrated that high-risk children could achieve positive outcomes through protective factors[7]. Suniya Luthar examined the interplay of risk and protective factors[8], while Michael Ungar developed a socio-ecological model emphasizing the role of context and culture[4].

Psychological resilience answers why some individuals experience trauma after events like earthquakes, while others adapt and recover. Despite numerous definitions, operational consensus remains elusive[9-10]. Resilience broadly refers to the ability to adapt positively to adversity, manage stress, and recover from trauma[11-13]. It involves dynamic processes fostering mental health and adaptation throughout life[14]. Positive psychology underpins resilience, focusing on human strengths like happiness and flourishing[15].

Resilience measurement tools included many scales[16-18]. Research on resilience surged during the COVID-19 pandemic, linking it to mental health and leading to resilience-building interventions[19-21]. Applications extend to military training[22], health contexts like cancer[23], and sports performance[24].

Trauma does not always result in post-traumatic stress disorder (PTSD) and can sometimes lead to post-traumatic growth, often linked to psychological resilience[25]. However, it was found that women exposed to physical, verbal, or sexual violence showed high post-traumatic growth but lower-than-expected resilience, with no association found between resilience and the type of violence[26]. A study defined resilience as a capacity, process, and outcome[27], while a different study described it as the ability to resist, bounce back from, or grow through stressors[28], which may explain Arabaci and colleagues' findings[26].

Resilience negatively correlates with psychiatric symptoms[29] and positively with social support, adaptive coping, and optimism[30]. Factors like secure attachment and purpose in life enhance resilience[12]. Men are reportedly more resilient than women[31]. Resilience also varies by age, with older adults excel-

ling in emotional regulation and problem-solving, while younger adults rely more on social support[32].

In a study, it was concluded that individuals with mood disorders are less resilient compared to psychologically normal participants[33]. Similarly, a different study found a medium-level negative correlation between resilience and depression[34]. A previous study observed that individuals with major depressive disorders in remission show lower resilience compared to the general population[35]. Additionally, a study provided empirical evidence of a positive association between psychological resilience and positive coping styles[36].

Resilience ensures well-being and supports self-confidence, healthy relationships, and achievement[37]. Researchers redefined resilience as a complex process involving pre-adversity functioning, challenging circumstances, post-adversity outcomes, and predictors. This multi-level process includes individual, family, and community factors, yet family and community resilience remain underexplored[38]. Resilience is a complex phenotype shaped by personality, mood, self-image, and adaptability[39-40]. Traits like optimism, attachment, intellectual functioning, and effective coping facilitate adaptation to adversity[41].

Neurologically, the medial prefrontal cortex and amygdala play key roles in stress and trauma reduction[41]. The left parietal lobule is linked to emotional control[42]. The central executive network (CEN) and salience network (SN) integrate sensory and emotional information for cognitive control and emotional regulation[43,44]. The hypothalamic-pituitary-adrenal (HPA) axis regulates stress via cortisol secretion, and its malfunction can reduce resilience[37].

Genetic factors also influence resilience. Serotonergic and dopaminergic systems regulate mood and stress. Genetic variations in dopamine and serotonin pathways impact resilience[45]. The NRG1 gene's rs10503920 polymorphism correlates with resilience, while serotonin (SERT) and dopamine transporter (DAT1) genes contribute to individual resilience differences[39,46]. Genetic variability plays a crucial role in determining vulnerability to stress and resilience. Monoamine oxidase A (MAOA) and the serotonin transporter gene (5-HTT) influence susceptibility to depression and stress-related behaviors[41]. Specific genetic combinations, such as the two long alleles of the 5-HTT gene, are linked to higher resilience[41]. Contributing genes include the serotonin transporter gene (SLC6A4), brain-derived neurotrophic factor (BDNF), and catechol-O-methyltransferase (COMT), which impact neurotransmitter function and stress responses[47]. Epigenetic mechanisms, such as DNA methylation and histone modification, mediate gene expression in response to environmental stress, affecting biological pathways related to emotional regulation[48]. Gene-

environment interactions further shape resilience; early-life adversity may amplify vulnerability in genetically predisposed individuals, while supportive environments can mitigate these effects[49].

A previous study highlighted the potential for identifying genetic markers for PTSD to develop early treatments, focusing on neurotransmitters like serotonin, dopamine, and cortisol[50]. Studies have observed increased medial prefrontal activity in at-risk siblings of bipolar patients, suggesting a compensatory resilience mechanism[41]. Furthermore, changes in beta coherence patterns in patients with Major Depressive Disorder (MDD) indicate impairments in emotional regulation and heightened psychological vulnerability[42]. Researchers explored resilience in children, noting short-lasting white matter changes after trauma[51]. Their recent work links resilience to resting-state brain connectivity, suggesting protective effects during concussion recovery[52].

Mindfulness practices enhance the central executive and salience networks, promoting cognitive control and emotional regulation[53]. They regulate the HPA axis, lower cortisol levels, and support neurotransmitter balance, enhancing resilience[54,55]. Mindfulness also induces beneficial epigenetic changes, potentially modifying gene expression associated with stress regulation[56]. By fostering adaptive coping strategies, mindfulness reduces the impact of genetic predispositions to stress[57].

Brain regions such as the prefrontal cortex and amygdala are closely associated with resilience, as identified through functional MRI and PET scans. Twin studies and genome-wide association studies (GWAS) have explored genetic markers like 5-HTTLPR and BDNF polymorphisms. Psychometric tools such as the Connor-Davidson Resilience Scale (CD-RISC) and the Resilience Scale for Adults (RSA) are commonly used, though cultural adaptability remains a challenge. High-resolution imaging and large genetic datasets enhance accuracy, but limitations include a lack of longitudinal designs and cultural biases in assessment tools.

Case studies illustrate resilience across diverse contexts. For example, during the COVID-19 pandemic, an anesthesiologist-maintained resilience through social networks, including managerial and peer support, which helped manage work stress[58]. In another study, resilience emerged from internal and external factors like benefit finding, empathy, self-belief, and gratitude in individuals facing adversities such as domestic violence, health challenges, and loss[59]. A coastal community repeatedly impacted by climate change achieved resilience through flood defenses, education, and social capital, highlighting the importance of adaptive capacity and mental health support[60]. Economically, small

businesses survived financial hardships by diversifying products and building strong stakeholder relationships, driven by leadership resilience, innovation, and stress management[61].

The biological underpinnings of resilience include neural networks like the Central Executive Network (CEN) and Salience Network (SN), which are critical for emotional regulation. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, however, lowers resilience. Genetic factors, particularly variations in serotonin transporter genes (5-HTTLPR), influence stress resilience. Epigenetic modifications shaped by early adversity also affect resilience.

Clinically, resilience-enhancing interventions, including mindfulness-based therapies, show promise but require further evidence. Digital tools like mobile apps and online programs are gaining traction for resilience training, offering accessible resources for stress management. Cultural sensitivity is crucial for tailoring interventions to diverse populations.

Strategies to enhance resilience include expressive writing for emotional processing, gradual fear exposure to build competence, and self-compassion to reduce self-criticism. Techniques like mindfulness, meditation, physical activity, and cognitive restructuring improve emotional regulation and stress relief. However, integrating multiple strategies should be approached cautiously, as some individuals may struggle with unfamiliar practices like mindfulness and self-distancing, potentially increasing frustration or anxiety and reducing adherence. General perspectives for studies about psychological resilience were given in Table 1.

Table 1. Perspectives about psychological resilience

Author(s)	Year	Focus	Key Findings
Rutter	1987	Protective Factors	Protective factors buffer against adversity
Masten	2001	Ordinary Magic	Resilience is common and can be nurtured
Southwick et al.	2014	Models of Resilience	Multiple dynamic models proposed
Maul et al.	2019	Neurobiology of Resilience	HPA axis, amygdala, and genetic factors crucial
Recent Studies	2020-2024	Mindfulness and Genetics	Mindfulness enhances CEN, epigenetic modulation

Pharmacists have been shown to play a vital role in enhancing psychological resilience at both the individual and community levels. Through their close interaction with patients, pharmacists contribute to the provision of emotional

support, health literacy, medication adherence, and stress management, which are critical elements for fostering resilience. By providing clear guidance on treatment plans, addressing concerns empathetically, and offering preventive health advice, pharmacists help patients feel more empowered and better equipped to manage chronic illnesses, mental health conditions, and acute crises. Furthermore, community pharmacists, who frequently function as the primary healthcare access point, contribute to the reduction of healthcare access barriers, thereby promoting social support – a recognised protective factor in resilience development[28]. Interventions led by pharmacists, encompassing medication therapy management, mental health first aid, and counselling for chronic disease management, have been demonstrated to reduce patient anxiety and enhance coping mechanisms[19]. The integration of mindfulness-based interventions and psychoeducational programs into pharmacy practice has the potential to enhance resilience among vulnerable populations[51]. The multifaceted role of the pharmacist has been demonstrated to enhance patient resilience on an individual basis, as well as augmenting the community's broader capacity to contend with and recuperate from public health challenges.

Limitations

Significant gaps in resilience research include the scarcity of longitudinal studies crucial for understanding causal relationships and resilience progression[62]. Factors like age, gender, ethnicity, and education affect resilience, with poverty reducing it[63]. Genetic influences, such as 5-HTTLPR and BDNF, are understudied despite exceptions like previous researches[49,64]. Additionally, research in low- and middle-income countries and digital interventions requires further exploration. Moreover, pharmacy-specific resilience interventions remain underexplored.

In conclusion, psychological resilience is crucial for mental health and adapting to adversity. Electroencephalography (EEG) biomarker-based cognitive retraining enhances resilience and cognitive function, particularly in high-stress environments like space missions[64]. Neurofeedback targeting beta coherence shows promise in improving emotional coping and neural patterns[42]. Resilience stems from biological, genetic, and environmental factors, yet cultural and temporal variations are underexplored. Future research should prioritize longitudinal studies and validate digital resilience training for diverse populations.

STATEMENT OF ETHICS

Not applicable as no human or animal subjects were involved in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare there is no conflict of interest associated with this study.

AUTHOR CONTRIBUTIONS

All authors were equally involved in the literature investigation and preparation of the manuscript draft. All authors read and approved the manuscript.

FUNDING SOURCES

No funding or other financial support was received for this study.

ACKNOWLEDGMENTS

None.

REFERENCES

1. Maul S, Giegling I, Fabbri C, Corponi F, Serretti A, Rujescu D. Genetics of resilience: implications from genome-wide association studies and candidate genes of the stress response system in posttraumatic stress disorder and depression. Am J Med Genet Part B, 2020;183:77-94. Doi: 10.1002/ajmg.b.32763

2. Rutter M. Psychosocial resilience and protective mechanisms. Am J Orthopsychiatry, 1987;57(3):316-331. Doi: 10.1111/j.1939-0025. 1987.tb03541.x

3. Masten AS. Ordinary magic: resilience processes in development. Am Psychol, 2001;56(3):227-238. Doi: 10.1037/0003-066X.56.3.227

4. Ungar M. The social ecology of resilience: addressing contextual and cultural ambiguity of a nascent construct. Am J Orthopsychiatry, 2011;81(1):1-17. Doi: 10.1111/j.1939-0025.2010. 01067.x

5. Southwick SM, Bonanno GA, Masten AS, Panter-Brick C, Yehuda, R. Resilience definitions, theory, and challenges: interdisciplinary perspectives. Eur J Psychotraumatol, 2014;5(1):1-14. Doi: 10.3402/ejpt. v5.25338

6. Garmezy N. Resiliency and vulnerability to adverse developmental outcomes associated with poverty. Am Behav Sci, 1991;34(4):416-430. Doi: 10.1177/0002764291034004003

7. Werner EE, Smith RS. Overcoming the odds: high-risk children from birth to adulthood. New York: Cornell University Press; 1992.

8. Luthar SS. Resilience in development: a synthesis of research across five decades. In: Cicchetti D, Cohen DJ, editors. Developmental psychopathology: risk, disorder, and adaptation. 2nd ed. John Wiley & Sons, Inc; 2006. p. 739-795. Doi: 10.1002/9780470939406.ch20

9. Sisto A, Vicinanza F, Campanozzi LL, Ricci G, Tartaglini D, Tambone V. Towards a transversal definition of psychological resilience: a literature review. Medicina (Kaunas), 2019;55(11):745. Doi: 10.3390/medicina55110745

10. Troy AS, Willroth EC, Shallcross AJ, Giuliani NR, Gross JJ, Mauss IB. Psychological resilience: an affect-regulation framework. Annu Rev Psychol, 2023;74:547-576. Doi: 10.1146/annurev-psych-020122-041854

11. Ghulam A, Bonaccio M, Costanzo S, Bracone F, Gianfagna F, de Gaetano G, Iacoviello L. Psychological resilience, cardiovascular disease, and metabolic disturbances: a systematic review. Front Psychol, 2022;13:817298. Doi: 10.3389/fpsyg.2022.817298

12. Herrman H, Stewart DE, Diaz-Granados N, Berger EL, Jackson B, Yuen T. What is resilience? Can J Psychiatry, 2011;56(5):258-265. Doi: 10.1177/070674371105600504

13. Ran L, Wang W, Ai M, Kong Y, Chen J, Kuang L. Psychological resilience, depression, anxiety, and somatization symptoms in response to COVID-19: a study of the general population in China at the peak of its epidemic. Soc Sci Med, 2020;262:113261. Doi: 10.1016/j.socscimed.2020.113261

14. Rutten BP, Hammels C, Geschwind N, Menne-Lothmann C, Pishva E, Schruers K, van den Hove D, Kenis G, van OJ, Wichers M. Resilience in mental health: linking psychological and neurobiological perspectives. Acta Psychiatr Scand, 2013;128(1):3-20. Doi: 10.1111/acps.12095

15. Vella SLC, Pai NB. A theoretical review of psychological resilience—defining resilience and resilience research over the decades. Arch Med Health Sci, 2019;7(2):233-239. Doi: 10.4103/amhs.amhs_119_19

16. Block J, Kremen AM. IQ and ego-resiliency: conceptual and empirical connections and separateness. J Pers Soc Psychol, 1996;70(2):349-361. Doi: 10.1037/0022-3514.70.2.349

17. Friborg O, Hjemdal O, Rosenvinge JH, Martinussen M. A new rating scale for adult resilience: what are the central protective resources behind healthy adjustment? Int J Methods Psychiatr Res, 2003;12(2):65-76. Doi: 10.1002/mpr.143

18. Connor KM, Davidson JRT. Development of a new resilience scale: the Connor-Davidson Resilience Scale (CD-RISC). Depress Anxiety, 2003;18(2):76-82. Doi: 10.1002/da.10113

19. Hou WK, Tong H, Liang L, Li TW, Liu H, Ben-Ezra M, Goodwin R, Lee TM. Probable anxiety and components of psychological resilience amid COVID-19: a population-based study. J Affect Disord, 2021;282:594-601. Doi: 10.1016/j.jad.2020.12.127

20. Killgore WDS, Taylor EC, Cloonan SA, Dailey NS. Psychological resilience during the COVID-19 lockdown. Psychiatry Res, 2020;291:113216. Doi: 10.1016/j.psychres.2020.113216

21. Song S, Yang X, Yang H, Zhou P, Ma H, Teng C, Chen H, Ou H, Li J, Mathews CA, Nutley S, Liu N, Zhang X, Zhang N. Psychological resilience as a protective factor for depression and anxiety among the public during the outbreak of COVID-19. Front Psychol, 2021;11:618509. Doi: 10.3389/fpsyg.2020.618509

22. Meredith LS, Sherbourne CD, Gaillot SJ, Hansell L, Ritschard HV, Parker AM, Wrenn G. Promoting psychological resilience in the U.S. military. Rand Health Q, 2011;1(2):2.

23. Min JA, Yoon S, Lee CU, Chae JH, Lee C, Song KY, Kim TS. Psychological resilience contributes to low emotional distress in cancer patients. Support Care Cancer, 2013;21(9):2469-2476. Doi: 10.1007/s00520-013-1807-6

24. Sarkar M, Fletcher D. How should we measure psychological resilience in sport performers? Meas Phys Educ Exerc Sci, 2013;17(4):264-280. Doi: 10.1080/1091367X.2013.805141

25. Brooks S, Amlot R, Rubin GJ, Greenberg N. Psychological resilience and post-traumatic growth in disaster-exposed organisations: overview of the literature. BMJ Mil Health, 2020;166(1):52-56. Doi: 10.1136/jramc-2017-000876

26. Arabaci LB, Dikec G, Buyukbayram A, Uzunoglu G, Ozan E. Traumatic growth and psychological resilience status of female victims of violence inpatients in a district psychiatric hospital. Arch Psychiatr Nurs, 2018;32(4):568-573. Doi: 10.1016/j.apnu.2018.03.017

27. Choi KW, Stein MB, Dunn EC, Koenen KC, Smoller JW. Genomics and psychological resilience: a research agenda. Mol Psychiatry, 2019;24(12):1770-1778. Doi: 10.1038/s41380-019-0457-6

28. Den Hartigh RJ, Hill Y. Conceptualizing and measuring psychological resilience: what can we learn from physics? New Ideas Psychol, 2022;66:100934. Doi: 10.1016/j.newideapsych.2022.100934

29. Cuhadar D, Tanriverdi D, Pehlivan M, Kurnaz G, Alkan S. Determination of the psychiatric symptoms and psychological resilience levels of hematopoietic stem cell transplant patients and their relatives. Eur J Cancer Care, 2016;25(1):112-121. Doi: 10.1111/ecc.12219

30. de Terte I, Stephens C, Huddleston L. The development of a three-part model of psychological resilience. Stress Health, 2014;30(5):416-424. Doi: 10.1002/smi.2625

31. Gök A, Koğar Yılmaz E. A meta-analysis study on gender differences in psychological resilience levels. Cyprus Turk J Psychiatry Psychol, 2021;3(2):132-143. Doi: 10.35365/ctjpp.21.2.15

32. Gooding PA, Hurst A, Johnson J, Tarrier N. Psychological resilience in young and older adults. Int J Geriatr Psychiatry, 2012;27(3):262-270. Doi: 10.1002/gps.2712

33. Imran A, Tariq S, Kapczinski F, de Azevedo Cardoso T. Psychological resilience and mood disorders: a systematic review and meta-analysis. Trends Psychiatry Psychother, 2024;46:e20220524. Doi: 10.47626/2237-6089-2022-0524

34. Karaşar B, Canli D. Psychological resilience and depression during the COVID-19 pandemic in Turkey. Psychiatr Danub, 2020;32(2):273-279. Doi: 10.24869/psyd.2020.273

35. Wang Q, Gong L, Huang H, Wang Y, Xu T, Dong X. Psychological resilience and related influencing factors in patients diagnosed with major depressive disorder in remission: a cross-sectional study. J Psychiatr Ment Health Nurs, 2023;30(3):492-500. Doi: 10.1111/jpm.12884

36. Wu Y, Yu W, Wu X, Wan H, Wang Y, Lu G. Psychological resilience and positive coping styles among Chinese undergraduate students: a cross-sectional study. BMC Psychol, 2020;8:79. Doi: 10.1186/s40359-020-00444-y

37. American Association for Adult and Continuing Education. The mind's design: the neuroscience of stress and resilience. In: The American Association for Adult and Continuing Education 2020 Conference; 2020, Oct 27-30. Ashford University; 2021. Available from: https://files.eric.ed.gov/fulltext/ED611654.pdf

38. Bonanno GA, Romero SA, Klein SI. The temporal elements of psychological resilience: an integrative framework for the study of individuals, families, and communities. Psychol Inq, 2015;26(2):139-169. Doi: 10.1080/1047840X.2015.992677

39. Cho SH, Chung JY, Bang YW, Joo E. Investigation into the possible genetic role of serotonin and dopamine transporters in psychological resilience. Psychiatry Investig, 2018;15(5):452-458.

40. Moreno-López L, Ioannidis K, Askelund AD, Smith AJ, Schueler K, van Harmelen AL. The resilient emotional brain: a scoping review of the medial prefrontal cortex and limbic structure and function in resilient adults with a history of childhood maltreatment. Biol Psychiatry Cogn Neurosci Neuroimaging, 2020;5(4):392-402. Doi: 10.1016/j.bpsc.2019.12.008

41. He Z, Lin Y, Xia L, Liu Z, Zhang D, Elliott R. Critical role of the right VLPFC in emotional regulation of social exclusion: a tDCS study. Soc Cogn Affect Neurosci, 2018;13(4):357-366. Doi: 10.1093/scan/nsy026

42. Evans ID, Sharpley CF, Bitsika V, Vessey KA, Jesulola E, Agnew LL. Functional network connectivity for components of depression-related psychological fragility. Brain Sci, 2024;14(8). Doi: 10.3390/brainsci14080845

43. Liu H, Zhang C, Ji Y, Yang L. Biological and psychological perspectives of resilience: is it possible to improve stress resistance? Front Hum Neurosci, 2018;12:326. Doi: 10.3389/fnhum.2018.00326

44. Altinok DC, Rajkumar R, Nießen D, Sbaihat H, Kersey M, Shah NJ, Veselinović T, Neuner I. Common neurobiological correlates of resilience and personality traits within the triple resting-state brain networks assessed by 7-Tesla ultra-high field MRI. Sci Rep, 2021;11(1):1-15. Doi: 10.1038/s41598-021-91056-y

45. Azadmarzabadi E, Haghighatfard A. Detection of six novel de novo mutations in individuals with low resilience to psychological stress. PLoS ONE, 2021;16(9):1-13. Doi: 10.1371/journal.pone.0256285

46. Zsuzsanna E, Rónai Z, Réthelyi J, Kiss EC, Tiringer I, Hupuczi E, Martin L, Kállai J. Biopsychologically based resilience traits in a group of healthy undergraduate students: the role of NRG1 gene. Int J Cogn Behav, 2020;3(1). Doi: 10.23937/2690-3172/1710006

47. Cahill S, Chandola T, Hager R. Genetic variants associated with resilience in human and animal studies. Front Psychiatry, 2022;13:840120. Doi: 10.3389/fpsyt.2022.840120

48. Smeeth D, Beck S, Karam EG, Pluess, M. The role of epigenetics in psychological resilience. Lancet Psychiatry, 2021;8(7):620-629.

49. Niitsu K, Rice MJ, Houfek JF, Stoltenberg SF, Kupzyk KA, Barron CR. A systematic review of genetic influence on psychological resilience. Biol Res Nurs, 2019;21(1):61-71. Doi: 10.1177/1099800418800396

50. Bowirrat A, Chen JH, Blum K, Madigan M, Bailey AJ, Chen LC, Downs WB, Braverman ER, Radi S, Waite RL, Kerner M, Giordano J, Morse S, Oscar-Berman M, Gold M. Neuro-psychopharmacogenetics and neurological antecedents of posttraumatic stress disorder: unlocking the mysteries of resilience and vulnerability. Curr Neuropharmacol, 2010;8(4):335-358. Doi: 10.2174/157015910793358123

51. Brown O, Healey K, Fang Z, Zemek R, Smith A, Ledoux AA. Associations between psychological resilience and metrics of white matter microstructure in pediatric concussion. Hum Brain Mapp, 2023;44(10):3986-3997. Doi: 10.1002/hbm.26321

52. Brown O, Fang Z, Smith A, Healey K, Zemek R, Ledoux AA. Associations between changes in psychological resilience and resting-state functional connectivity throughout pediatric concussion recovery. Brain Connect, 2024;14(7):357-368. Doi: 10.1089/brain.2023.0096

53. Tang YY, Hölzel BK, Posner MI. The neuroscience of mindfulness meditation. Nat Rev Neurosci, 2015;16(4):213-225. Doi: 10.1038/nrn3916

54. Kross E, Ayduk O. Making meaning out of negative experiences by self-distancing. Curr Dir Psychol Sci, 2011;20(3):187-191. Doi: 10.1177/0963721411408883

55. Davidson RJ, McEwen BS. Social influences on neuroplasticity: stress and interventions to promote well-being. Nat Neurosci, 2012;15(5):689-695. Doi: 10.1038/nn.3093

56. Kaliman P, Álvarez-López MJ, Cosín-Tomás M, Rosenkranz MA, Lutz A, Davidson RJ. Rapid changes in histone deacetylases and inflammatory gene expression in expert meditators. Psychoneuroendocrinology, 2014;40:96-107. Doi: 10.1016/j.psyneuen.2013.11.004

57. Boyce WT, Levitt P, Martinez FD, McEwen BS, Shonkoff JP. Genes, environments, and time: the biology of adversity and resilience. Pediatrics, 2021;147(2):e20201651. Doi: 10.1542/peds.2020-1651

58. Cross R, Dillon K, Greenberg D. The secret to building resilience [Internet]. Harvard Business Review; 2021 [May 14, 2025]. Available from: https://hbr.org/2021/01/the-secret-to-building-resilience

59. Soni S, Deb A. Resilience and well-being: case studies of four individuals who have undergone adversities. In: Deb S, Gerrard BA, editors. Handbook of health and well-being: challenges, strategies and future trends. Springer Nature Singapore, 2022. p. 669-690. Doi: 10.1007/978-981-16-8263-6_28

60. Blunden J. Highlights from 'state of the climate in 2023' [Internet]. NOAA Climate.gov; 2024 [May 14, 2025]. Available from: https://www.climate.gov/news-features/understanding-climate/highlights-state-climate-2023

61. International Monetary Fund. Building shared prosperity and collective resilience. In: The managing director's global policy agenda annual meetings. International Monetary Fund, 2023 [May 14, 2025]. Available from: https://www.imf.org/-/media/Files/Publications/PP/2023/English/PPEA2023043.ashx

62. van der Meulen E, van der Velden PG, van Aert RCM, van Veldhoven MJPM. Longitudinal associations of psychological resilience with mental health and functioning among military personnel: a meta-analysis of prospective studies. Soc Sci Med, 2020;255:112814. Doi: 10.1016/j.socscimed.2020.112814

63. Riehm KE, Brenneke SG, Adams LB, Gilan D, Lieb K, Kunzler AM, Smail EJ, Holingue C, Stuart EA, Kalb LG, Thrul J. Association between psychological resilience and changes in mental distress during the COVID-19 pandemic. J Affect Disord, 2021;282:381-385. Doi: 10.1016/j.jad.2020.12.071

64. de Terte I, Stephens C, Huddleston L. The development of a three-part model of psychological resilience. Stress Health, 2014;30(5):416-424. Doi: 10.1002/smi.2625

65. Smeeth D, Beck S, Karam EG, Pluess M. The role of epigenetics in psychological resilience. Lancet Psychiatry, 2021;8(7):620-629.

66. Cripe C, Cooper R, Weedn S. Improving psychological resilience with cognitive retraining methods using EEG brain network biomarkers: example from UND/NASA Lunar/Martian Habitat. Am J Aerosp Eng, 2021;8(1):14-26. Doi: 10.11648/j.ajae.20210801.13

ORIGINAL ARTICLES

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6332

Assessing the anticancer potential of propolis and polyphenolic compounds in combination: An *in vitro* approach

Damla KIRCI[1*], Elif İNCE ERGÜÇ[2]

1 Izmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmacognosy, Izmir, Türkiye
2 Izmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Izmir, Türkiye

ABSTRACT

This study aimed to evaluate the in vitro anticancer activity and possible synergism of ethanolic propolis extract combined with quercetin, hesperidin, and hesperetin. Propolis (EEP) was extracted with ethanol and analyzed via high-performance liquid chromatography (HPLC), confirming major phenolic constituents. Quercetin, hesperidin, and hesperetin were tested individually and in combination with the extract. Cytotoxicity was assessed on MCF-7 using the MTT assay. The chemical composition of the EEP was characterized by the identification of 17 phenolic and flavonoid compounds. Among these, caffeic acid phenethyl ester was the most abundant constituent, with a concentration of 5733.58 μg/mL. When applied alone, quercetin reduced MCF-7 cell viability to 59% at 31.25 μg/mL, 38% at 62.50 μg/mL, and 11% at 125.00 μg/mL. Co-administration with EEP significantly enhanced the cytotoxic effect, reducing viability to 28%, 22%, and below 10% at the respective concentrations. The lowest combination index (CI) value, calculated as 1.06 (50 μg/mL propolis + 31.25 μg/mL quercetin), indicated a nearly additive interaction, while higher concentrations resulted in antagonistic effects.

Keywords: polyphenolic compound, propolis, anticancer effect, quercetin, hesperetin

* Corresponding author: Damla KIRCI
E-mail: damla.kirci@ikc.edu.tr
ORCIDs:
Damla KIRCI: 0000-0002-3479-3999
Elif İNCE ERGÜÇ: 0000-0003-0764-7694
(Received 25 Mar 2025, Accepted 21 Apr 2025)

INTRODUCTION

Propolis is a resinous substance collected by bees from plant buds and exudates, enriched with beeswax and enzymes. It has been used since ancient times for wound healing, as an antiseptic, and as an anti-inflammatory agent, with reports of its application in embalming rituals in Ancient Egypt[1]. Today, propolis is widely used in both traditional medicine and pharmaceutical products due to its immunomodulatory, anticancer, antimicrobial, antioxidant, and anti-inflammatory properties[2,3]. These biological activities are attributed to its rich chemical composition, which typically consists of approximately 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and other trace substances[1]. In the beehive, propolis serves as a natural sealant and protective agent, helping prevent microbial contamination and infection within the colony[1,4].

Polyphenols are a large group of plant-derived secondary metabolites widely present in fruits, vegetables, tea, coffee, and red wine[5]. Structurally, they consist of one or more aromatic rings with hydroxyl groups, and over 10,000 distinct compounds have been identified[6]. These natural compounds are mainly classified into flavonoids, phenolic acids, lignans, and stilbenes, with flavonoids being the most abundant in the human diet[7]. Flavonoids are further subdivided into six major classes: flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanidins[8].

Polyphenols have attracted considerable scientific interest due to their wide range of biological activities, including antioxidants, anti-inflammatory, antiviral, and anticancer properties. Several studies have demonstrated that polyphenols exert antiproliferative effects on various cancer cells with minimal toxicity to normal tissues. Their anticancer activity is thought to involve modulation of oxidative stress, inflammation, and cell signaling pathways. Moreover, epidemiological data suggest that polyphenol-rich diets may help reduce cancer risk[8,9].

Despite the well-documented biological effects of propolis and various polyphenolic compounds, limited data are available on their combined cytotoxic activity and potential synergistic interactions. Understanding such interactions may offer promising insights for the development of more effective natural compound-based therapies with enhanced efficacy and reduced toxicity. In this study, we aim to evaluate the individual and combined anticancer effects of propolis and selected flavonoids (quercetin, hesperidin, and hesperetin) on MCF-7 using the MTT assay. Furthermore, this study explored the nature of these combinations (synergistic, additive, or antagonistic) through Chou–Ta-

lalay combination index analysis using CompuSyn software. To the best of our knowledge, this is one of the few studies systematically assessing the interaction between a chemically characterized propolis extract and individual flavonoids, offering a novel perspective on the combined therapeutic potential of bee-derived and plant-derived bioactive.

METHODOLOGY

Chemicals and reagents

Quercetin, hesperidin, and hesperetin (purity≥98%) were purchased from Sigma-Aldrich, Alfa-Aesar and Santa Cruz, respectively. All solvents used were of analytical grade. RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] powder was also obtained from Sigma-Aldrich.

Preparation of propolis extract

Raw propolis samples were obtained from Türkiye and stored at 4°C until extraction. Ethanolic extract of propolis (EEP) was prepared by macerating 1 g of ground raw propolis in 3 mL of 70% ethanol at room temperature. The EEP was stored at −20°C for further use.

HPLC analysis of propolis constituents

The identification and quantification of phenolic compounds in the ethanolic propolis extract were performed by high-performance liquid chromatography (HPLC) following the method described, with minor modifications. A Shimadzu HPLC system (LC-20AD/SPD-M20A) equipped with a vacuum degasser, binary pump, autosampler, and diode-array detector (DAD) was employed.

Chromatographic separation was achieved using a C18 reverse-phase column (Inertsil ODS-3, 5 µm, 4.6 × 150 mm) maintained at 30°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). A gradient elution was applied as follows: 0–3 min, 10–25% B; 3–15 min, 25–30% B; 15–60 min, 30–50% B; 60–70 min, 50–60% B; 70–80 min, 60–90% B; 80–85 min, 90–60% B; 85–90 min, 60–25% B; and 90–95 min, 25–10% B. The column was equilibrated at 30°C for 15 minutes before each injection. The flow rate was 1.0 mL/min, and the injection volume was 5 µL. Each sample was injected twice under the same conditions[10].

Cell culture

Human Breast Cancer Cells (MCF-7) (ATTC HTB-22) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin under standard conditions (37°C, 5% CO_2, humidified incubator). Cells were subculture every 2–3 days and used for experiments at 70–80% confluency.

Cytotoxicity assay

This method is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases in viable cells, resulting in the formation of purple formazan crystals. To assess the effect of propolis extract and phenolic compounds on cell viability, MCF-7 cells were seeded at a concentration of 6,000 cells per well and incubated for 24 hours at 37°C. Subsequently, the cells were treated with various concentrations of phenolic compounds, either alone or in combination with propolis extract, for 24 hours.

After the treatment period, the wells were washed with PBS and incubated with MTT solution at 37°C for 2 hours. Following incubation, the medium was removed, and the formazan crystals were dissolved in DMSO. The optical density was then measured at 550 nm using the multiplate reader[11].

Combination index (CI) analysis

The interaction between propolis and polyphenolic compounds (quercetin, hesperidin, and hesperetin) was evaluated using the Chou–Talalay method with CompuSyn software (Cambridge, UK). Cells were treated with each compound individually and in combination at fixed doses of propolis (50 µg/mL) and varying concentrations of each polyphenolic compounds. The cytotoxic effect was assessed using the MTT assay, and the percentage of inhibition was converted to fractional effect values (Fa), ranging from 0 (no effect) to 1 (complete inhibition).

Combination index (CI) values were calculated by the software based on the median-effect equation. CI<1 indicates synergism, CI=1 denotes an additive effect, and CI>1 represents antagonism. Statistical analysis[12,13].

Statistical analysis

Statistical analysis was performed using GraphPad Prism9.0 software (GraphPad Software, La Jolla, CA). The data was analyzed using one-way Anova, with post hoc Tukey's multiple comparisons test. P-values<0.05 were considered to indicate significance[14].

RESULTS and DISCUSSION

Chemical characterization of propolis by HPLC

The chemical composition of the ethanolic propolis extract was determined using high-performance liquid chromatography (HPLC), revealing a total of 17 phenolic and flavonoid compounds. The results are summarized in Table 1. These compounds include a wide range of phenolic acids, flavones, flavonols, chalcones, and flavanones, indicating a chemically diverse and biologically active extract.

Table 1. Phenolic compounds identified in ethanolic propolis extract by HPLC analysis

Compound	Amount (μg/mL extract)
Gallic acid	19.70
Epigallocatechin gallate	150.62
Caffeic acid	704.91
p-Coumaric acid	479.46
trans- Ferulic acid	209.65
trans-Isoferulic acid	388.62
3,4-Dimetoxycinnamic acid	675.01
Quercetin	1216.20
trans-Cinnamic acid	38.63
Naringenin	1550.39
Apigenin	447.99
Kaempferol	90.36
Chrystin	2721.28
Pinocembrin	3330.40
Galangin	3645.52
Caffeic acid phenethyl ester	5733.58
trans-Chalcone	1644.20

Among the quantified compounds, caffeic acid phenethyl ester (CAPE) was identified as the most abundant constituent, with a concentration of 5733.58 μg/mL extract. CAPE is a well-known bioactive component of propolis with documented anti-inflammatory and anticancer activities. Other major constituents included galangin (3645.52 μg/mL), pinocembrin (3330.40 μg/mL), and chrysin (2721.28 μg/mL) - all of which are flavonoids known for their cytotoxic and antioxidant potential. These high concentrations suggest that flavonoids major the chemical profile of the extract.

Several phenolic acids were also detected in considerable amounts, particularly caffeic acid (704.91 µg/mL), 3,4-dimethoxycinnamic acid (675.01 µg/mL), and p-coumaric acid (479.46 µg/mL). In addition, several flavones and flavanols such as quercetin (1216.20 µg/mL), naringenin (1550.39 µg/mL), apigenin (447.99 µg/mL), and kaempferol (90.36 µg/mL) were identified.

Cytotoxic effects of propolis, quercetin, hesperidin, and hesperetin on MCF-7

In the present study the cytotoxic effects of propolis extract and phenolic compounds, both individually and in combination, on MCF-7 breast cancer cells were investigated by using the MTT assay (Figures 1 and 2).

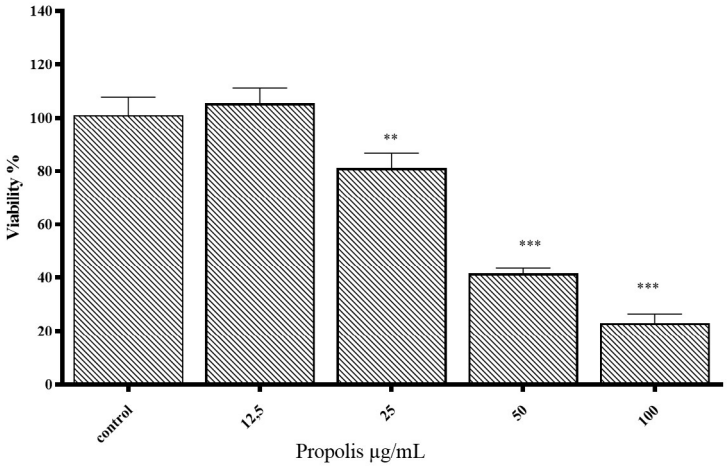


Figure 1. Effects of propolis extract on MCF-7 cell viability as determined by MTT assay

Bars represent the percentage of viable cells relative to the control group, based on three independent experiments. Statistical significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001.

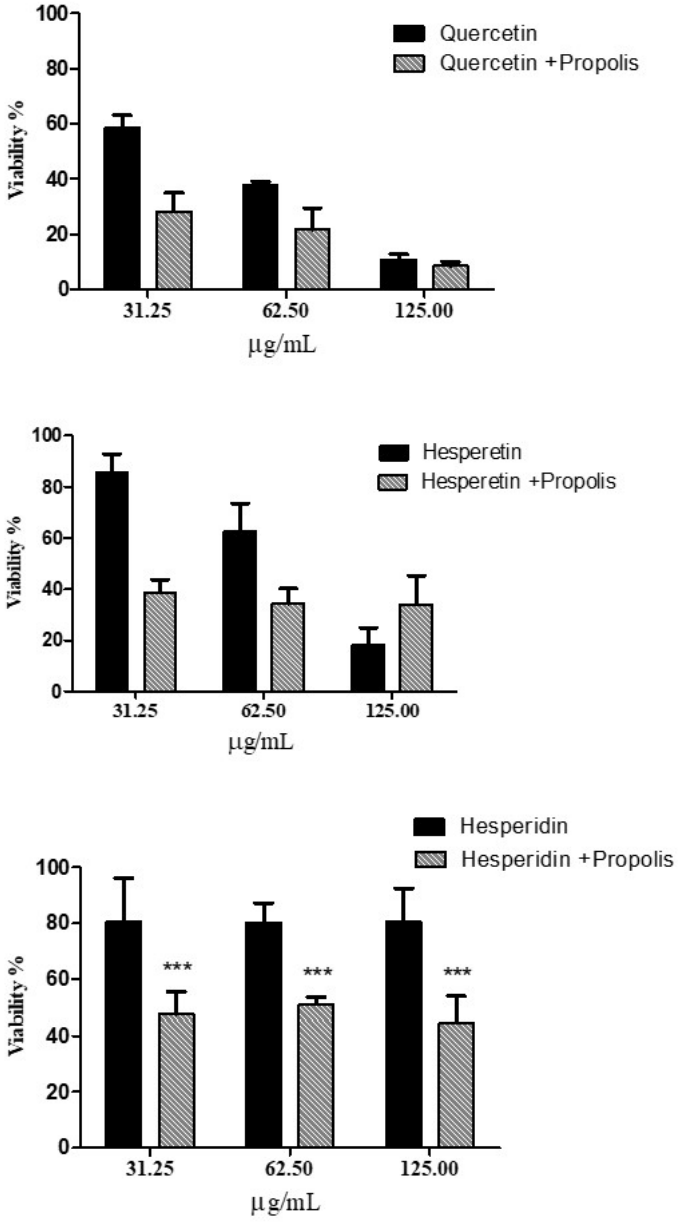


Figure 2. Effects of phenolic compounds on MCF-7 cell viability either alone or combination with 50 µg/mL propolis extract

Bars represent the percentage of viable cells relative to the control group, based on three independent experiments. Statistical significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001.

The results showed that propolis extract exerted a dose-dependent cytotoxic effect on MCF-7 cells. At the 25 μg/mL concentration, cell viability was reduced to approximately 85% compared to the untreated control group. As the concentration increased to 50 μg/mL, cell viability decreased further to around 50%. A more pronounced reduction was observed at 100 μg/mL, where cell viability dropped to approximately 23%. Statistical analysis indicated that the reduction in cell viability was significant at all concentrations tested, except for 12.5 μg/mL, suggesting a potent cytotoxic effect of propolis extract on MCF-7 cells.

Subsequently, the cytotoxic effects of quercetin, hesperetin, and hesperidin on MCF-7 breast cancer cells were evaluated at three different concentrations (31.25, 62.50, and 125.00 μg/mL), both individually and in combination with 50 μg/mL of propolis extract.

We found that quercetin exhibited the highest cytotoxicity among the tested compounds. When used alone, quercetin reduced cell viability to 59% at 31.25 μg/mL,38% at 62.50 μg/mL, and 11% at 125.00 μg/mL. The combination of quercetin with propolis extract significantly enhanced the cytotoxic effect, resulting in cell viability reductions to 28%, 22%, and below 10% at the respective concentrations.

Hesperetin, in contrast to quercetin, showed relatively high cell viability when used alone, especially at lower concentrations. At the lowest concentration of 31.25 μg/mL, cell viability remained around 90%. As the concentration increased to 62.50 μg/mL, viability dropped to 63%, and at 125.00 μg/mL, viability decreased to about 18%. The combination of hesperetin with propolis extract significantly enhanced the cytotoxic effect only at the highest two concentrations (62.50 and 125.00 μg/mL), where cell viability decreased to 39% and 34%, respectively. At the lowest concentration (31.25 μg/mL), the combination did not significantly reduce cell viability compared to hesperetin alone.

Hesperidin displayed the lowest cytotoxicity among the three compounds, with cell viability remaining around 80% at increasing concentrations. However, the combination with propolis extract significantly reduced viability to 47%, 51%, and 45%, respectively ($p<0.001$), demonstrating a marked enhancement in cytotoxicity compared to hesperidin alone.

Among the three phenolic compounds tested, quercetin exhibited the strongest cytotoxic effect, followed by hesperetin and hesperidin. The addition of propolis extract significantly increased the cytotoxicity of all three compounds, highlighting a potential synergistic or additive interaction.

Combination analysis and synergistic evaluation

The potential synergistic or antagonistic effects of propolis in combination with quercetin, hesperidin, and hesperetin were analyzed using the Chou–Talalay method via CompuSyn software. The results are presented in Table 2 and Figure 3, showing the calculated combination index (CI) values for different dose ratios.

Table 2. Combination index (CI) values of different propolis-polyphenol combinations in MCF-7

Combination	Propolis (μg/mL)	Polyphenolic compound (μg/mL)	Effect (Fa)	CI Value
P+Q	50	31.25	0.72	1.06
P+Q	50	250	0.91	1.94
P+Hd	50	31.25	0.61	1.17
P+Hd	50	125	0.65	2.11
P+Ht	50	31.25	0.53	1.38
P+Ht	50	62.50	0.49	3.19

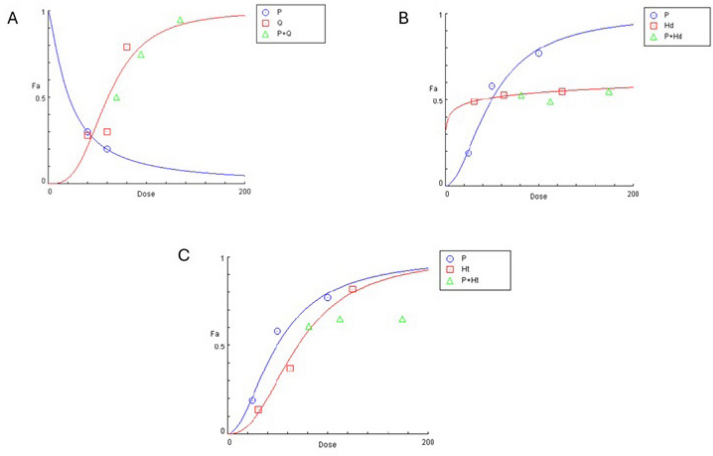


Figure 3. Dose–effect curves in propolis-polyphenol combinations

A: Propolis (P) and quercetin (Q); B: Propolis and hesperidin (Hd); C: Propolis and hesperetin (Ht)

Propolis + quercetin combination

The combination of propolis with quercetin exhibited antagonistic effects at all tested concentrations. The CI values were consistently greater than 1, indi-

cating that the combination was less effective than the individual compounds alone. The lowest CI value was 1.06 at 50 μg/mL Propolis + 31.25 μM Quercetin, suggesting a nearly additive effect. However, as quercetin concentration increased, CI values increased (e.g., CI=1.94 at 50 μg/mL Propolis + 250 μM Quercetin), confirming strong antagonism at higher doses (Figure 3A).

Propolis + hesperetin combination

Similarly, the combination of propolis with hesperetin resulted in antagonistic effects (CI>1 at all tested doses). The highest antagonism was observed at 50 μg/mL Propolis + 125 μM Hesperetin (CI=2.11). A mild reduction in antagonism was noted at lower hesperetin doses, but no synergy was observed (Figure 3B).

Propolis + hesperidin combination

The combination of propolis with hesperidin also failed to exhibit synergistic effects, with all CI values above 1. The strongest antagonistic interaction was observed at 50 μg/mL Propolis + 62.5 μM Hesperidin (CI=3.19). A relatively lower CI value (1.38 at 50 μg/mL Propolis + 31.25 μM Hesperidin) was noted, but the combination remained antagonistic (Figure 3C).

In a recent LC-HRMS-based study, the chemical profiles of seven ethanol–water extracts of propolis collected from different regions of Cyprus were comprehensively characterized. The analysis revealed notable variation in compound composition and abundance among samples. The most prominent flavonoids identified across the samples included isosakuranetin, naringenin, rhamnocitrin, diosmetin, chrysin, and acacetin, while chlorogenic acid and verbascoside stood out among phenolic acids. Isosakuranetin was especially abundant in propolis from Tirmen (102.75 mg/g), and diosmetin was detected at high levels in most samples (18.13–81.91 mg/g), except Tirmen. Compared to the present study, which also employed ethanol-based extraction and HPLC analysis, several overlapping compounds were detected, particularly chrysin, caffeic acid, hesperidin, and quercetin. However, the Cypriot samples showed higher chemical diversity and concentration ranges, which may be attributed to botanical origin, regional flora, or extraction differences. Notably, Tirmen propolis, identified as the richest in flavonoids and phenolics, was suggested to have stronger cytotoxic and antioxidant potential, aligning with the biological relevance of similar compounds evaluated in our study[15].

In comparison to the present study, where the chemical composition and cytotoxic effects of a propolis extract were analyzed along with selected polyphenols, a comprehensive LC-HRMS-based investigation of 39 Turkish propolis

samples revealed a broad chemical diversity. A total of 31 compounds were simultaneously identified, including major flavonoids such as isosakuranetin, diosmetin, chrysin, and naringenin, as well as phenolic acids like caffeic acid and chlorogenic acid. Similarly, our study identified chrysin, quercetin, caffeic acid, and hesperidin as dominant constituents, supporting previous findings. However, the Turkish propolis samples exhibited much wider quantitative ranges, with diosmetin levels reaching over 100 mg/g, while in our sample, such high concentrations were not observed. Additionally, Turkish samples displayed high triterpene content—notably oleanolic and tormentic acids-which were not predominant in our extract. These differences can be attributed to botanical origin, extraction solvent composition, and geographical factors, highlighting the chemical variability of propolis across regions. Despite these compositional differences, the presence of shared bioactive compounds strengthens the rationale for evaluating combined anticancer effects, as explored in the current study[16].

In contrast to the current study, which identified a diverse range of flavonoids and phenolic acids such as chrysin, quercetin, caffeic acid, and hesperidin, the chemical profiling of Brazilian green propolis revealed a more standardized composition. Using UPLC-ESI-QTOF-MS and HPLC, seven phenolic acids—including chlorogenic acid, caffeic acid, and artepillin C—were identified and quantified. Among these, artepillin C was found to be the most abundant compound (2.48 ± 0.94%), while isochlorogenic acid B had the lowest content (0.08 ± 0.04%) (Brazilian study). Unlike the chemical variability observed in our study, particularly in propolis samples from different regions, Brazilian green propolis showed minimal variation in phenolic acid content across samples, which the authors attribute to the use of a consistent plant resin source. Our results, in contrast, revealed noticeable differences in the concentration of major flavonoids depending on sample origin, suggesting a greater influence of regional flora. Furthermore, artepillin C, a characteristic marker of Brazilian green propolis, was not detected in our sample, underscoring botanical and geographical differences in propolis composition[17].

The cytotoxic effect of our propolis extract on MCF-7 cells was found to be dose-dependent, with viability decreasing from ~85% at 25 μg/mL to ~23% at 100 μg/mL. These findings are in line with a study on Moroccan propolis (PNM), which also demonstrated dose-dependent antiproliferative effects in MCF-7 cells, reporting an IC_{50} of 479.22 μg/mL. Notably, the propolis used in our study showed a more pronounced cytotoxic effect at lower concentrations, suggesting possible differences in chemical composition. While both studies

identified chrysin and quercetin as major constituents, the higher potency observed in our extract may be attributed to the presence and concentration of additional active compounds such as hesperidin or pinocembrin. These differences underscore the role of geographical origin and phytochemical variability in the bioactivity of propolis and highlight the necessity of standardizing propolis extracts for therapeutic applications[18].

The cytotoxic activity of the propolis extract on MCF-7 breast cancer cells demonstrated a clear dose-dependent trend. Cell viability decreased from ~85% at 25 μg/mL to ~50% at 50 μg/mL and reached ~23% at 100 μg/mL, indicating a potent antiproliferative effect. These findings are consistent with previous studies demonstrating the anticancer activity of propolis in hormone-dependent breast cancer models. Importantly, fibroblast cells exhibited significantly less sensitivity at equivalent concentrations, suggesting a degree of selectivity toward cancer cells. In comparison to quercetin and paclitaxel, propolis showed a milder but more gradual cytotoxic profile, which may be advantageous in minimizing off-target effects. The observed selective toxicity supports the potential of propolis as a natural, multitarget anticancer agent, particularly when considering its complex composition rich in flavonoids such as chrysin and quercetin. These results highlight the therapeutic relevance of propolis and provide a rationale for further investigation into its use in combination with standard chemotherapeutics[19].

In the current study, hesperetin exhibited moderate cytotoxic activity on MCF-7 cells, with a dose-dependent reduction in cell viability. These findings agree with previous research demonstrating the pro-apoptotic potential of hesperetin in breast cancer models. Palit et al. (2015) reported that hesperetin induces apoptosis in MCF-7 cells via activation of the ASK1/JNK signaling pathway, increasing the Bax/Bcl-2 ratio, promoting cytochrome c release, and subsequently activating caspase-9 and -3. Although the present study did not explore mechanistic pathways, the observed reduction in cell viability upon hesperetin treatment may reflect the activation of similar intrinsic apoptotic cascades. The relatively lower cytotoxicity of hesperetin compared to quercetin observed in our study may be attributed to differences in hydroxylation patterns and cell permeability. Nonetheless, our data support hesperetin's potential as a naturally derived antiproliferative agent, warranting further mechanistic investigations in future studies[20].

Quercetin markedly inhibits the nuclear translocation of Y-box binding protein-1 (YB-1), thereby enhancing the chemosensitivity of both MCF-7 and doxorubicin-resistant MCF-7/dox cells to multiple chemotherapeutic agents[21].

In the referenced study, hesperidin demonstrated significant cytotoxic activity against the MCF-7 breast cancer cell line. At a concentration of 80 μg/mL, cell viability decreased to 11.25% and remained relatively low (15.6%) even at 160 μg/mL, indicating a plateau in response. The calculated IC_{50} was approximately 10 μg/mL, confirming potent antiproliferative effects. These effects were attributed to secondary cytotoxic mechanisms, primarily the induction of apoptosis. The findings are consistent with the analyses conducted and are well-supported[22].

In conclusion, this study demonstrated that an ethanolic propolis extract, along with selected polyphenolic compounds (quercetin, hesperidin, and hesperetin) exerts dose-dependent cytotoxic effects on MCF-7 breast cancer cells. HPLC analysis confirmed a flavonoid-rich chemical profile, aligning with previous findings from both regional and international propolis samples. While each compound displayed significant antiproliferative activity individually, combination index analysis revealed primarily additive or antagonistic interactions, rather than synergistic ones. These results suggest that the complex interplay among bioactive constituents in natural extracts can influence therapeutic efficacy. Moreover, comparisons with propolis from different geographic origins underscored the pivotal role of botanical source and extraction methods in shaping chemical diversity and biological activity. To the best of our knowledge, this is the first study to investigate the combined cytotoxic effects of propolis and individual polyphenols in a cancer model, offering novel insights into natural product-based anticancer strategies. Overall, these findings highlight the need for further mechanistic research to optimize the therapeutic potential of polyphenol-propolis combinations.

STATEMENT OF ETHICS

No need for ethical approval for this study.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study.

FUNDING SOURCES

The authors did not receive any funding for the study.

ACKNOWLEDGMENTS

The authors would like to thank Ergüç Gıda Sanayi Limited (Çanakkale, Türkiye).

REFERENCES

1. Sforcin JM. Propolis and the immune system: a review. J Ethnopharmacol, 2007;113(1):1-14. Doi: 10.1016/j.jep.2007.05.012

2. Bankova VS, de Castro SL, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie, 2000;31(1):3-15. Doi: 10.1051/apido:2000102

3. Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. Phytother Res, 2001;15(7):561-571. Doi: 10.1002/ptr.1029

4. Salatino A, Teixeira ÉW, Negri G, Message D. Origin and chemical variation of Brazilian propolis. Evid Based Complement Alternat Med, 2005;2(1):33-38. Doi: 10.1093/ecam/neh060

5. Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li HB. Resources and biological activities of natural polyphenols. Nutrients, 2014;6(12):6020-6047. Doi: 10.3390/nu6126020

6. González-Vallinas M, González-Castejón M, Rodríguez-Casado A, Ramírez de Molina A. Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives. Nutr Rev, 2013;71(9):585-599. Doi: 10.1111/nure.12051

7. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev, 2009;2(5):270-278. Doi: 10.4161/oxim.2.5.9498

8. Abbaszadeh H, Keikhaei B, Mottaghi S. A review of molecular mechanisms involved in anticancer and antiangiogenic effects of natural polyphenolic compounds. Phytother Res, 2019;33(8):2002-2014. Doi: 10.1002/ptr.6403

9. Alam MN, Almoyad M, Huq F. Polyphenols in colorectal cancer: current state of knowledge including clinical trials and molecular mechanism of action. Biomed Res Int, 2018;2018:4154185. Doi: 10.1155/2018/4154185

10. Oruç HH, Çayci M, Sorucu A, Uzabacı E, Nyandwi R. Characterization of commercially available propolis products in Turkey based on individual phenolic compounds. J Apic Res, 2023;62(5):1225-1232. Doi: 10.1080/00218839.2021.1962110

11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983;65(1-2):55-63. Doi: 10.1016/0022-1759(83)90303-4

12. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev, 2006;58(3):621-681. Doi: 10.1124/pr.58.3.10

13. Buettner R, Nguyen LXT, Morales C, Chen MH, Wu X, Chen LS, et al. Targeting the metabolic vulnerability of acute myeloid leukemia blasts with a combination of venetoclax and 8-chloro-adenosine. J Hematol Oncol, 2021;14:1-16. Doi: 10.1186/s13045-021-01076-4

14. Kırcı D, Batur ÖÖ, Demirci B, Demirci F. Synergistic antimicrobial effects of *Melaleuca alternifolia* essential oil and kojic acid combinations. Curr Microbiol, 2025;82(5):1-8. Doi: 0.1007/s00284-025-04175-4

15. Nalbantsoy A, Sarıkahya NB, Özverel CS, Barlas AB, Kırcı D, Akgün İH, et al. Chemical composition and biological activities of Cypriot propolis. J Apic Res, 2022;61(2):233-245. Doi: 10.1080/00218839.2021.1977028

16. Sarıkahya NB, Gören AC, Okkalı GS, Çöven FO, Orman B, Kırcı D, et al. Chemical composition and biological activities of propolis samples from different geographical regions of Turkey. Phytochem Lett, 2021;44:129-136. Doi: 10.1016/j.phytol.2021.06.008

17. Sun S, Liu M, He J, Li K, Zhang X, Yin G. Identification and determination of seven phenolic acids in Brazilian green propolis by UPLC-ESI-QTOF-MS and HPLC. Molecules, 2019;24(9):1791. Doi: 10.3390/molecules24091791

18. Touzani S, Embaslat W, Imtara H, Kmail A, Kadan S, Zaid H, et al. *In vitro* evaluation of the potential use of propolis as a multitarget therapeutic product: physicochemical properties, chemical composition, and immunomodulatory, antibacterial, and anticancer properties. Biomed Res Int, 2019;2019:4836378. Doi: 10.1155/2019/4836378

19. Misir S, Aliyazicioglu Y, Demir S, Turan I, Hepokur C. Effect of Turkish propolis on miRNA expression, cell cycle, and apoptosis in human breast cancer (MCF-7) cells. Nutr Cancer, 2020;72(1):133-145. Doi: 10.1080/01635581.2019.1616100

20. Palit S, Kar S, Sharma G, Das PK. Hesperetin induces apoptosis in breast carcinoma by triggering accumulation of ROS and activation of ASK1/JNK pathway. J Cell Physiol, 2015;230(8):1729-1739. Doi: 10.1002/jcp.24818

21. Oršolić N, Jembrek MJ. Potential strategies for overcoming drug resistance pathways using propolis and its polyphenolic/flavonoid compounds in combination with chemotherapy and radiotherapy. Nutrients, 2024;16(21):3741. Doi: 10.3390/nu16213741

22. Noori SD, Kadhi MS, Najm MA, Oudah KH, Qasim QA, Al-Salman HNK. In-vitro evaluation of anticancer activity of natural flavonoids, apigenin and hesperidin. Mater Today Proc, 2022;60:1840-1843. Doi: 10.1016/j.matpr.2021.12.506

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6333

A novel multi-layer mucoadhesive buccal film containing liposomal Sumatriptan: Development and *in vitro* drug release kinetics evaluation

Zeynab AHMADIFARD[1,2], Mohammad Hossein ABDANI[1], Nima ASKARPOUR[1], Nooshin TASHARROFI[1,2*]

1 Lorestan University of Medical Sciences, School of Pharmacy, Khorramabad, Iran
2 Lorestan University of Medical Sciences, Razi Herbal Medicines Research Center, Khorramabad, Iran

ABSTRACT

This study introduces a new three-layered buccal film for the controlled drug delivery of Sumatriptan. Sumatriptan was loaded in cationic liposomes and embedded in a hyaluronic acid film. This film was sandwiched between a mucoadhesive layer of carbopol® 934P/HPMC K4M and an ethylcellulose backing layer. The systems' characteristics were evaluated, including thickness, weight uniformity, swellability, mucoadhesive strength, etc. Also, the in vitro release kinetics of Sumatriptan were assessed using DDSolver software. The nanoliposomes showed a spherical shape with average size, zeta potential, and entrapment efficiency of 138.3 ± 3.99 nm, 17.3 ± 2.7 mv and 75% ± 4.16, respectively. The final system exhibited a suitable mucoadhesive strength (1225 Pascal) by altering the swelling and disintegration of layers, the backing layer facilitated the unilateral drug release toward the mucus, resulting in prolonged drug release. About 90% of Sumatriptan was released within 24 h through predominant diffusion and polymer relaxation mechanisms.

Keywords: buccal drug delivery, drug release, liposomal Sumatriptan, mucoadhesive film

* Corresponding author: Nooshin TASHARROFI
E-mail: n_tasharrofi@yahoo.com
ORCIDs:
Zeynab AHMADIFARD: 0000-0002-8634-8467
Mohammad Hossein ABDANI: 0009-0009-1421-2032
Nima ASKARPOUR: 0009-0004-1068-4503
Nooshin TASHARROFI: 0000-0002-6443-0961
(Received 8 Apr 2024, Accepted 24 Sept 2024)

INTRODUCTION

Oral drug delivery is one of the most preferred medicine administration routes; however, it must be formulated in a way to overcome absorption restrictions. There are advantageous strategies that assist with this. Among them are oral mucoadhesive films, which enhance the chance of drug absorption through adhesion to the oral cavity mucosa and increase the drug residence time in the absorption site[1]. They enable extended or rapid drug release for local or systemic activities[2-4]. These dosage forms can also be single- or multi-layered to fulfill diverse medicinal purposes. Besides, they help the drugs to bypass the hepatic first-pass effect or prevent them from degradation by enzymatic activity and severe pH changes in the gastrointestinal tract, which leads to increased bioavailability of susceptible medications[5,6]. The ease of drug administration and improved patient compliance are their other benefits[7,8]. The limited surface area in the mouth, the continuous washing by saliva, and the low penetration of hydrophilic molecules into biological membranes are among the main challenges with oral mucoadhesive systems. So, increasing the loading capacity of the drug into the dosage form, providing enough adhesion strength, and exploiting penetration-enhancing strategies must be considered.

The restricted surface area in the mouth, the continuous washing by saliva, and the low penetration of hydrophilic molecules into biological membranes are among the main challenges with oral mucoadhesive systems. So, increasing the loading capacity of the drug into the dosage form, providing enough adhesion strength, and exploiting penetration-enhancing strategies must be considered.

Generally, mucoadhesive polymers have a hydrophile nature with multiple polar functional groups that interact with mucus components through physical entanglements and/or secondary chemical bonds, creating weak mutual networks. These interactions maintain long-term contact between the formulation and the oral mucosa. For example, the carboxyl and sulfate functional groups have shown high mucoadhesive performance because they can make hydrogen bonds with mucin oligosaccharide chains[9,10].

Polyacrylic acid (PAA) is a well-known mucoadhesive polymer containing carboxylic groups. Polycarbophil and carbopol are PAA derivatives used as mucoadhesive platforms for drug delivery. They also have advantages in sustained-release drug delivery systems due to their ability to form a good gel[9-11].

Hydroxypropyl methylcellulose (HPMC) is a polysaccharide polymer. The high presence of –OR groups in HPMC plays a significant role in mucoadhesive

strength through hydrogen bond interactions. Many studies have demonstrated that HPMC-containing films had appropriate mucoadhesive and adhesion times on oral membranes while also displaying the desired release of drugs at the appropriate time[10,12,13].

Hyaluronic acid (HA) is a natural anionic polymer made of repeating units of glucuronic acid and N-acetylglucosamine. Due to its high biocompatibility and low immunogenicity, it has gained significant attention in the pharmaceutical industry, particularly in film systems, over the last few years. HA is an excellent choice for oral drug administration because of its significant adhesive properties, which enable the loaded drug to be delivered in a continuous pattern[14-16].

When systemic drug absorption is required guiding the drug molecules toward the mucosa will increase the therapeutic yield[17,18]. For example, by placing a backing layer, the bilateral medication release can be limited to unilaterally toward the mucosal membrane[19]. Ethylcellulose (EC) is a water-insoluble derivative of cellulose; having hydrophobicity, moderate flexibility, and drug impermeability, EC can be employed as a polymer for constructing the backing layer in bioadhesive formulations to ensure unidirectional drug release[20,21].

Nanoparticulate vehicles are shown to assist drug penetration through mucous membranes. They can be tailor-made to offer benefits such as excellent cellular crossing, deep tissue penetration, and sustained drug release[4,22,23]. These colloidal systems, such as micelles, liposomes, nanoemulsions, and polymeric nanoparticles, can alter the drugs' distribution in the body, increasing their effectiveness and decreasing their toxicity[24].

Among nanoparticles, liposomes (LPs) have received significant attention for their ability to carry both lipophilic and hydrophilic drugs[25,26] and their easy crossing through the cell membranes due to their similarity to biological membranes[27]. By incorporating liposomes in mucoadhesive films, the advantages of both can be taken; i.e., mucoadhesive buccal films extend retention time and modify the drug release profile, and liposomes enclose the drug and improve their release and permeability[3,4].

Different mechanisms and rates are involved in drug release which can affect the absorbed amounts of drug per time unit and duration of therapeutic effect. The release assessment is among the main tests that must be done for a newly designed product to ensure its quality. Generally, a constant and extended release is desired, while varied release rates make systemic concentration predictions difficult.

In this study, a three-layer mucoadhesive system was designed, synthesized, and characterized. It includes the carbopol® 934P-HPMC mucoadhesive layer, the hyaluronic acid middle layer containing Sumatriptan nanoliposomes, and the ethylcellulose impermeable backing layer. The three layers were separately synthesized and characterized in appearance, morphology, thickness, surface pH, mucoadhesion, folding endurance, swellability, film disintegration time, and content uniformity. Finally, the *in vitro* release mechanism of the Sumatriptan from the final three-layer formulation was evaluated by fitting the data to different kinetics models using DDSolver software.

METHODOLOGY

Material

Carbopol® 934P and ethylcellulose (EC) were gained from Sigma, Germany. Hydroxypropyl methylcellulose (HPMC) K4M was acquired from Alfa Aesar, United Kingdom. Hyaluronic acid (HA), octadecyl amine (stearyl amine), propylene glycol, glycerol, acid citric, chloroform, acetone, methanol, and agar were purchased from Merck, Germany. Tehran Chemie Pharmaceutical Co., Tehran, Iran, provided Sumatriptan. Other reagents and chemicals were of the analytical grade.

Preparation of nanoliposomes

For preparing liposomes, the thin film hydration method was used. Briefly, lipophilic compounds, namely phosphatidylcholine (17 or 25 mg), stearyl amine (2 or 7 mg), and cholesterol (3 or 13 mg), were dissolved in a 4 mL solvent mixture of 3:1 chloroform: methanol and delivered to a round bottom flask. To form a thin lipid film on the flask wall, the organic solvent was evaporated in a rotary evaporator (IKA® RV 10 basic/digital) under the condition of 58°C, 100 rpm, and slow declining pressure from 470 to 50 mbar to completely remove all the solvent[28,29]. The dried lipid layer was then hydrated by a 4 mL aqueous phase (phosphate-buffered saline, PBS, pH=7.4) containing Sumatriptan at different concentrations of 0 (blank), 0.1, 0.55, 1, or 1.25% w/v for one hour under slowly rotating in the rotary evaporator (temperature 58°C; 50 rpm; with opened vacuum screw to reach the ambient pressure). Subsequently, this dispersion was sonicated in an ultrasonic bath for 10 minutes to become homogeneous. It was then kept at room temperature for about one hour to allow intermolecular forces to form and strengthen the liposome membrane. For further size reduction, the liposomal solution was sonicated for a 10-second cycle by an ultrasonic probe (120 W) and freeze-thawed for at least ten cycles. Afterward, the liposome dispersion was centrifuged (15,000 rpm for 1.5 hours

at four °C); the supernatant was used in the next steps to determine the encapsulation efficiency and drug loading, and the precipitate (nanoliposomes) was washed three times by distilled water and collected freshly for formulation preparation.

Based on the liposomes evaluation, the formulation consisting of 17 mg phosphatidylcholine, 7 mg stearyl amine, 13 mg cholesterol, and 0.55% w/v Sumatriptan was chosen.

Characterization of nanoliposomes

Liopsome size, PDI, and zeta potential studies

The surface charge, hydrodynamic diameter, and polydispersity index (PDI) of drug-loaded nanoliposomes were assessed upon investigation with a zeta sizer (DLS; HORIBA Scientific SZ-100, CA, USA).

Evaluation of the liposome morphology

Nanoliposome morphology and size were evaluated using scanning electron microscopy (FE-SEM, TESCAN MIRA3, and the Czech Republic).

Entrapment efficiency (EE%) and drug loading (DL%) measurement

The supernatant obtained from nanoliposome centrifugation was subjected to the determination of the unloaded drug. The concentration of Sumatriptan was measured using spectrophotometry at a wavelength of 227 nm. The calibration curve for Sumatriptan was acquired by plotting the absorbance against the different drug concentrations in artificial saliva (pH=6.8).

Entrapment efficiency (EE%) and drug loading (DL%) were calculated using equations 1 and 2, respectively:

$$EE\,(\%) = \frac{W_t - W_f}{W_t} \times 100 \quad \text{(Equation 1)}$$

$$DL\,(\%) = \frac{W_t - W_f}{W_s} \times 100 \quad \text{(Equation 2)}$$

Where W_t, W_f, and W_s are the initial amount of drug, the amount of unloaded drug measured in the supernatant, and the total amount of the nanoliposomal system, respectively.

Stability evaluation of liposomes

The stability of prepared liposomes was examined at three temperatures of -20, 4, and 25 for three weeks. Briefly, liposomes, with or without (blank) drug, were kept in a freezer (-20), refrigerator (4), and room temperature (25) for a defined duration, namely three weeks, and the amounts of Sumatriptan that were released in this period measured. Then the percentage of the drug remaining in liposomes was statistically compared between these three groups to find out the effect of storage temperature on premature drug release.

Preparation of multi-layer mucoadhesive film

The designed final dosage form consisted of three layers: a mucoadhesive layer, a layer containing drug-loaded nanoliposomes, and an impermeable backing layer. Each layer was prepared using the solvent casting and then attached to construct the final system.

The mucoadhesive layer was prepared according to the following steps. First, carbopol® 934P (0.5, 1.5, or 3% w/v) and HPMC K4M (0.5, 1, or 1.5% w/v) polymers were dispersed in 7 mL distilled water. The mixture was stirred until it became a perfectly homogeneous solution. Propylene glycol (300 µL) and glycerol (200 µL) with a ratio of 3:2 v/v were then added to the solution as plasticizers. The resulting solution was held stationary until all of its bubbles had disappeared, then it was poured into a glass Petri dish with a diameter of 7 cm and dried for 24 hours in a 40°C oven[30]. Based on the produced mucoadhesive film's physical appearance, elasticity, and homogeneity, the optimal concentrations of 1.5% w/v and 0.5% w/v were ultimately chosen for carbopol® 934P and HPMC, respectively.

The middle layer was constructed by homogeneously dispersing various concentrations of HA polymer (1.5 or 2% w/v) in 7 mL of distilled water, followed by adding Sumatriptan-containing nanoliposomes (containing an equivalent of 16.5 mg of Sumatriptan) and 300 µL propylene glycol and 200 µL glycerol. The bubble-free solution was then poured on top of the formed mucoadhesive layer and dried entirely for 24 hours in an oven set at 40°C.

HA in a 1.5% w/v concentration was chosen based on superior physical characteristics, flexibility, and consistency.

In order to create the EC impenetrable layer, different amounts of the polymer (1.5, 3, or 5% w/v) were dissolved in 7 mL of acetone, and the polymer solution was supplemented with 300 µL castor oil and 100 µL propylene glycol, as plasticizers; it was then poured onto the previously prepared two-layer and then

dried in the oven for 24 hours. The EC concentration greatly influences the creation of a uniform impermeable barrier and the homogeneity of the final formulation. It was determined that the 3% w/v EC was the best concentration based on its physical characteristics, flexibility, and uniformity compared to other concentrations.

Characterization of mucoadhesive system

The physical appearance of the film's layers

The appearance of films was evaluated visually for having a smooth surface and flexibility and being free of bubbles and wrinkles.

Weight and thickness uniformity

As layers must be uniform throughout, they undergo weight and thickness uniformity. A digital scale (Mettler Toledo, ME303, Switzerland) was used to weigh at least three different pieces of each film with an area of one cm^2; the mean weight SD was then calculated.

A digital micrometer was used to measure the thickness of three separated pieces, one in the center and two in the corners, of a film with a one cm^2 surface area.

Folding endurance

The film's folding strength was measured by manually folding a one cm^2 piece several times and counting the number of folds until cracking happened.

Surface pH

An agar plate (1% w/v of agar in artificial saliva, pH=6.8, as solvent) was used to measure the film's surface pH.

The artificial saliva consisted of 1.2 g of potassium chloride, 0.85 g of sodium chloride, 0.05 g of magnesium chloride, 0.13 g of calcium chloride, and 0.13 g of di-potassium hydrogen orthophosphate in one liter of distilled water; the final pH was set to 6.8[31].

Agar powder was dissolved in the artificial saliva at 100°C; it was then put onto a petri dish and allowed to cool down and gelled. Then, one cm^2 piece of the film was placed on the agar gel's surface, and after 10 minutes, the pH was determined by placing the pH indicator paper on the swollen film's surface.

Disintegration time

The films were placed in a beaker containing 10 mL of artificial saliva (pH=6.8) and shaken at a rate of 400 rpm at 37°C. The disintegration time was verified visually when the film started to fragment.

Swellability study

The degree of swelling of the films was also determined in the agar plate (1% w/v). The initial weight (W_1) of one cm² piece of film was first measured. The agar plate's surface was moistened with artificial saliva, and the samples were then placed on it. The excess water was then removed from the surface of the films with filter paper after ten minutes, and the weight of the swollen films was measured (W_2). The swelling percentage of the films was calculated using Equation 3:

$$\text{Swelling}(\%) = \frac{W_2 - W_1}{W_1} \times 100 \qquad \text{(Equation 3)}$$

In vitro mucoadhesive strength study

The mucoadhesion strength was tested for the final three-layered film and the mucoadhesive layer alone. The examination was handled using a self-built instrument (Figure 1)[32]. Sodium alginate (10% w/v) gel was employed as a mucosal model. As illustrated in Figure 1, a piece of thread with appropriate length was affixed to the surface of carbopol® 934P -HPMC film (or, in the case of three layers, to the backing layer), and the other end was attached to a light plastic cup (container). This connection was at a perpendicular angle using a glass roller connector. The film's surface was moistened with artificial saliva, gently adhered to the gel, and then allowed to set for two minutes. After releasing the container from the lab jack stage, water was slowly added at a constant rate until the film separated from the sodium alginate gel's surface. The weight of the water-containing container was determined, and the film's mucoadhesive strength was calculated using Equation 4:

$$\text{Mucoadhesive strength}\left(N/cm^2\right) = \frac{W\,(Kg) \times g\left(m/s^2\right)}{A\left(cm^2\right)} \qquad \text{(Equation 4)}$$

Where the W is the weight of the container plus the weight of water, g is the acceleration of gravity (9.8 m/s²) and A is the surface area of the films.

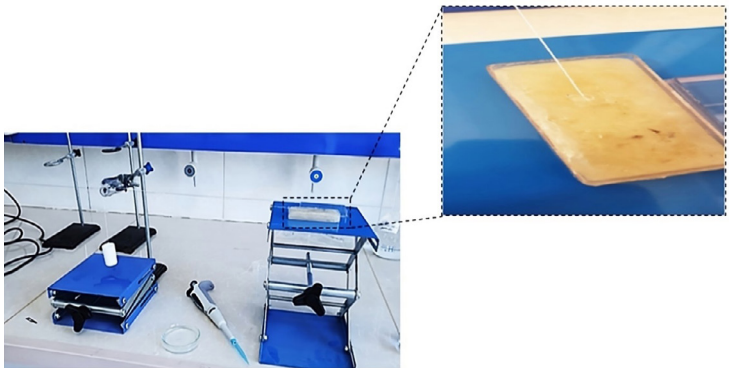


Figure 1. The apparatus designated for measuring mucoadhesive strength. Sodium alginate gel (10% W/V) was molded into a rectangular plate and moistened with artificial saliva. The film was then adhered to sodium alginate and connected to a thread. A lightweight container is fastened to the thread at a perpendicular angle. The container is then gradually filled with water until such a point that the film becomes detached. Ultimately, the weight of the container and water is measured to determine mucoadhesive strength.

In vitro drug release evaluation

The drug release test was studied using a two-sided modified Franz cell and dialysis bag (cut off=12 kDa). The apparatus consisted of two identical chambers between which the holding area is located (Figure 2). A piece of film with an area equal to the cross-sectional area of the sample hold area (4.5 cm^2 containing an equivalent of 1.9 mg of Sumatriptan) was sandwiched between two dialysis bags with the same dimensions. Each chamber was fully filled with 10 mL of artificial saliva. The device was placed in a shaker incubator and shaken at 100 rpm at 37°C. During 48 h in defined intervals, 500 µL of the release medium was withdrawn from both chambers and replaced with the same amount of artificial saliva. The released Sumatriptan amounts at each time point were plotted graphically to find the release behavior of the drug.

The final formulation without the drug was used as a blank sample to measure Sumatriptan concentration more accurately.

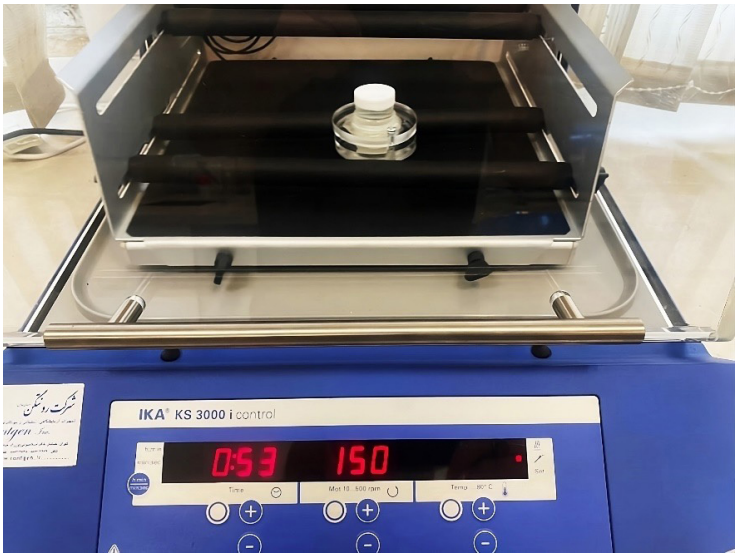


Figure 2. The modified Franz cell instrument for drug-release test

The mechanism of drug release

Sumatriptan release kinetics was analyzed by fitting the release data into different kinetic models: zero-order, first-order, and Higuchi. Other models, namely Makoid-Banakar, Korsmeyer-Peppas, Weibull, and Peppas-Sahlin, were also employed to find the involved mechanisms in Sumatriptan release. The untransformed release results were analyzed using DDSolver (v 1.0; an Excel add-in software). The best model was selected based on the highest adjusted and model selection criterion (MSC) and the lowest Akaike information criterion (AIC).

Statistical analysis

All experiments were repeated thrice, and the findings were presented as mean ± SD. The ANOVA was used to compare groups, with p-values≤0.05 indicating statistically significant differences. SPSS software (SPSS 16.0 Version) was utilized for Statistical analysis.

RESULTS and DISCUSSION

Characterization of Sumatriptan-loaded nanoliposomes

Sumatriptan-loaded nanoliposomes were characterized based on their morphology (Figure 3), size, polydispersity index (PDI), zeta potential, drug loading (DL%), and entrapment efficiency (EE%) (Table 1).

Table 1. Characteristics of the optimized Sumatriptan-loaded nanoliposomes

Characteristic	Hydrodynamic size ± SD	PDI ± SD	Zeta potential	DL* (%) ± SD	EE* (%) ± SD
Value	138.3 ± 3.99	0.34 ± 0.01	17.3 ± 2.7	33% ± 1.83	75% ± 4.16

*DL and EE stand for drug loading and entrapment efficiency, respectively.

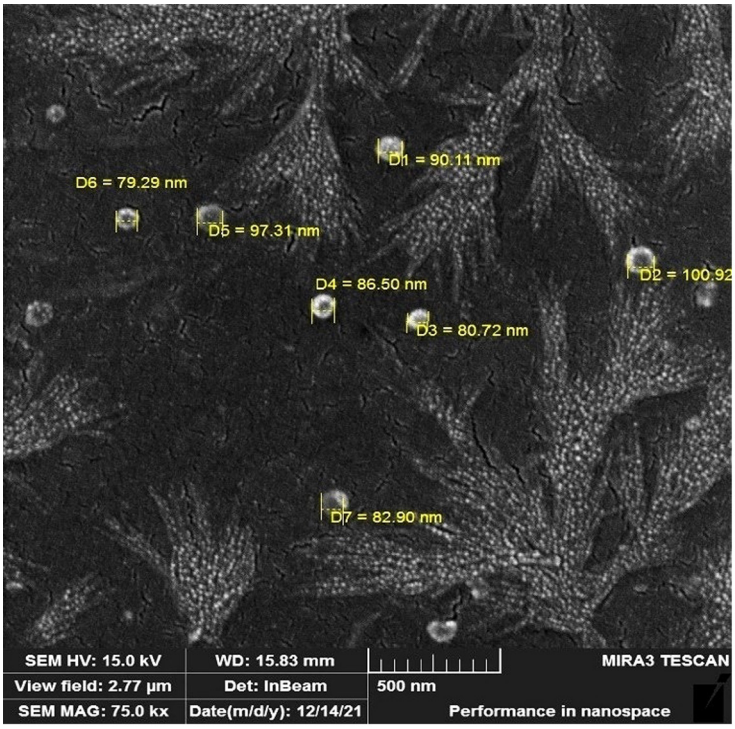


Figure 3. SEM image of Sumatriptan-loaded nanoliposomes

The Sumatriptan calibration curve (Figure 4) was used to measure its concentration.

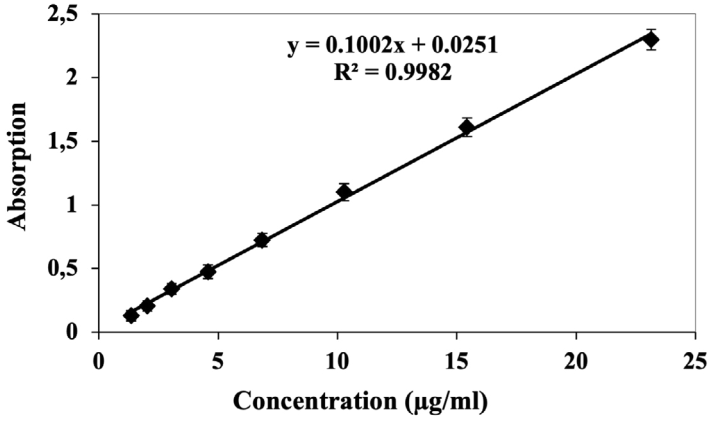


Figure 4. Calibration curve of Sumatriptan in artificial saliva (pH=6.8)

Data from the dynamic light scattering (DLS) method showed that the liposomal Sumatriptan had an average particle size (z-average) ± SD of 138.3 ± 3.99. The nanoliposome's observed size and morphology under the scanning electron microscope (SEM) photograph (Figure 3) displayed the particle's average size was 88.25 nm with spherical shapes. The difference between the DLS and SEM results is due to the presence of the double layer of charge on the surface of nanoliposomes formed in the aqueous medium, i.e., the hydrodynamic diameter is determined through DLS, whereas the projected area diameter can be seen in SEM image[1].

The value of PDI is an index for determining nanoliposome size uniformity. This parameter is important because, in nanometre size, the behavior of particles profoundly depends on their size and effective surface; in this sense, a wide PDI reduces the control over the drug release rate and penetration[19]. The PDI of 0.34 ± 0.01 is acceptable for our Sumatriptan-loaded nanoliposome. This size uniformity can also be seen in SEM images. Therefore, the release of Sumatriptan from all particles is expected to be uniform.

The vesicle charge is an important parameter in drug permeability. Generally, positively charged compounds interact more with the biosurfaces compared to neutral or negatively charged ones. For instance, it was shown that coating phosphatidylcholine and cholesterol-based liposomes with chitosan can enhance Sumatriptan nasal absorption[33,34].

The optimum entrapment efficiency (EE%) and drug loading (DL%) were 75% and 33%, respectively. The key parameters in nanoliposome synthesis are the water-to-oil phase ratio, temperature, drug concentration, the ratio of the different types of lipids, and other manufacturing process conditions[29]. In the

case of water-soluble drugs, high EE% depends on the ability of liposomes to entrap them during the vesicle formation. Actually, the hydrophilic drugs can enter the interior compartment of the liposome along with the aqueous phase, so this compartment's volume plays an important role in imported medicine. For example, single-wall liposomes have more water core volume than multilamellar vesicles of the same size. It was shown that lamellarity can also affect Sumatriptan EE% and unilamellar liposome was favored[29,35]. The freeze-thawing can reduce the number of walls and lead to an increased internal volume.

For increasing the EE%, the temperature comes to the transition temperature of amphiphiles during liposome manufacturing. This elevated temperature gives energy to lipids and disrupts their interactions, so lets more drugs enter. However, the temperature must be decreased again then.

On the other hand, the liposome must be able to confine and keep the entrapped drug. The drug's log p, the interactions between liposome membrane components (lipid constituents), and the production temperature are of important parameters for drug retention inside the vesicle[36]. Drugs with affinity to both lipid and water can easily escape the liposome, while drugs with little partitioning to the lipid membrane can retain more. The lamellae packing depends on the interaction of its components. Based on the therapeutic goal, the type and ratio of the constituents must be chosen. For instance, cholesterol can increase the lipoid molecules' interactions and rigidify the membrane, reducing the bilayer permeability and hence drug scaping. However flexible vesicles are favorable when it comes to penetrating the biological membrane. So, the cholesterol content must be optimized.

The interaction of the enclosed drug with liposome constituents is also important. By changing the membrane components, the affinity and distribution of the drug would also be changed. For example, it was shown that depending on the liposome charge, Sumatriptan EE% varies. Compared to neutral or negatively charged liposomes, the positively charged liposome containing stearylamine (SA) had the highest EE%. Albeit the charge density is important, by increasing the SA concentration, the repulsive forces can reduce the membrane packing and reduce the EE%. Sumatriptan is mainly protonated in neutral pH and below, which can electrostatically interact with anionic compounds like the phosphate group in phosphatidylcholine or diacetyl phosphate (DCP). It can also form hydrophobic bonds with the tails of phospholipids. The interactions can cause the drug to be both in the core and bilayer membrane; the presence of the drug in the membrane can perturb the bilayer and lower the EE%. Their results also confirmed that positively charged liposomes had smaller sizes, narrower size distribution, and more stability[29,35].

Stability of liposomes at different temperatures

The percentage of drug remaining in liposomes after three weeks of storage in -20, 4, and 25 is presented in Figure 5. As is evident in Figure 5 in three groups more than 90% of the drug remained in liposomes.

Statistical evaluation (using GraphPad Prism® 9 software) revealed that there are no significant differences between these three groups; in other words, liposomes can be stably stored in these three temperatures.

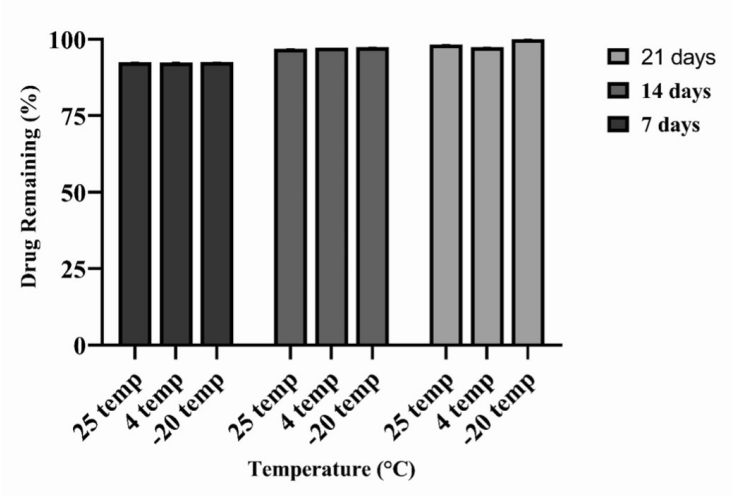


Figure 5. The percentage of Sumatriptan remaining in liposomes after three storage at -20, 4 and 25

Characterization of the film layers

The weight, thickness, folding endurance, surface pH, swelling, and disintegration time were evaluated for the mucoadhesive system; Table 2 shows the physical characteristics of its three layers.

Table 2. Physical characteristics of the three layers and final formulation

Layer	Weight (mg)	Thickness (μm)	Folding endurance	Surface pH	Swellability (%)	Disintegration time (min)	Mucoadhesion strength (N/cm^2)
Mucoadhesive	18.42 ± 2.07	23.09 ± 1.47	No cracks seen	5	192.42 ± 4.80	38.25 ± 2.2	1225 ± 40
Nanoliposome-loaded hyaluronic acid	14.33 ± 0.82	60.21 ± 2.5	No cracks seen	6	203.05 ± 31.2	60 ± 1.46	-
Impermeable	15.26 ± 0.57	19.66 ± 0.47	No cracks seen	7	0	Not disintegrated	
Final formulation	45.37 ± 0.58	99.67 ± 4.73	No cracks seen	7	-	-	1274 ± 80.01

According to the results, all three layers of the optimized formulation have desirable physical features. The required dose should be placed in the intended dimensions; to reduce the sensation of foreign bodies on the oral mucosa and to enhance patient compliance, oral mucoadhesive films should be flexible and have appropriate thickness. Therefore, it is necessary to determine the thickness of the mucoadhesive film. The final thickness of the synthesized mucoadhesive film was 99.67 ± 4.73 μm. The mucoadhesive film prepared in our study has less than 100 μm thickness which is in the range of patient acceptance[5].

The oral mucoadhesive film must have strong strength and endurance against the mechanical stresses in the mouth. The folding endurance test in this investigation demonstrated the appropriate films' flexibility.

Having a similar pH to the oral environment is another characteristic of an ideal mucoadhesive film[7,8,37]. The surface pHs of the films were between 5 and 7, which is within the range of saliva's pH (5.6 to 7.4) and is compatible with the buccal mucosa with no irritation or mucosal damage.

The swellability of the layers also has a favorable value. As EC is an insoluble polymer, it cannot absorb water to its chains and swell; it cannot be disintegrated in an aqueous medium. Our data is consistent with this; no signs of degradation were seen, even after three days of testing, nor did this layer swell. These features of EC help this layer prevent the release of the drug in the oral environment, and the whole drug diffusion is toward the mucosa[2]. The swelling of the mucoadhesive layer shows the tendency of polymers to absorb water; this is required because by drawing water to their strands, polymers can extend in the aqueous medium and expose their functional groups for binding with the mucin. In addition, the water can easily diffuse into hydrophilic polymers, dissolve the drug, and facilitate its release out of the system. Although exces-

sive water absorption might cause the loosening of the film attachment and wash it away with water, so highly hydrophilic polymers are not ideal for being mucoadhesive[3,4]. The strength of the mucoadhesion has a significant effect on the retention of the system on the mucus. The mucoadhesive strength should be sufficient without damaging the mucosal membrane. Using a modified balancing method, the mucoadhesive strength of the three-layered mucoadhesive film and the carbopol® 934P -HPMC layer were studied separately and compared. Based on the findings, the mucoadhesion was strong enough. Besides, no statistically significant difference was seen between the single-layer carbopol® 934P-HPMC and the final three-layer system mucoadhesive strength. Therefore, adding HA and EC layers to the carbopol® 934P -HPMC mucoadhesive layer did not affect its mucoadhesive strength (Table 1).

Sumatriptan release from the three-layered mucoadhesive film

The release of Sumatriptan from the final multi-layer system in artificial saliva (pH=6.8) was followed up for 48 hours. Both the film matrix and the liposome features could affect the release. As a carrier, the liposome can aid the bioabsorption of the drug molecules. The drug-containing liposomes can interact with the mucus and resist washing by saliva which can enhance the chance of the drug, alone or encapsulated in the liposome, to cross biological membranes[36]. So, the drug should remain inside the vesicle when it reaches the mucosa.

Generally, extended drug release is preferred by patients because the frequency of dose uptake is reduced. However, for this issue, zero-order release with a constant rate is preferential. The Sumatriptan release data demonstrate a biphasic behavior in which 67% of the drug was released during the first four hours, followed by a sustained release phase. As shown in Figure 6, approximately 90% of the drug is released from the system within 24 h and reaches the maximum (100%) after 48 h. It means the drug release rate is fast at first and reduced gradually. The fast release of surface-bound drugs and those in the bilayer membrane can be the reasons for the biphasic nature of the release[38]. The rate constant (k) determines the maximum cumulative drug release time. It is governed by the drug permeability across the liposome, which itself relies upon the liposome constituents and drug physicochemical features.

In this study, the drug did not release from the EC layer side, which indicates the optimal performance of this layer as an impermeable back layer of the film.

Based on the disintegration results, the carbopol® 934P -HPMC and HA layers degradation are almost fast, however, the backing layer can change the disin-

tegration behavior of the final film and pronounce the roll of film matrices in drug release as well.

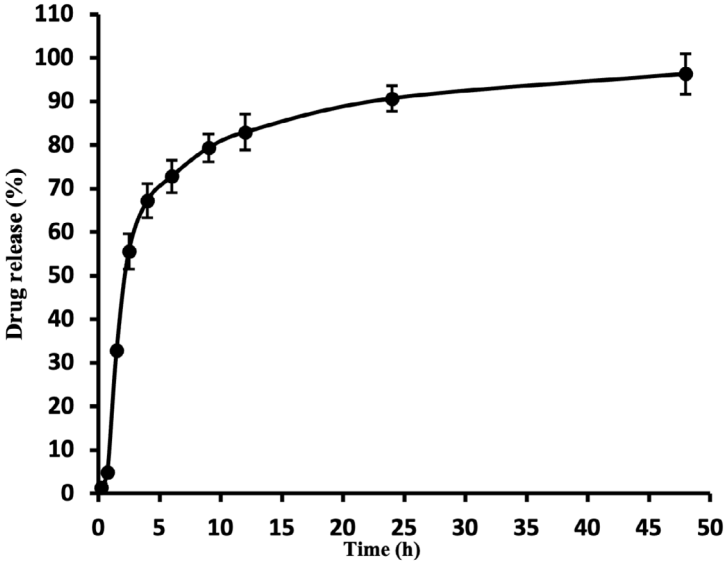


Figure 6. *In vitro* release profile of Sumatriptan from the three-layered mucoadhesive film

Data was further fitted in various kinetics models to better understand the release mechanism.

Drug release kinetics

As drugs must reach the desired place in an appropriate and predictable amount, realizing the drug release mechanism and rate for each newly designed drug delivery system is necessary. A well-defined behavior of drug release kinetics is necessary for drug dosing. Drug release depends on the system's characteristics and environmental conditions, like temperature, pH, contents, etc. So, for the in vitro study of drug release, the experiment conditions must be strictly selected in a way to be similar to the real situation in the body.

System parameters, like the dosage form geometry, the accessible surface for drug release, uniformity of the drug distribution in the entire system, interactions between ingredients, and the way the dosage form is introduced to the body, all contribute to drug release behaviour[39]. In polymeric matrices, polymer microstructure, and crystallinity, swelling potency, polymer chain relaxation, polymer chain disentanglement (especially in non-cross-linked polymers), and polymer dissolution can control drug release.

Generally, diffusion, erosion, and swelling, alone or in combination, are the main drug release mechanisms. For example, in hydrophilic matrices, swelling is supposed to play a role in drug transport, and the water content of the system can affect drug diffusivity. Still, other factors must also be considered. For instance, the presence of a backing layer in mucoadhesive films can prevent water diffusion into the system, hamper the swelling of the matrix, and cause a unidirectional release of the article through a diffusion mechanism. In this case, the swelling and polymer dissolution contribution may be less substantial.

In our study, Sumatriptan is loaded in the liposome, and this nanoparticle is embedded in a hydrophilic matrix, i.e., HA film. Therefore, the drug must pass through the liposome bilayer and polymer gel to be released. A backing layer was also applied to make the molecules' movements unilateral toward the mucous.

To find the kinetics of drug release, data were graphically fitted in zero-order, first-order, Higuchi, and Korsmeyer-Peppas models, Figure 7. The best kinetic model was determined based on the coefficient of determination (R^2).

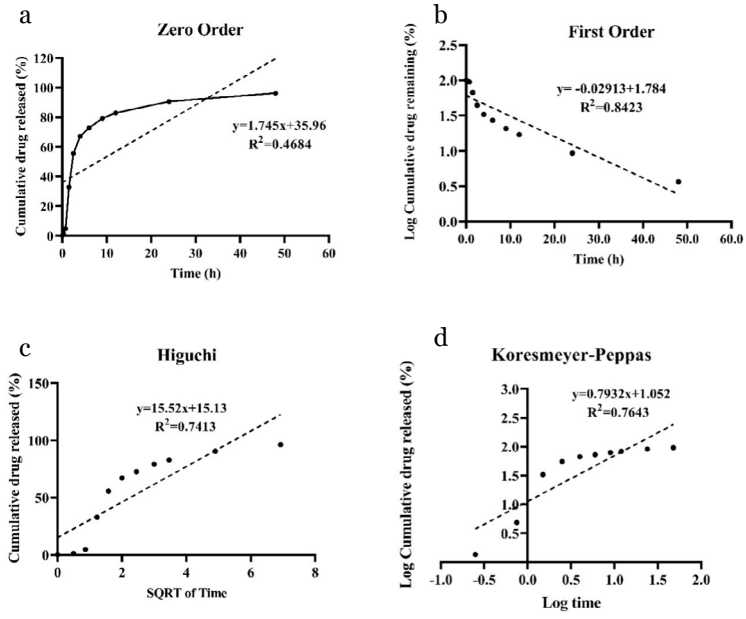


Figure 7. The Sumatriptan release data fitted in (a) Zero-order, (b) Higuchi, (c) First-order, and (d) Korsmeyer-Peppas kinetic models; GraphPad Prism is used for data graphing.

Zero-order, first-order, and Higuchi are related to diffusion and Fickian release. If other mechanisms are involved, other models, like Korsmeyer-Peppas and Weibull, can reveal them. Generally, Higuchi governs the molecule release from polymeric insoluble matrices where the dimensions of the polymer remain constant, for example, in non-eroding polymeric films. The penetrated medium into the matrix can dissolve the embedded drug hence Fickian drug release happens.

As mentioned, polymer chain glassy/rubbery transitions, relaxation, disentanglement, polymer dissolution, and surface erosion can also happen.

When a membrane (like a liposome bilayer) is in the way of molecule movement, the zero- or first-order is possible, depending on the molecule concentration. Zero-order rate is commonly seen in depot systems, where the amount is beyond release ability and the concentration gradient remains constant. In the first-order phenomenon, the rate depends on the concentration gradient; so the rate would be reduced as the drug is released over time.

In our designed system, all of these phenomena are likely to occur, which must be evaluated.

The best kinetic model was chosen based on the highest R^2 values, determined by the regression. According to Figure 7, R^2 had the highest value in the first-order model in which the rate is concentration gradient-dependent.

If the polymeric film does not limit the drug release and the release of Sumatriptan from the nanoliposome is the rate-limiting step, then it can be concluded that the drug concentration decreases inside the nanocarrier with time; consequently, the rate of drug release from the system decreases. Indeed, the R^2 is not satisfactorily large, i.e., less than 0.9. It can be concluded that the simple Fickian diffusion does not lonely govern the drug release, and probably the HA matrix also collaborates in drug release.

DDSolver (v 1.0; an Excel add-in software) software was used to analyze the untransformed data. Zero-order, first-order, Higuchi, Makoid-Banakar, Korsmeyer-Peppas, Weibull, and Peppas-Sahlin models were considered. The best model was selected based on the highest adjusted R^2 and model selection criterion (MSC), and lowest Akaike information criterion (AIC). As shown in Table 3, among the diffusion-based models, drug release behavior is based on the first-order mechanism (adjusted R^2=0.95, AIC=72.69, and MSC=2.76), which is in accordance with the graphical result.

Table 3. Sumatriptan release evaluation by fitting release data to mathematical kinetics models; the model parameters are calculated using DDSolver to find out the goodness of fit.

Model	Equation*	Rate constant	Model parameters
Zero Order	$F = k_0 \times t$ $D_t = D_0 + k_0 t$	$k_0 = 2.972$	R^2_adj = -0.22 AIC = 108.98 MSC = -0.61
First-Order	$F = 100\ (1-e^{-k\_1})$ $\log C = \log C_0 - k_1\ t/2.303$	$k_1 = 0.234$	R^2_adj = 0.95 AIC = 72.69 MSC = 2.68
Higuchi	$F = k\_{H} \times t^{\wedge 1/2}$	$k_H = 19.287$	R^2_adj = 0.66 AIC = 94.58 MSC = 0.69

*k_o, k_1 and k_H are zero-order, first-order, and Higuchi rate constants, respectively; t is the time; F is the fraction of the drug that is released at the time t; D_t is the amount of the drug that is released at the time t; D_o is the initial amount of the drug; C is the drug concentration at the time t and C_o is the initial concentration of the drug in the medium.

Among the models for finding the underlying release mechanism, Table 4, the Weibull model showed the best data fitting (R^2_adj=0.95, AIC=73.69, and MSC=2.59). Since the shape parameter (β) in the Weibull model is 0.864, which is between 0.75 and one, it indicates the release rate is reduced over time, and a combined mechanism, swelling along with diffusion, plays a role in drug release[40,41]. The presence of the backing layer and the mucoadhesive layer can delay the fairly fast disintegration time of the HA film and reinforce its effect on drug release. This finding was also confirmed by n=0.56 in the Makoid-Banakar model (R^2_adj=0.91). The n=0.563 (between 0.5 and one) implies the anomalous mechanism.

Table 4. The used mechanism models for Sumatriptan release evaluation; DDSolver calculated the model parameters to find out the goodness of fit.

Model	Equation*	Rate constant	Other parameters	Model parameters
Makoid-Banakar	$F = k_{MB}\, t^n e^{-kt}$	$k_{MB} = 27.719$	n= 0.563 k= 0.021	R^2_adj = 0.91 AIC = 81.06 MSC = 1.92
Korsmeyer-Peppas	$F = k_{KP}\, t^n$	$k_{KP} = 34.787$	n= 0.303	R^2_adj = 0.76 AIC = 80.84 MSC = 1.18
Weibull	$F = 100\ (1-e^{-\frac{t^\beta}{\alpha}})$		α=3.655 β=0.864	R^2_adj = 0.95 AIC = 73.69 MSC = 2.59
Peppas-Sahlin	$F = k_1\, t^m + k_2 t^{2m}$	$k_1 = 30.741$ $k_2 = -2.313$	m= 0.558	R^2_adj = 0.91 AIC = 81.5 MSC = 1.88

*k_{MB}, k_{KP}, are Makoid-Banakar, and Korsmeyer-Peppas rate constants, respectively; k_1 and k_2 are Peppas-Sahlin constants; t is the time; F is the fraction of the drug that is released at the time t; n is exponent power; m is Fickian diffusional coefficient; α is the scale factor, and β is the shape factor.

The data did not satisfactorily fit in the Korsmeyer-Peppas (R^2_adj=0.76).

Peppas-Sahlin (R^2_adj=0.91) approximates the contribution of diffusion and polymer relaxation in an anomalous drug release. The model equation consists of two terms: $F = k_1t^m + k_2t^m$; the m exponent in this equation is the Fickian diffusion exponent. The contribution of diffusion (F) and polymer relaxation (R) can be calculated using the model equation or $\frac{R}{F} = \frac{k_2 t^m}{k_1}$. For our system, the k_1 and k_2 coefficients were 30.741 and -2.313, respectively, and m was equal to 0.558[42]. Figure 8 shows the contribution of each mechanism over time; the diffusion is prominent in the early hours and reduced to 60% at the end of drug release. As approximately 80% of the drug is released during nine hours, the involvement of diffusion at this time is still about 80%.

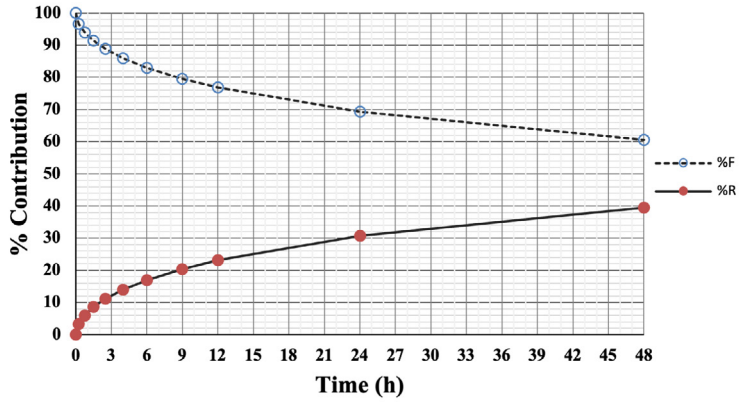


Figure 8. The % contribution of diffusion and polymer relaxation in drug release, based on the Peppas-Sahlin model.

The correlation between the observed amount of Sumatriptan released and the anticipated amounts by different kinetics models are shown in Figure 9 which is in line with mathematical analysis data.

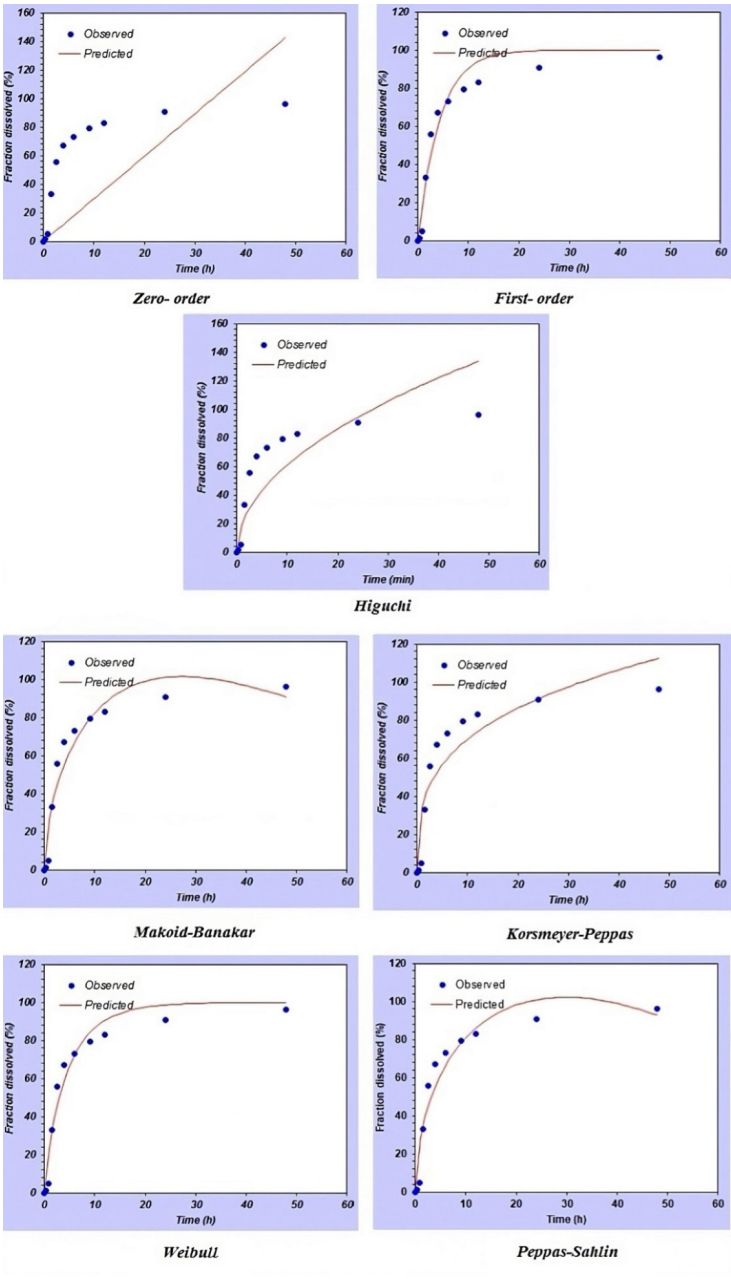


Figure 9. The correlation between the observed amount of Sumatriptan released, and the anticipated amount is based on different kinetics models.

From all these observations, it can be deduced that both the film and liposome can control the drug transport; probably drug was released from the liposome by diffusion (possibly by first-order mechanism), and film swelling at the next step let the drug leach out. As the role of diffusion is more predominant, exiting from liposome is the rate-limiting step. It is preferred the drug does not leave the liposome readily; this is confirmed by extended drug release, which is more dependent on the drug leaving the liposome.

It must be noticed that the liposomes with unreleased drugs can also penetrate biomembranes, while in our release test, the dialysis film was in the way of liposomes, and only the released drugs were evaluated.

In this study, a three-layered mucoadhesive buccal film for the delivery of Sumatriptan was designed and characterized. A positively charged liposome was used as the drug carrier as it can interact with mucus and enhance drug permeation. The system showed a good mucoadhesive feature which is helpful to keep the drug in contact with the mucus and give it enough time to be absorbed. The backing layer made the drug transport unilateral and also affected drug release behavior. The results showed that a predominant diffusion and polymer relaxation mechanisms are involved in drug release which is mainly dependent on the drug diffusion through the liposome bilayer (by first-order mechanism) rather than being controlled by the polymeric matrices. In other words, the system can help keep the drug sufficiently in contact with the mucosa, whereas liposomes can control the drug release and aid its penetration. As the zero-order mechanism has the advantage of releasing drugs at a constant rate, changing the first-order to zero-order can be achieved by increasing the drug loading in liposomes and turning them into drug depots.

As our system showed desirable features, it can also be used for other hydrophilic drugs. Although ex vivo and in vivo studies, like cytotoxicity, mucoadhesion using animal models, and drug penetration through epithelial cells, need to be performed to empower the data, which are now underway in our laboratory.

STATEMENT OF ETHICS

This study has received the Ethics Code of IR.LUMS.REC.1400.147 from the Ethics Committee of Lorestan University of Medical Sciences on September 9, 2021.

CONFLICT OF INTEREST STATEMENT

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: NT; Methodology: MHA; Formal analysis and investigation: MHA, NA; Writing - original draft preparation: ZA; Writing - review and editing: NT; Supervision: NT.

FUNDING SOURCES

No funding was received for this study.

ACKNOWLEDGMENTS

The authors declare that no acknowledgments are applicable for this work.

REFERENCES

1. Silva BM, Borges AF, Silva C, Coelho JF, Simões S. Mucoadhesive oral films: the potential for unmet needs. Int J Pharm, 2015;494(1):537-551. Doi: 10.1016/j.ijpharm.2015.08.038

2. Gilhotra RM, Ikram M, Srivastava S, Gilhotra N. A clinical perspective on mucoadhesive buccal drug delivery systems. J Biomed Res, 2014;28(2):81-97. Doi: 10.7555/JBR.27.20120136

3. Abd El Azim H, Nafee N, Ramadan A, Khalafallah N. Liposomal buccal mucoadhesive film for improved delivery and permeation of water-soluble vitamins. Int J Pharm, 2015;488(1-2):78-85. Doi: 10.1016/j.ijpharm.2015.04.052

4. Chen J, Duan H, Pan H, Yang X, Pan W. Two types of core/shell fibers based on carboxymethyl chitosan and Sodium carboxymethyl cellulose with self-assembled liposome for buccal delivery of carvedilol across TR146 cell culture and porcine buccal mucosa. Int J Biol Macromol, 2019;128:700-709. Doi: 10.1016/j.ijbiomac.2019.01.143

5. Chen J, Pan H, Yang Y, Xiong S, Duan H, Yang X, et al. Self-assembled liposome from multi-layered fibrous mucoadhesive membrane for buccal delivery of drugs having high first-pass metabolism. Int J Pharm, 2018;547(1-2):303-314. Doi: 10.1016/j.ijpharm.2018.05.062

6. Salamat-Miller N, Chittchang M, Johnston TP. The use of mucoadhesive polymers in buccal drug delivery. Adv Drug Deliv Rev, 2005;57(11):1666-1691. Doi: 10.1016/j.addr.2005.07.003

7. Abruzzo A, Bigucci F, Cerchiara T, Cruciani F, Vitali B, Luppi B. Mucoadhesive chitosan/gelatin films for buccal delivery of propranolol hydrochloride. Carbohydr Polym, 2012;87(1):581-588. Doi: 10.1016/j.carbpol.2011.08.024

8. Teubl BJ, Absenger M, Fröhlich E, Leitinger G, Zimmer A, Roblegg E. The oral cavity as a biological barrier system: design of an advanced buccal in vitro permeability model. Eur J Pharm Biopharm, 2013;84(2):386-393. Doi: 10.1016/j.ejpb.2012.10.021

9. Andrews GP, Laverty TP, Jones DS. Mucoadhesive polymeric platforms for controlled drug delivery. Eur J Pharm Biopharm, 2009;71(3):505-518. Doi: 10.1016/j.ejpb.2008.09.028

10. Russo E, Selmin F, Baldassari S, Gennari C, Caviglioli G, Cilurzo F, et al. A focus on mucoadhesive polymers and their application in buccal dosage forms. J Drug Deliv Sci Technol, 2016;32:113-125. Doi: 10.1016/j.jddst.2015.06.016

11. Singla AK, Chawla M, Singh A. Potential applications of carbomer in oral mucoadhesive controlled drug delivery system: a review. Drug Dev Ind Pharm, 2000;26(9):913-924. Doi: 10.1081/ddc-100101318

12. Chen X, Yan J, Yu S, Wang P. Formulation and *in vitro* release kinetics of mucoadhesive blend gels containing matrine for buccal administration. AAPS PharmSciTech, 2018;19(1):470-480. Doi: 10.1208/s12249-017-0853-7

13. Kraisit P, Limmatvapirat S, Luangtana-Anan M, Sriamornsak P. Buccal administration of mucoadhesive blend films saturated with propranolol loaded nanoparticles. Asian J Pharm Sci, 2018;13(1):34-43. Doi: 10.1016/j.ajps.2017.07.006

14. Alemdar N. Fabrication of a novel bone ash-reinforced gelatin/alginate/hyaluronic acid composite film for controlled drug delivery. Carbohydr Polym, 2016;151:1019-1026. Doi: 10.1016/j.carbpol.2016.06.033

15. Tighsazzadeh M, Mitchell JC, Boateng JS. Development and evaluation of performance characteristics of timolol-loaded composite ocular films as potential delivery platforms for treatment of glaucoma. Int J Pharm, 2019;566:111-125. Doi: 10.1016/j.ijpharm.2019.05.059

16. Ding J, He R, Zhou G, Tang C, Yin C. Multilayered mucoadhesive hydrogel films based on thiolated hyaluronic acid and polyvinylalcohol for insulin delivery. Acta Biomater, 2012;8(10):3643-3651. Doi: 10.1016/j.actbio.2012.06.027

17. Birudaraj R, Mahalingam R, Li X, Jasti BR. Advances in buccal drug delivery. Crit Rev Ther Drug Carr Syst, 2005;22(3). Doi: 10.1615/critrevtherdrugcarriersyst.v22.i3.20

18. Rossi S, Sandri G, Caramella CM. Buccal drug delivery: a challenge already won? Drug Discov Today Technol, 2005;2(1):59-65. Doi: 0.1016/j.ddtec.2005.05.018

19. Landová H, Daněk Z, Gajdziok J, Vetchý D, Stembirek J. Mucoadhesive films as perspective oral dosage form. Ceska Slov Farm, 2013;62(1):4-11.

20. Cavallari C, Fini A, Ospitali F. Mucoadhesive multiparticulate patch for the intrabuccal controlled delivery of lidocaine. Eur J Pharm Biopharm, 2013;83(3):405-414. Doi: 10.1016/j.ejpb.2012.10.004

21. He C, Cui F, Yin L, Qian F, Tang C, Yin C. A polymeric composite carrier for oral delivery of peptide drugs: bilaminated hydrogel film loaded with nanoparticles. Eur Polym J, 2009;45(2):368-376. Doi: 10.1016/j.eurpolymj.2008.11.004

22. Lai SK, Wang Y-Y, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev, 2009;61(2):158-171. Doi: 10.1016/j.addr.2008.11.002

23. Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang Y-Y, Cone R, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. PNAS, 2007;104(5):1482-1487. Doi: 10.1073/pnas.0608611104

24. Tan Y-l, Liu C-G. Preparation and characterization of self-assemblied nanoparticles based on folic acid modified carboxymethyl chitosan. J Mater Sci Mater Med, 2011;22(5):1213-1220. Doi: 10.1007/s10856-011-4302-y

25. Kumar A, Badde S, Kamble R, Pokharkar VB. Development and characterization of liposomal drug delivery system for nimesulide. Int J Pharm Pharm Sci, 2010;2(4):87-89.

26. Xu X, Khan MA, Burgess DJ. Predicting hydrophilic drug encapsulation inside unilamellar liposomes. Int J Pharm, 2012;423(2):410-418. Doi: 10.1016/j.ijpharm.2011.12.019

27. Gregoriadis G. Liposome technology: interactions of liposomes with the biological milieu. 3rd ed. Boca Raton (FL), CRC Press; 2006.

28. Zhang H. Thin-film hydration followed by extrusion method for liposome preparation. In: Gregoriadis G, editor. Liposomes. New York: Springer; 2017. p. 17-22.

29. Villasmil-Sánchez S, Drhimeur W, Ospino SCS, Rabasco Alvarez AM, González-Rodríguez M. Positively and negatively charged liposomes as carriers for transdermal delivery of sumatriptan: in vitro characterization. Drug Dev Ind Pharm, 2010;36(6):666-675. Doi: 10.3109/03639040903419640

30. Kim TH, Ahn JS, Choi HK, Choi YJ, Cho CS. A novel mucoadhesive polymer film composed of carbopol, poloxamer and hydroxypropylmethylcellulose. Arch Pharm Res, 2007;30(3):381-386. Doi: 0.1007/BF02977622

31. Nowakowska-Toporowska A, Malecka K, Raszewski Z, Wieckiewicz W. Changes in hardness of addition-polymerizing silicone-resilient denture liners after storage in artificial saliva. Journal Prosthet Dent, 2019;121(2):317-321. Doi: 10.1016/j.prosdent.2018.05.002

32. Akbari J, Nokhodchi A, Farid D, Adrangui M, Siahi-Shadbad MR, Saeedi M. Development and evaluation of buccoadhesive propranolol hydrochloride tablet formulations: effect of fillers. Il Farmaco, 2004;59(2):155-161. Doi: 10.1016/j.farmac.2003.11.011

33. Assadpour S, Shiran MR, Akhtari J. Chitosan coating of anionic liposomes containing sumatriptan succinate: a candidate for nasal administration. Nanomed J, 2021;8(2):132-139. Doi: 10.22038/NMJ.2021.08.006

34. Assadpour S, Akhtari J, Shiran MR. Pharmacokinetics study of chitosan-coated liposomes containing sumatriptan in the treatment of migraine. Caspian J Intern Med, 2022;13(1):90-99. Doi: 10.22088/cjim.13.1.90

35. Villasmil-Sánchez S, Rabasco AM, González-Rodríguez ML. Thermal and ^{31}P-NMR studies to elucidate sumatriptan succinate entrapment behavior in phosphatidylcholine/cholesterol liposomes. Comparative ^{31}P-NMR analysis on negatively and positively-charged liposomes. Colloids Surf B Biointerfaces, 2013;105:14-23. Doi: 10.1016/j.colsurfb.2012.12.019

36. Tasharrofi N, Nourozi M, Marzban A. How liposomes pave the way for ocular drug delivery after topical administration. J Drug Deliv Sci Technol, 2022;67:103045. Doi: 10.1016/j.jddst.2021.103045

37. Patel VF, Liu F, Brown MB. Advances in oral transmucosal drug delivery. J Control Release, 2011;153(2):106-116. Doi: 10.1016/j.jconrel.2011.01.027

38. Jain A, Jain SK. *In vitro* release kinetics model fitting of liposomes: an insight. Chem Phys Lipids, 2016;201:28-40. Doi: 10.1016/j.chemphyslip.2016.10.005

39. Peppas NA, Narasimhan B. Mathematical models in drug delivery: how modeling has shaped the way we design new drug delivery systems. J Control Release, 2014;190:75-81. Doi: 10.1016/j.jconrel.2014.06.041

40. Paolino D, Tudose A, Celia C, Di Marzio L, Cilurzo F, Mircioiu C. Mathematical models as tools to predict the release kinetic of fluorescein from lyotropic colloidal liquid crystals. Materials, 2019;12(5):693. Doi: 10.3390/ma12050693

41. Papadopoulou V, Kosmidis K, Vlachou M, Macheras P. On the use of the Weibull function for the discernment of drug release mechanisms. Int J Pharm, 2006;309(1-2):44-50. Doi: 10.1016/j.ijpharm.2005.10.044

42. Luciano M. Mathematical models of drug release. In: Bruschi ML, editors. Strategies to modify the drug release from pharmaceutical systems. 2015. p. 63-86. Doi: 10.1016/B978-0-08-100092-2.00005-9

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6334

Antibacterial activity and UPLC analysis of *Hypericum perforatum* L. extracts

Elif GÜZEL[1,2*], Mukhlesur RAHMAN[3]

1 Istanbul Medipol University, Institute of Health Sciences, Istanbul, Türkiye

2 Istanbul Medipol University, School of Medicine, Department of Medical Pharmacology, Istanbul, Türkiye

3 University of East London, School of Health, Sport and Bioscience, Department of Bioscience, Medicines Research Group, London, UK, United Kingdom

ABSTRACT

Hypericum perforatum L. has been used for centuries as a herbal remedy against variety of diseases for its biological activities. It has been also used as an antibacterial agent against different bacteria. The aim of this study is to investigate the antibacterial properties of the *H. perforatum* L. extracts against several clinical isolates and explore their chemical composition. In terms of *S. aureus* strains, *n*-hexane extract demonstrated the best activity against XU212 strain, with the MIC of 256 μL/mL. The MIC was 512 μL/mL for other *S. aureus* strains for *n*-hexane and dichloromethane extracts. *n*-hexane extract demonstrated activity against *E. coli* ATCC 25922 with the MIC of 1 μL/mL. UPLC was performed for dichloromethane and methanol extracts. The main compounds were identified as catechin, hyperforin, and rutin from methanol extract, and hypericin and luteoskyrin from dichloromethane extract. *S. aureus* demonstrated good antibacterial activity against tested MRSA and *Bacillus subtilis* strains.

Keywords: *Hypericum perforatum* L., extract, antibacterial activity, MRSA, UPLC

* Corresponding author: Elif GÜZEL
E-mail: elif.guzel@medipol.edu.tr
ORCIDs:
Elif GÜZEL: 0000-0002-2737-0568
Mukhlesur RAHMAN: 0000-0001-8180-7970
(Received 9 Jan 2025, Accepted 12 Mar 2025)

INTRODUCTION

Staphylococcus aureus commonly presents in the mucosal surfaces and the skin of the human body, and it is a Gram-positive bacterium[1]. When there is a breach on the mucosal surfaces or the skin, *S. aureus* enters the body, and the infection is initiated. For centuries, *S. aureus* has been one of the widespread and life-threatening causes of infections in health-care settings, because the bacteria mostly spread to the adjacent organs and cause severe invasive infections such as bacteremia or pneumonia. *S. aureus* has been identified as one of the six bacteria that are the most dangerous nosocomial infections in many countries, including the USA, the UK, and Canada, by The Infectious Diseases Society of America[2]. According to a report published by WHO in 2014, which is about antibiotic resistance surveillance, *S. aureus* spread is increasing in all continents. Some countries reported the number of *S. aureus* infections up to 80% which results in longer hospital stays or the use of a second-line antibiotic treatment[3]. Significant number of the bacteria that have been isolated from the patients mostly in ventilators or from the surgical site of the patient, approximately 43-58%, was identified as Methicillin Resistant *Staphylococcus aureus* (MRSA)[4]. According to a survey which was carried out by the European Centre for Disease Control and Prevention (ECDC), which includes 33 different countries in Europe, it has been recorded that *E. coli* is the first and *S. aureus* is the second most common cause of nosocomial infections[5].

Bacteria can acquire resistance against antibacterial therapeutics via different routes such as efflux pump activation, enzymatic destruction of the bacteria, modification of antibacterial agent's enzymes, and target site alteration of the antibacterial agents. Efflux pumps have a crucial role in bacterial resistance mechanisms as they work as an export system for antibacterial agents. Throughout the efflux pumps, an antibacterial agent is pushed out of the bacteria faster than it gets in, as a result, antibiotic resistance is seen in bacteria. Because of this mechanism, efflux pumps are one of the most important target sites for potential antibacterial therapeutics against multidrug-resistant bacteria. Therefore, developing novel antibacterial therapeutics that can prevent bacteria to efflux the antibacterial agent is an emerging issue today.

Several synthetic antibacterial agents are widely used around the world; however, plant-based antibacterial agents still attract many of the researchers[6]. Antibacterial compounds derived from natural sources have shown significant results against several multidrug-resistant bacteria[7]. Depending on the chemical structures of these naturally derived antibacterial compounds, they can be mainly classified as alkaloids, terpenoids, polyphenols, and sulphur containing compounds[8].

Hypericum perforatum L. is a perennial shrub and has yellow flowers. It has five sepals and petals in its flowers. The plant has opposite leaves, and, in its stamen, it has five bundles[9]. *H. perforatum* L. is in the family *Clusiaceae,* which includes around 400 different species worldwide[10]. *Hypericum perforatum* L. is mainly native to Western Asia, North Africa, and around Europe, however, it is also distributed among Australia and North America[11]. For centuries, *Hypericum perforatum* L. has been used as a herbal remedy for several diseases such as skin lesions, gastrointestinal tract diseases, anxiety and depression, mucosal lesions, and superficial injuries, and as a nursing remedy[11]. Today, different preparations of dried and fresh *Hypericum perforatum* L. are used for various purposes. Fresh plant species are used as a mother tincture in homeopathy as drops. Also, oil of the plant species is used for ointments and capsules. Dried extract of *Hypericum perforatum* L. is used for tablets and capsules, fluid extract is used for ointments and tinctures, and dried raw preparations are used as tea[10]. The aim of this study is to investigate the antibacterial properties of *H. perforatum* L. extracts against clinical isolates of MRSA and determine its chemical composition by Ultra Performance Liquid Chromatography (UPLC) analysis.

METHODOLOGY

Plant material

Hypericum perforatum L. plant's dried aerial parts were obtained from Herbal Apothecary, UK, with batch number 15522, in October 2012.

Preparation of plant extracts

14 grams of plant material was grinded and used for extraction, and the Soxhlet extraction method was used. Increasing polarity of 150 mL of 3 different solvents were used to obtain extracts, including *n*-hexane, dichloromethane, and methanol, respectively. After the extraction, the residual solvent was evaporated with a rotary evaporator (Heidolph, Germany).

Bacterial strains

The standard Gram-positive bacteria (*S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633) and Gram-negative bacteria (*E. coli* ATCC 25922) were used in this study. Additionally, a series of MRSA strains such as *S. aureus* SA 1199B and *S. aureus* XU212 were used. *S. aureus* XU212 is a tetracycline resistant strain as it overexpresses *tetK* efflux pump[12], and *S. aureus* SA 1199B is an Muti Drug Resistant (MDR) strain that overexpresses *norA* MDR efflux pump[13].

Antibacterial assay

Recommended protocol by the British Society for Antimicrobial Chemotherapy (BSAC) was followed to determine the Minimum Inhibitory Concentration (MIC) of the plant extracts and the antibiotic, against all the tested bacteria[14]. The broth microdilution technique was performed in duplicate.

The bacteria were sub-cultured on nutrient agar (Oxoid) prior to the antibacterial assay and incubated at 37°C for 18 hours. Cation levels of Mueller-Hinton Broth (Oxoid) were adjusted to include 20 mg/L of Ca^{2+} (Acros Organics) and 10 mg/L of Mg^{2+} (Acros Organics). Norfloxacin (Sigma Chemical Co.) was used as an antibiotic for positive control. To prepare the stock solution, Norfloxacin was dissolved in DMSO (Dimethyl Sulfoxide) (Sigma-Aldrich), and then further dilution was done with Mueller-Hinton Broth to obtain a final concentration of 128 µL/mL. Extracts were dissolved in DMSO to prepare the stock solutions for the extracts. Then, further dilution was done with Mueller-Hinton Broth to obtain a final concentration of 512 µL/mL. Test organisms were prepared in saline water (0.9% NaCl) with 5 x 10^5 cfu inoculum density and compared with 0.5 MacFarland turbidity standard.

96 well plate was used for the determination of MIC against each bacterium. 100 µL of Mueller-Hinton Broth was dispensed to the wells from columns 1 to 11. 100 µL of samples were dispensed to the first column as follows; wells A and B for *n*-hexane extract, wells C and D for dichloromethane extract, wells E and F for methanol extract, and wells G and H for Norfloxacin. Serial dilution was done starting from the first column up to column 12 by skipping column 11 which was used as growth control. 100 µL of bacterial suspension was dispensed to all wells except column 12 which was used as sterility control. All the plates were incubated at 37°C for 18 hours. After that, 5 mg/mL methanolic MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Alfa Aesar) solution was prepared, and 20 µL was dispensed to all wells. Then incubated for 20 minutes at 37°C to observe the color change. The blue color indicated bacterial growth, and the MIC of all the extracts and antibiotics was recorded. The antibacterial activity of extracts was evaluated in comparison with the positive control, Norfloxacin. The MIC of the extracts and the antibiotic were determined by looking at the lowest concentration where no bacterial growth was seen. The results of the antibacterial assay are provided in the "Table 1".

UPLC analysis

Chromatographic separation was done with normal phase Ultra Performance Liquid Chromatography technique for dichloromethane and methanol extracts. Sample concentrations were prepared as 1 mg/mL initially with HPLC grade methanol, then further diluted with the ratio of 1:4 with the same solvent. A 0.22 µm pore size filter was used to filter samples before the analysis. Agilent Technologies 1260 Infinity Series was used with Agilent Technologies Poroshell 120 EC-C18 column with the column size of 3 x 50 mm, and 2.7 µm particle size. Two elution binary gradients were used. As a mobile phase, HPLC-grade water was used as an aqueous, and HPLC-grade acetonitrile was used as an organic phase. They were acidified with Trifluoroacetic acid (TFA) with 0.01% ratio, and then further filtered with a 0.22 µm pore size filter. The flow rate was 0.750 mL/min, and the pressure was 242.43 bars. Column temperature was between 18.61°C to 18.85°C. The injection volume was 5.00 µL, and full loop injection was used. From 0 to 2 min 100% A (0.01% TFA in water), from 2 to 3.5 min 100% A, from 3.5 to 5.5 min 100% B (0.01% TFA in acetonitrile), from 5.5 to 6 min 100% B and at the min 6 100% A was used. A Photodiode Array (PDA) detector was used to detect chemical compounds. Separation was performed under 3 different wavelengths which were 210 nm, 260 nm, and 350 nm, and all the chromatograms were recorded. The run time was 6 minutes for each sample.

RESULTS and DISCUSSION

Antibacterial assay

All the plant extracts were tested for *in vitro* antibacterial activity with broth micro-dilution assay to determine the MIC. All the antibacterial activity was evaluated in comparison with the positive control, Norfloxacin. Among all extracts, *n*-hexane extract demonstrated the highest antibacterial property against all the tested bacteria, whereas methanol extract did not have activity against any of the tested bacteria.

n-hexane extracts showed the best activity against the *S. aureus* XU212 strain which is the tetracycline resistant strain. MIC against *S. aureus* XU212 was 256 µL/mL for *n*-hexane extract. MIC of *n*-hexane extract against *S. aureus* ATCC 25923 standard strain and *S. aureus* SA 1199B strain, which overexpress *norA* MDR efflux pump, was same and 512 µL/mL. MIC of dichloromethane extract against all the tested *S. aureus* strains was the same, which was 512 µL/mL. The MIC of the Norfloxacin was recorded as 16 µL/mL for *S. aureus* XU212 strain, and 64 µL/mL for *S. aureus* ATCC 25923 standard strain and *S. aureus* SA 1199B. Methanol extract was not effective against any of the tested *S. aureus* strains.

n-hexane extract demonstrated the best antibacterial activity against tested *B. subtilis* ATCC 6633 strain. MIC was between 64 – 128 µL/mL. And the MIC of dichloromethane extract was between 256 – 512 µL/mL. The MIC of Norfloxacin was recorded as 8 µL/mL, and methanol extract was not effective against this bacterium. One bacterium was used as a Gram-negative bacterium which was *E. coli* ATCC 25922. *n*-hexane extract had the best activity with the MIC of 1 µL/mL. The MIC of Norfloxacin was recorded as 0.25 µL/mL. Dichloromethane and methanol extracts did not show any activity against this bacterium.

The summary of the results of the antibacterial assay is provided in "Table 1" below.

Table 1. MIC of the plant extracts and the antibiotic

Bacteria	Description	MIC (µL/mL)		
		n-hexane	Dichloromethane	Norfloxacin
S. aureus ATCC 25923	Standard strain	512	512	64
S. aureus XU212	*tetK* efflux pump, tetracycline-resistant	256	512	16
S. aureus SA 1199B	*norA* efflux pump, MDR strain	512	512	64
B. subtilis ATCC 6622	Commonly used strain	64 – 128	256 – 512	8
E. coli ATCC 25922	Commonly used strain	1	–	0.25

UPLC analysis

The chemical composition of dichloromethane and methanol extract of *Hypericum perforatum* L. was determined with normal phase UPLC analysis using a gradient mobile phase consisting of HPLC grade water as an aqueous phase and HPLC grade acetonitrile as an organic phase. The analysis was conducted with 3 different wavelengths including 210 nm, 260 nm, and 350 nm. The run time was 6 minutes for each extract and all wavelengths. UPLC analysis was not carried out for *n*-hexane extract because of its highly non-polar nature.

Different compounds were observed with different wavelengths for methanol extract. 4 peaks and 6 peaks were observed under 210 nm and 350 nm, respectively. However, the best separation for methanol extract was seen under 260 nm. There were 8 different compounds with different peaks. The highest peak was observed at the retention time (RT) of 3.659 min, and determined as the major compound, as Hyperforin, which is the fourth compound in the chromatogram.

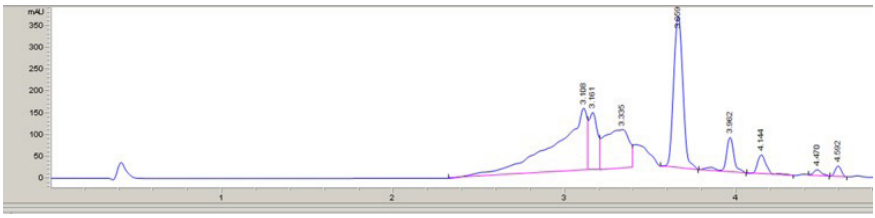


Figure 1. UPLC chromatogram of the methanol extract under 260 nm

Previous studies have been performed to investigate the chemical composition of the methanolic extracts of *H. perforatum*. Depending on those studies, compounds found with UPLC were determined by comparing the retention time. Previous studies have shown that the methanolic extract of the plant species includes catechin which was seen at 3.216 minutes under 275 nm. Compound number 2 in our analysis with the retention time of 3.108 min was determined as Catechin as the retention time and the observation wavelength is very close. The same study also determined Procyanidin B1 at the time of 3.383 min under 275 nm, and compound number 3 in our analysis is also believed to be Procyanidin B1 as the retention time is very close to 3.161 min, and our wavelength was 260 nm[15]. Another previous study was conducted to identify Hyperforin and its metabolites which are present in *Hypericum perforatum* L. by using UPLC, and it was reported that the standard Hyperforin was seen at the minute of 3.64. In our results, compound 4 has retention time of 3.659 min, which was determined as Hyperforin[16]. Similarly, Rutin was identified in the previous studies at 3.95 min under 255 nm. Our compound 5 with the retention time of 3.962 is identified as Rutin depending on the literature[15]. The same study also identified 2 derivatives of Quercetin which are Quercetin-3-O-galactoside (Hyperoside) and Quercetin-3-O-rhamnoside (Isoquercetrin) with the retention time of 4.083 min and 4.383 min, respectively. In our results, Compounds 6 and 7 have retention times of 4.144 min and 4.470, respectively. By comparing the literature, they were identified as Hyperoside and Isoquercetrin, respectively[15]. To determine the chemical composition of *Hypericum perforatum*, former studies were performed with HPLC for methanolic extract of the plant species as well. The study has reported that Quercetin was seen at 34.6 min for a 40 min run time HPLC analysis. By comparing the total run time and the time Quercetin was seen, our compound 8 was identified as Quercetin[17]. Compound 1 was unidentified as there is no proof of compound in the literature for the specific retention time and wavelength of this compound. The list of the compounds in the methanolic extract is provided below, in “Table 2”.

Table 2. Possible compounds found in the methanolic extract of *H. perforatum* L. via UPLC under 260 nm

#	RT (min)	Area %	Possible Compound	Reference
1	3.108	37.810	Unknown	–
2	3.161	8.886	Catechin	(15)
3	3.335	17.438	Procyanidin B1	(15)
4	3.659	25.519	Hyperforin	(16)
5	3.962	5.196	Rutin	(15)
6	4.144	3.081	Quercetin-3-O-galactoside (Hyperoside)	(15)
7	4.470	0.898	Quercetin-3-O-rhamnoside (Isoquercetrin)	(15)
8	4.592	1.172	Quercetin	(17)

The dichloromethane extract was observed under 3 different wavelengths as well. No peak was observed under 350 nm, and 14 peaks were observed under 210 nm. However, the best separation was observed under 260 nm again, with 13 peaks. The highest peak was seen at the retention time of 3.667 min.

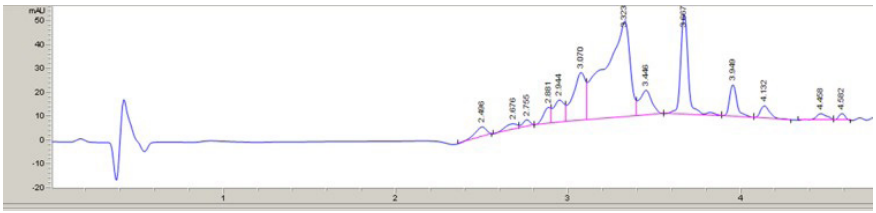


Figure 2. UPLC chromatogram of the dichloromethane extract under 260 nm

Because of the limited number of previous studies of HPLC and UPLC analysis of dichloromethane extract of *H. perforatum*, the majority of the compounds could not be determined by comparing the literature. Therefore, the main compounds at the retention time of 3.323 min and 3.667 min could not identified. A recent UPLC-MS study was carried out to determine the naphthodianthrones, emodin, skyrin, and bisanthrones in the *H. perforatum* L. extracts. The extraction was performed with several solvents including methanol, ethanol, ethyl acetate, acetone, and dichloromethane with changing ratios. This UPLC-MS analysis suggested that the extracts of the plant species are rich in the naphthodianthrones and bisanthrones. UPLC run time was 13 minutes in the mentioned study and

depending on the ratio of the UPLC run time and the time where compounds are observed, some possible compounds are determined in our analysis. Depending on the ratio, Luteoskyrin was seen at 2.673 min, and our compound 2 was determined as Luteoskyrin as it has retention time of 2.676 min. Similarly, depending on the ratio, the study identified Protohypericin at 2.876, and compound 4 was determined as Protohypericin as it has very close retention time of 2.881 min. And lastly, the previous study identified Hypericin at 3.165 min, and our compound 6 has a close retention time of 3.07 min, as a result, it was identified as Hypericin[18]. Due to the lack of previous studies, the rest of the compounds could not be identified. The list of the identified compounds and the retention times of the unknown compounds are provided below, in "Table 3".

Table 3. Possible compounds found in the dichloromethane extract of *H. perforatum* L. via UPLC under 260 nm

#	RT (min)	Area %	Possible Compound	Reference
1	2.496	2.321	Unknown	–
2	2.676	1.432	Luteoskyrin	(18)
3	2.755	0.940	Unknown	–
4	2.881	2.428	Protohypericin	(18)
5	2.944	4.602	Unknown	–
6	3.07	11.879	Hypericin	(18)
7	3.323	46.102	Unknown	–
8	3.446	5.688	Unknown	–
9	3.667	12.225	Unknown	–
10	3.949	5.173	Unknown	–
11	4.132	4.013	Unknown	–
12	4.458	2.176	Unknown	–
13	4.582	1.021	Unknown	–

The summary of the identified compounds by UPLC analysis has been shown below in "Table 4".

Table 4. Summary of the possible compounds found in the dichloromethane and methanolic extracts of *H. perforatum* L. via UPLC under 260 nm

Extract	#	RT (min)	Area %	Possible Compound	Reference
Methanolic Extract	**1**	3.161	8.886	Catechin	(15)
	2	3.335	17.438	Procyanidin B1	(15)
	3	3.659	25.519	Hyperforin	(16)
	4	3.962	5.196	Rutin	(15)
	5	4.144	3.081	Quercetin-3-O-galactoside (Hyperoside)	(15)
	6	4.470	0.898	Quercetin-3-O-rhamnoside (Isoquercetrin)	(15)
	7	4.592	1.172	Quercetin	(17)
Dichloromethane Extract	**1**	2.676	1.432	Luteoskyrin	(18)
	2	2.881	2.428	Protohypericin	(18)
	3	3.07	11.879	Hypericin	(18)

The findings from both the antibacterial assays and UPLC analyses provide a comprehensive overview of the bioactive potential of the tested extracts, particularly highlighting the remarkable activity of the n-hexane extract. These results are now explored in detail to assess their implications and alignments with existing research as they provide new insights into the antibacterial properties of less-polar extracts, such as n-hexane, a relatively underexplored area in the literature. The discussion contextualizes these findings within the broader framework of antimicrobial research.

In the current study, *n*-hexane extract is the one that demonstrated the best inhibitory effect against all the tested bacteria. The majority of the previous studies were carried out with methanolic, ethanolic, or aqueous extracts of the plant species. Therefore, the knowledge of the bacterial inhibitory property of the extracts obtained with less polar solvents, such as *n*-hexane is limited[19]. The *n*-hexane extract showed the strongest anti-staphylococcal activity against the MRSA strain *S. aureus* XU212, which carries the *tetK* efflux pump, with a MIC value of 256 μL/mL. The rest of the MIC of *n*-hexane and dichloromethane extracts against all the tested clinical isolates of *S. aureus* strains were the same which was 512 μL/mL. Even though there are considerable number of studies in the literature about the bacterial inhibitory effect of methanolic extracts of the plant species, in this study, methanol extract did not show activity against any of the tested *S. aureus* strains[19,20,21]. This can be explained with several reasons including the solvents used to obtain the extract. Many of the studies used methanol as a solvent either directly or with slightly less polar solvents before using methanol, such as acetone or ethanol[19]. However, in this study less polar compounds were first used then, methanol was used to obtain crude extracts. The compounds that exhibit the antibacterial activity in the methanolic extracts in the previous studies might be extracted within the first two solvents, as the activity was the strongest in the *n*-hexane extract. Hence the inhibitory effect of methanolic extract was not observed in the current study. Another reason can be the type of the bacteria used in the MIC assay. Previous studies have demonstrated promising antibacterial activity against several MRSA and PRSA strains with MIC and disc diffusion techniques. It has been proven that *H. perforatum* L. has activity against different bacterial strains that were used in the previous study, including *S. aureus* (PRSA) E12431, *S. aureus* (PRSA) E12398, and *S. aureus* (MRSA) RV5 strains. It was also demonstrated that the plant has activity against *S. aureus* ATCC 25923 strain which is the standard strain used in the current study[22]. Even though the previous study has demonstrated inhibitory activity against the standard strain of *S. aureus* ATCC 25923, no activity was observed in the current study, and this can be explained by the reason mentioned above as the extraction was done with 3 different solvents, as opposed to the study performed previously, as the antibacterial activity was observed for both *n*-hexane and dichloromethane extracts against all the tested *S. aureus* strains with MIC ranging between 256 – 512 μL/mL. Finally, one of the reasons for the lack of antibacterial activity of methanolic extracts can be the extraction type used. In the current study, Soxhlet extraction was used to obtain the crude extracts. However, the extracts were obtained with different techniques including percolation methods, decoction, or supercritical fluid ex-

traction in the previous studies where the antibacterial activity was seen[23,24,25]. The type of extraction could affect the compounds within the extracts, hence the antibacterial activity. In terms of Gram-negative bacteria, *n*-hexane extract has a promising result, as it showed significant MIC of 1 µL/mL, which is the lowest concentration tested against *E. coli* ATCC 25922 strains. Previous studies demonstrated that hyperforin which was isolated from *H. perforatum* L. demonstrated antibacterial activity against the same bacterial strain with the MIC of 0.1 µL/mL[22]. The reason for the strong antibacterial activity of *n*-hexane extract against *E. coli* could be the presence of hyperforin. Thereby, further studies should be focused on this activity and isolating this compound specifically for definitive results. Regarding *B. subtilis*, again *n*-hexane extract had the highest inhibitory activity with the MIC ranging between 64 – 128 µL/mL. Previous studies have shown that extracts of *H. perforatum* L. including methanol extracts have an inhibitory effect against the same bacterial strain of *B. subtilis* ATCC 6633 with MIC ranging between 25 – 50 µL/mL[26]. The reason for the lack of inhibitory activity in the methanol extract could be again the solvents used for extraction. In the previous studies, extraction was done with several solvents with similar polarity index, together with methanol. However, in the current study, methanol was used as the last solvent. Thereby, the activity was the strongest for *n*-hexane extract, followed by dichloromethane extract with the MIC ranging between 256 – 512 µL/mL and no activity for methanol extract. The compounds that showed antibacterial activity within the methanol extract in the previous study might be extracted with the *n*-hexane and dichloromethane extracts. This can be identified by isolation and purification of the compounds in the extracts with further research.

UPLC is a modern technique for liquid chromatography as it provides more precise and reliable results by using a smaller particle size, which is 2 µm, than HPLC which is between 3 – 5 µm with a shorter run time, hence quick results. It was invented in 2004, which can be considered as a fairly new technique, and therefore it is not commonly used in the studies today[27]. Because of this, the reports from previous studies are very limited for UPLC analysis of *H. perforatum*, as the majority of the studies used the

HPLC technique, which is an older commonly used technique. The studies that performed UPLC for the extracts of *H. perforatum* L. have demonstrated some compounds that have antibacterial activity against various bacteria, and some of the compounds include Hypericin, Hyperforin, and Luteoskyrin[15]. In the current study, the compounds were identified depending on the retention times of the compounds in the previous studies. The run times of the samples

were different from some of the former studies. Therefore, the ratio of the retention time and the total run time was used to compare with the peaks of the extracts in this study to determine the compounds. Similarly, some of the compounds were identified by comparing previous HPLC results of the plant extracts in the same way. Further studies should focus on the identification of the compounds by isolation and purification of the compounds and perform UPLC with mass spectrometry for definitive results.

Although the present study evaluated a limited number of bacterial strains, the promising results suggest that future studies should expand the range of clinical isolates tested. Incorporating a broader spectrum of multidrug-resistant (MDR) pathogens could validate the findings and increase the generalizability of the antibacterial activity observed in *n*-hexane and dichloromethane extracts. The strong activity of the *n*-hexane extract against both Gram-positive and Gram-negative bacteria, particularly MDR strains like *S. aureus* XU212 and *E. coli* ATCC 25922, highlights its potential as a natural antibacterial agent. These findings could guide the development of plant-based inhibitors targeting efflux pumps or other resistance mechanisms in pathogenic bacteria. The results underscore the potential application of *H. perforatum*-derived compounds in pharmaceutical sciences. By isolating and characterizing the active components, these extracts could be optimized for therapeutic use, either as standalone agents or in synergy with existing antibiotics. The UPLC analysis revealed the presence of key bioactive compounds such as Hyperforin and Hypericin, which are known for their antimicrobial properties. Further studies employing advanced techniques like UPLC-MS or NMR spectroscopy are necessary to confirm these findings and to identify the unknown compounds detected in both methanolic and dichloromethane extracts. Given the relatively recent introduction of UPLC, its application in studying *H. perforatum* L. extracts remains underutilized. Expanding its use in combination with mass spectrometry could facilitate a deeper understanding of the plant's chemical profile and its link to antibacterial activity. While the MIC values of *n*-hexane extract were higher than those of the synthetic antibiotic Norfloxacin, the results remain significant given that the extract represents a crude mixture. Optimization and purification of the active components could potentially enhance their efficacy and position them as viable alternatives or adjuncts to synthetic antibiotics. The study not only advances our understanding of natural antibacterial agents but also provides a foundation for the integration of less-polar plant extracts into drug discovery pipelines. The demonstrated efficacy against resistant strains suggests a promising avenue for addressing the global challenge of antibiotic resistance.

The majority of the previous studies were focused on methanolic, ethanolic, and aqueous extracts. However, in this study, less polar solvents, such as *n*-hexane, were also used to examine the antibacterial properties of the plant species against variety of bacteria. In addition to the information about the antibacterial activity of methanolic and ethanolic extracts in the literature, it was demonstrated that *n*-hexane extract has a promising inhibitory result for some of the tested bacteria. Especially against *E. coli*, as the *n*-hexane extract inhibited the bacteria for the lowest concentration tested, which was 1 μL/mL. Additionally, as the UPLC technique is a recently discovered liquid chromatography technique, there were a limited number of UPLC analysis of the *H. perforatum* L. extracts. Although there are some UPLC chromatograms for the methanol extract of *H. perforatum* L., this study is the first report on the UPLC chromatograms of the dichloromethane extract. With further investigation, our findings will be helpful for the identification of the chemical composition of *H. perforatum* L.

STATEMENT OF ETHICS

This study does not require any ethical approval.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept: E.G., M.R.; Design: E.G., M.R.; Data Collection and Processing: E.G., M.R.; Analysis or Interpretation: E.G., M.R.; Literature Search: E.G.; Writing: E.G.

FUNDING SOURCES

No funding or other financial support was received.

ACKNOWLEDGMENTS

Not applicable.

REFERENCES

1. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis, 2005;5(12):751-762. Doi: 10.1016/S1473-3099(05)70295-4

2. Lee JH, Bae IK, Hee Lee S. New definitions of extended-spectrum β-lactamase conferring worldwide emerging antibiotic resistance. Med Res Rev, 2012;32(1):216-232. Doi: 10.1002/med.20210

3. World Health Organization. Antimicrobial resistance: global report on surveillance 2014 [Internet]. World Health Organization, Geneva; 2014 [Jan 9, 2025]. Available from: https://iris.who.int/bitstream/handle/10665/112642/9789241564748_eng.pdf

4. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. Infect Control Hosp Epidemiol, 2013;34(1):1-14.

5. The European Centre for Disease Prevention and Control. Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals: 2011 2012. [Internet]. LU: Publications Office; 2013. Available from: 10.1086/668770

6. Moloney MG. Natural products as a source for novel antibiotics. Trends Pharmacol Sci, 2016;37(8):689-701. Doi: 10.1016/j.tips.2016.05.001

7. Rossiter SE, Fletcher MH, Wuest WM. Natural products as platforms to overcome antibiotic resistance. Chem Rev, 2017;117(19):12415-12474. Doi: 10.1021/acs.chemrev.7b00283

8. Khameneh B, Iranshahy M, Soheili V, Fazly Bazzaz BS. Review on plant antimicrobials: a mechanistic viewpoint. Antimicrob Resist Infect Control, 2019;8(1):118. Doi: 10.1186/s13756-019-0559-6

9. Lee MR. Saint John's wort (Hupericum Perforatum). A balm for hurt minds? Proc R Coll Physicians Edinb, 1999;29(3):253-237.

10. Linde K. St. John's wort - an overview. Forsch Komplementmed, 2009;16(3):146-155. Doi: 10.1159/000209290

11. Saller R, Melzer J, Reichling J. Johanniskraut (Hypericum perforatum): ein plurivalenter rohstoff für traditionelle und moderne therapien. Complement Med Res, 2003;10(1):33-40. Doi: 10.1159/000071690

12. Smith ECJ, Kaatz GW, Seo SM, Wareham N, Williamson EM, Gibbons S. The phenolic diterpene totarol inhibits multidrug efflux pump activity in *Staphylococcus aureus*. Antimicrob Agents Chemother, 2007 Dec;51(12):4480-4483. Doi: 10.1128/AAC.00216-07

13. Abreu AC, Saavedra MJ, Simões LC, Simões M. Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. Biofouling, 2016;32(9):1103-1114. Doi: 10.1080/08927014.2016.1232402

14. Andrews JM. The development of the BSAC standardized method of disc diffusion testing. J Antimicrob Chemother, 2001;48(Suppl.1):29-42. Doi: 10.1093/jac/48.suppl_1.29

15. Farag M, Wessjohann L. Metabolome classification of commercial *Hypericum perforatum* (St. John's wort) preparations via UPLC-qTOF-MS and chemometrics. Planta Med, 2012;78(5):488-496. Doi: 10.1055/s-0031-1298170

16. Hokkanen J, Tolonen A, Mattila S, Turpeinen M. Metabolism of hyperforin, the active constituent of St. John's wort, in human liver microsomes. Eur J Pharm Sci, 2011;42(3):273-284. Doi: 10.1016/j.ejps.2010.12.002

17. Chandrasekera DH, Heinrich M, Ashton D, Welham KJ, Middleton R. Quantitative analysis of the major constituents of St John's wort with HPLC-ESI-MS. J Pharm Pharmacol, 2005;57(12):1645-1652. Doi: 10.1211/jpp.57.12.0015

18. Pradeep M, Kachlicki P, Franklin G. Simultaneous determination of naphtodianthrones, emodin, skyrin and new bisanthrones in *Hypericum perforatum* L. *in vitro* shoot cultures. Ind Crops Prod, 2020;144:112003. Doi: 10.1016/j.indcrop.2019.112003

19. Saddiqe Z, Naeem I, Maimoona A. A review of the antibacterial activity of *Hypericum perforatum* L. J Ethnopharmacol, 2010;131(3):511-521. Doi: 10.1016/j.jep.2010.07.034

20. Gibbons S, Ohlendorf B, Johnsen I. The genus *Hypericum*—a valuable resource of anti-Staphylococcal leads. Fitoterapia, 2002;73(4):300-304. Doi: 10.1016/s0367-326x(02)00082-5

21. Reichling J, Weseler A, Saller R. A current review of the antimicrobial activity of *Hypericum perforatum* L. Pharmacopsychiatry, 2001;34(Suppl.1):116-118. Doi: 10.1055/s-2001-15514

22. Schempp CM, Pelz K, Wittmer A, Schöpf E, Simon JC. Antibacterial activity of hyperforin from St John's wort, against multiresistant *Staphylococcus aureus* and gram-positive bacteria. The Lancet, 1999;353(9170):2129. Doi: 10.1016/S0140-6736(99)00214-7

23. Dadgar T, Asmar M, Saifi A, Mazandarani M, Bayat H, Moradi A, et al. Antibacterial activity of certain Iranian medicinal plants against methicillin-resistant and sensitive *Staphylococcus aureus*. Asian J Plant Sci, 2006;5(5):861-866.

24. Quave CL, Plano LRW, Pantuso T, Bennett BC. Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol, 2008;118(3):418-428. Doi: 10.1016/j.jep.2008.05.005

25. Voss A, Verweij PE. Antibacterial activity of hyperforin from St John's wort. The Lancet, 354(9180):777. Doi: 10.1016/S0140-6736(05)76018-9

26. Avato P, Raffo F, Guglielmi G, Vitali C, Rosato A. Extracts from St John's wort and their antimicrobial activity. Phytother Res, 2004;18(3):230-232. Doi: 10.1002/ptr.1430

27. Swartz ME. UPLC™: an introduction and review. J Liq Chromatogr Relat Technol, 2005;28(7-8):1253-1263. Doi: 10.1081/JLC-200053046

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6335

Potential of anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of the marine-associated bacterial extracts

Rachow KHAWCHAMNAN[1], Monthon LERTCANAWANICHAKUL[2], Patchara PEDPRADAB[3*]

1 Prince of Songkla University, Faculty of Natural Resources, Department of Aquatic Sciences, Hat-Yai, Songkhla, 90110 Thailand

2 Walailak University, School of Allied Health Sciences, 222 Thaiburi, Thasala District, Nakhonsithammarat, 80161 Thailand

3 Rajamangala University of Technology Srivijaya, Faculty of Sciences and Fishery Technology, Department of Marine Science, Trang Campus, Sikao 92150 Thailand

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global health threat, highlighting the urgent need for new treatments as antibiotic effectiveness wanes. Marine microbes are valuable sources of potential anti-MRSA compounds. In this study, marine bacteria were isolated, cultured in marine broth, and extracted with ethyl acetate. The extracts were tested against clinical MRSA 142. Among four bacteria isolated from sponges and sediment, isolates S6.2 (sponge-derived) and SK3 (sediment-derived actinobacteria) showed the strongest anti-MRSA activity, with MIC values of 0.156 mg/mL and 0.078 mg/mL, respectively. HPLC analysis revealed key peaks at Rt 25.58 and 29.02 minutes for S6.2 and a prominent peak at Rt 29.18 minutes for SK3. The SK3 isolate exhibited robust growth and high metabolite production on marine agar, ISP no. 2, 5, and 7, indicating it a promising candidate for anti-MRSA compound production.

Keywords: anti-MRSA, marine associated bacteria, extracts, culture, potential

* Corresponding author: Patchara PEDPRADAB
E-mail: ppedpradab@gmail.com
ORCIDs:
Rachow KHAWCHAMNAN: 0000-0002-0473-1789
Monthon LERTCANAWANICHAKUL: 0000-0001-7777-8062
Patchara PEDPRADAB: 0000-0001-9382-9085
(Received 8 Aug 2024, Accepted 21 Mar 2025)

INTRODUCTION

Staphylococcus aureus is a pandemic pathogen that causes a wide spectrum of pyogenic lesions involving several organs, resulting in nosocomial outbreaks and community-acquired infections. In the 1940s, the medical treatment of *S. aureus* infection became successful and routine due to the discovery of antibiotic agents such as penicillin[1]. Nonetheless, *S. aureus* has rapidly developed resistance to penicillin and methicillin, an antibiotic commonly used for *S. aureus* treatment since the late 1950s. In 1961, scientists identified the first methicillin-resistant *S. aureus* (MRSA) in England[2]. MRSA strains are highly variable in different geographical areas and can rapidly mutate to acquire resistance to commercially available antibiotics, with the exception of vancomycin and teicoplanin[3]. A recent report by both the World Health Organization and the Centers for Disease Control highlighted that MRSA infection accounts for over 94,000 cases and approximately 18,650 deaths annually in the United States[4]. In European countries, MRSA has been reported in more than 25% of infections, and its prevalence in African countries (except South Africa) has increased since 2000[5].

Since the 1980s, MRSA detection in healthcare settings in asian nations currenly has increased[6]. MRSA virulence and spread in Thailand have led to the infection rate increasing to 45% since 1999; furthermore, the national antimicrobial resistance surveillance centre surveyed 46 hospitals and found vancomycin resistance in 98% of MRSA strains in 2023[7]. The emergence of vancomycin-resistant *S. aureus* and intermediate MRSA strains has recently been recognized and leaves physicians with few available options for antibiotics to treat MRSA infection[8]. Thus, the discovery of new anti-MRSA antibiotic agents is urgent. Bioactive natural products are one of the main sources of antibiotic drugs[3]; many antimicrobial agents and resistance-modifying compounds have been the prototype of molecules isolated from natural resources, particularly marine environments, including microorganisms and other invertebrates. Among microorganisms, marine bacteria are a rich source of anti-MRSA compounds[8,9].

Herein, we report the isolation and cultivation of marine-derived microorganisms and screened the anti-MRSA properties of their extracts, aiming to identify the promising candidates for the development of new antimicrobial compounds.

METHODOLOGY

Marine sponges and sediment collection

Samples were collected from the intertidal zone of Sarai Island (6°39′.97″ N, 99°51′.32.01″ E), Satun Province, Thailand (Figure 1), during low tide. Sponge and sediment samples were collected by hand and kept in iceboxes at 4°C during transfer to the laboratory and immediate isolation of the associated microorganisms.

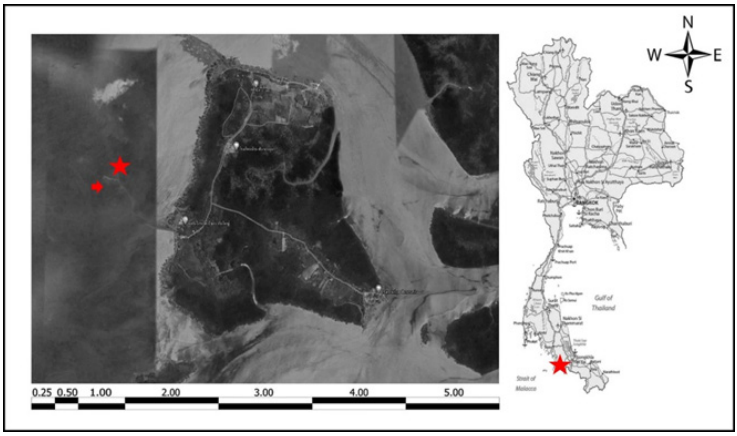


Figure 1. Area of sample collection

Isolation, cultivation and purification of marine-associated bacteria

Sponge-associated bacteria

The sponge samples were washed with sterile seawater until clean, and then their surfaces were sterilized by rapidly swabbing with 70% ethanol, followed by immersion in sterile seawater for 2 min. Approximately 1 cm^3 samples of the central core tissues were cut and ground in 3 mL of sterile sea water. The ground tissue was serially diluted in sterile seawater to 10^{-6} before 100 µl aliquots were spread on Zobell Marine Agar (Himedia, New Delhi, India). The plates were placed in the incubation chamber at 30°C for 7 days, with observation on each day. Clear colonies with different characteristics were picked with a needle, restreaked on other MA plates to obtain pure strains, and maintained in a marine agar slant at 4°C for further experiments. For long-term preservation, 15% glycerol was added, followed by freezing at -80°C. The sponge samples were identified by Dr. Pedpradab S. using the standard reference guide for sponge identification[10].

Sediment-associated bacteria

Three grams of sediment were collected using a gravity core sampling instrument (Dormer, Sydney, Australia). The samples were stored in sterile sample bags made of low-density polyethylene (LDPE) and kept at 4°C during transport to the laboratory for subsequent experiments. Marine-associated bacteria were isolated using the serial dilution method. Sediment samples were serially diluted (10^{-1} to 10^{-9}), and the dilutions were plated onto marine agar medium. The inoculated plates were incubated at 37°C for 24 to 72 hours. Morphologically distinct bacterial colonies were observed and picked from the 10^{-5} to 10^{-7} dilution plates. These colonies were further purified using the streak plate technique. All experiments were conducted in triplicate. A pure bacterial strain was subsequently cultivated in marine broth medium for 72 hours before extraction with ethyl acetate.

Cultivation of bacteria and crude extract preparation

Seed cultures were prepared by inoculating pure colonies of the isolated bacteria in 5 mL of marine broth and incubating them at 30°C on a shaker for 10 days. The seed cultures were examined for anti-MRSA activity by the agar well diffusion and agar overlay method[11]. The cultures that showed anti-MRSA properties were transferred to 180 mL of marine broth in a 250 mL conical flask and incubated in the previously described conditions for 10 days. The cell suspension was lysed using an ultrasonic bath at 30°C for 5 minutes, followed by centrifugation at 5,000 rpm for 5 minutes. The supernatant (liquid part) was extracted three times with ethyl acetate in a separatory funnel. This extraction process resulted in the formation of two distinct liquid layers: the organic phase (ethyl acetate) and the aqueous phase (broth). The organic phase was then concentrated by evaporation using a rotary evaporator set to a water bath temperature of 45°C, yielding the crude extract.

The organic layer was separated and concentrated under a vacuum to yield the crude extract. The extract showing the most potent anti-MRSA activity was further studied for growth in different media, including ISP no. 1-7, Mueller Hinton Agar (MHA), Marine Agar, and Luria Bertani (LB).

Isolation and purification of methicillin-resistant *Staphylococcus aureus*

The MRSA strain was isolated from Thasala Hospital, Thailand, using the method[12,13]. The method consisted of two procedures. First, the cefoxitin disc screen test method was used for the detection of MRSA. The susceptibility of *S. aureus* isolates to 30 µg of cefoxitin was determined by the disc diffusion

method on Mueller-Hinton agar plates using a bacterial suspension equivalent to a 0.5 McFarland standard. The MRSA-inoculated plates were incubated at 35°C for 24 hrs. The results were interpreted according to CLSI guidelines. For MRSA sensitivity, the inhibition zone was ≤21 mm. Second, for multiplex PCR for the SCC*mecA* gene, DNA was extracted from MRSA strains using an extraction kit (Qiagen, USA) according to the manufacturer's instructions. Multiplex PCR was performed to detect three loci (A, B and C) of the *mecA* gene for SCC types I, II, and III, respectively. The primers and amplification method[12].

Anti-MRSA activity determination

The agar well diffusion and agar overlay methods were used to examine the extracts' anti-MRSA activity. The agar well diffusion method was performed by using MHA. The MRSA suspension was equally diluted to a 0.5 McFarland standard with sterile sodium chloride and spread on an MHA medium. Then, wells with a diameter of 6 mm were punched aseptically with a sterile borer. Eighty microlitres of MRSA suspension was transferred to the wells and incubated at 37°C for 24 h to measure the inhibition zone. The agar overlay method was performed by spotting marine-associated strains on Zobell marine agar plates and allowing them to grow in an incubation chamber for 3-5 days. The tested MRSA strain in a soft medium was gently overlaid on the marine strain and then incubated at 37°C for 24 h to measure the inhibition zone. Medium and vancomycin were used as negative and positive controls, respectively. Mass culturing of bacteria was performed in 500 mL conical flasks on a shaker, and the cultured organisms were screened daily for anti-MRSA properties.

Bioautography analysis

Direct thin-layer chromatography (TLC) bioautography was examined by following the method[14]. Briefly, the developed chromatogram was sprayed with bacterial suspension (10^6 CFU/mL), incubated at 25°C for 48 h under humid conditions, and then sprayed with tetrazolium salt solution and reincubated at 25°C for 24 h. The antimicrobial activity was visualized as a clear, bright zone against a purple background on the TLC image.

Chromatographic analysis of the crude extract

Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used to analyse the components in crude extracts. TLC was performed by using an ALUGRAM® Xtra SIL G/UV_{254} precoated TLC sheet (Macherey-Nagel, Düren, Germany). A mixture of chloroform, ethyl acetate, and methanol (6:3:1, v/v) was used as an eluent (mobile phase). HPLC chromatograms were obtained by a Dionex UltiMate 3000 (Thermo Scientific,

Waltham, MA, USA). The separation column was achieved using an Acclaim 120 C18 reversed-phase column (5 μm, 4.6 x 150 mm, Thermo Scientific, Waltham, MA, USA). Gradient elution was programmed to progress from 100% water to 100% methanol within 30 min with a flow rate of 1 mL/min, and a photodiode array detector was set at wavelengths of 230, 254, 285 and 366 nm.

RESULTS and DISCUSSION

MRSA isolation

Three MRSA strains (MRSA 142, MRSA 1096, and MRSA 2468) were isolated from patients in Thasala Hospital. All strains were examined for *mecA*-mediated oxacillin-resistant *Staphylococcus* and inducible clindamycin-resistant *Staphylococcus* (Table 1). All clinically isolated MRSA strains showed oxacillin resistance and retained *mecA*; only strain 142 exhibited a D-shaped zone in the clindamycin inducible resistance examination. On the basis of the results, MRSA 142 was selected as the index strain for further experiments.

Table 1. The isolated MRSA and drug-resistant examination

Strain code	Oxacillin resistance	mecA gene	Clindamycin resistance
			Inducible resistance (D-zone of inhibition)
MRSA 142	✓	✓	✓
MRSA 1096	✓	✓	-
MRSA 2468	✓	✓	-

Isolation and culture of marine-associated bacteria and anti-MRSA assay

Several marine sponges (Figure 2) were used to isolate associated bacteria. Three of them, namely, *Cacospongia* sp., *Mycale grandis*, and *Paratetilla bacca*, yielded bacterial strains S1.2, S6.2, and SL9, respectively.

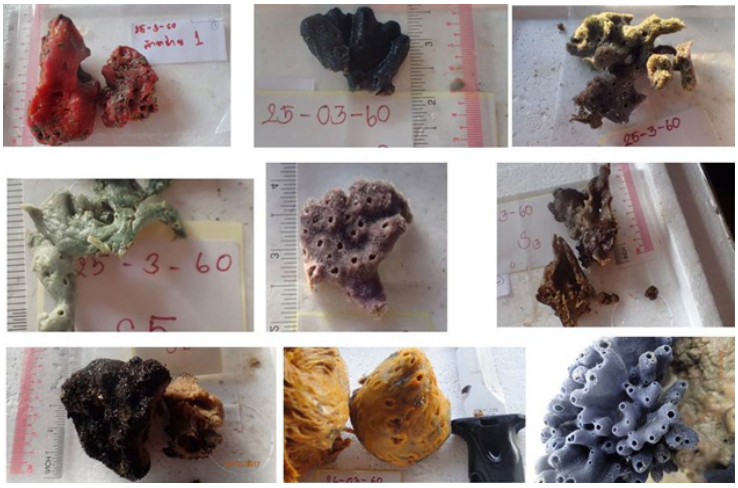


Figure 2. Marine sponge samples using for isolating associated bacteria

In addition, a starin of bacteria, namely SK3 (Figure 3), was isolated from marine sediment. The isolated bacteria were cultivated in marine broth for crude extract preparation.

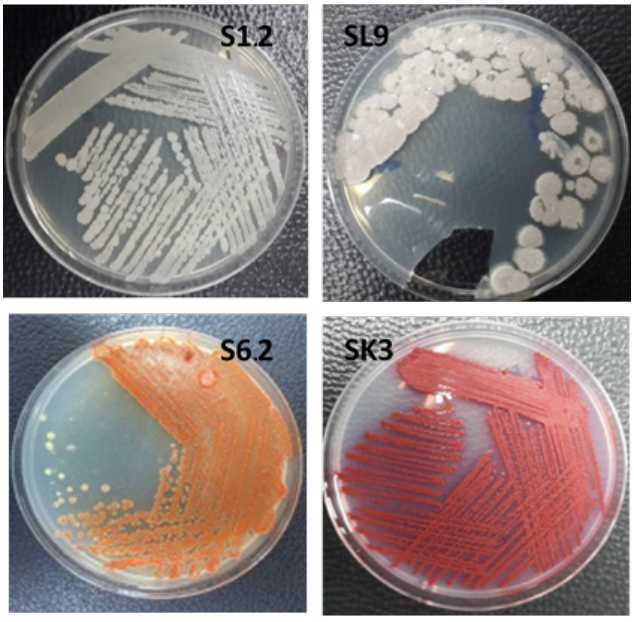


Figure 3. Colony feature on solid media (marine agar) of the isolated bacteria

The cultures were harvested in the late lag phase and then extracted with ethyl acetate to obtain the crude extracts. The anti-MRSA bioactivity of the extracts was subsequently determined (Table 2 and Figure 4).

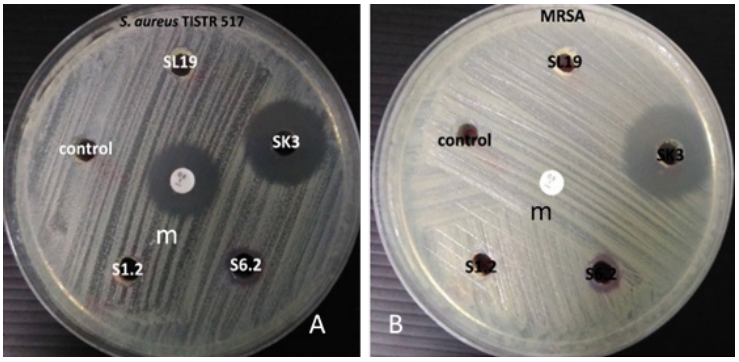


Figure 4. Inhibition zones of bacterial extracts against S. aureus (A) and MRSA (B). Marine broth and methicillin (m) were used as negative and positive control, respectively.

Table 2. Anti-*S.aureus* and anti-MRSA screening of the bacteria extracts

Test organisms	Extract code	Inhibition zone (cm)
S. aureus TISTR 517	SK3	0.032 ± 0.01
	S6.2	0.007 ± 0.01
	S1.2	0.003 ± 0.02
	SL19	0.003 ± 0.05
	control	0.002 ± 0.01
MRSA	SK3	0.037 ± 0.01
	S6.2	0.0060 ± 0.02
	S1.2	0.002 ± 0.01
	SL19	0.001 ± 0.04
	control	0.002 ± 0.01

The S6.2 extract exhibited weak inhibition against the tested microorganisms, whereas the SK3 extract showed strong inhibition. The SK3 extract was further checked for potential anti-MRSA bioactivity by using MIC and MBC methods, and the colony features were studied using different types of media. The SK3 extract exhibited the greatest potential for anti-MRSA bioactivity, indicated using MIC and MBC values (0.156 ± 0.001 and 0.625 ± 0.003 mg/mL, respectively; Table

3). Moreover, our study revealed that the SK3 strain displayed different colony features and colours when cultured on various solid media. The colonies were pink with white spots on ISP media No. 2, 3, and 4, whereas they appeared dark orange on ISP No. 5 and 7 and marine agar. Furthermore, cream-coloured colonies were observed when the SK3 strain was cultured on ISP No. 6, MHA, and LB media.

Table 3. MIC and MBC values of the extracts from marine associated bacteria

Codes	Test organisms	MIC mg/mL	MBC (mg/mL)
S6.2	MRSA 142	0.156	0.625
	S.aureus 517	0.312	1.25
SK3	MRSA 142	0.0078	>0.031
	S.aureus 517	0.0625	>0.25

Chemical analysis of the extract

The SK3 extract was subsequently analysed for the allocation of active compounds by using TLC-directed bioautography and for its chemical constituents by using HPLC methods. According to TLC-directed bioautography, the positions of the bright spots (Figure 5) indicated the presence of anti-MRSA metabolites within the extract.

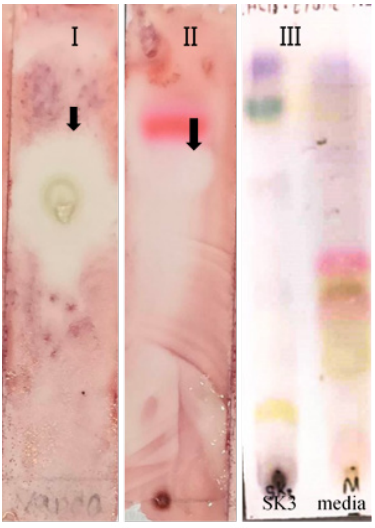


Figure 5. Bioautogram (I, II) and TLC (III) of the extract from a bacterium SK3 demonstrate inhibition against MRSA and chemical profile. I represent a positive control (Vancomycin), while II and III are the extract and its chemical profile, respectively. The brilliant spots observed on II correspond to the active secondary metabolites containing.

These active metabolites did not react with the anisaldehyde reagent, suggesting that they were not steroids or terpenoid compounds present in the extract. According to HPLC (Figure 6), the main active compounds were observed at an Rt of 29.187 min in the SK3 extract, with the remaining peaks identified as components of the culture media. The S6.2 extract contained minor metabolites at Rt values of 25.583 min and 29.023 min, with the other peaks attributed to components of the culture media.

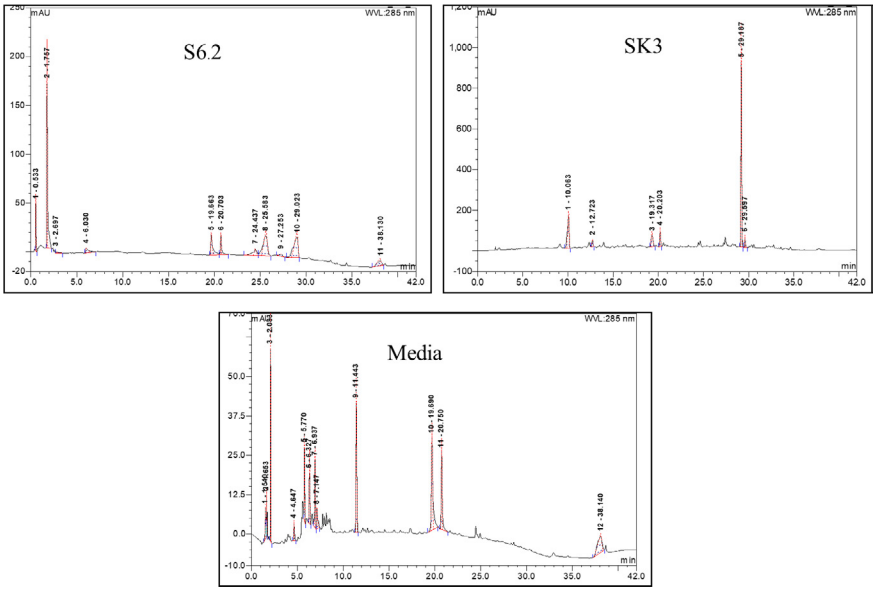


Figure 6. HPLC chromatograms of the active extracts show peaks at Rt ranging 0 - 20.750 minute, which are attributed to the media composition. The active metabolites of S6.2 are expected at Rt 25.583 and 29.023 minute, while SK3 exhibits a main peak at Rt 29.187.

A MRSA outbreak in Thailand was observed 15 years ago. Since 2012, the National Antimicrobial Resistance Surveillance Center (NARSC) has reported that the rate of MRSA vancomycin resistance has increased to 98%[7]. In this work, three MRSA strains were isolated from the mucous of patients in Thasala Hospital, Nakhon Sri Thammarat Province, Thailand. The isolated MRSA strains were determined for their drug resistance induction by using the *mec*A gene, oxacillin, and clindamycin as indicators (Table 1). All strains tested positive for anti-oxacillin resistance and carried the *mec*A gene, but only MRSA142 showed a D-shaped zone on clindamycin resistance testing. A D-shaped zone on agar well plates can indicate the presence of inducible antibiotic resistance. It occurs when bacteria carry genes that can be turned on or off, depending on the type of certain antibiotics. In this case, MRSA142 exhibited inducible

resistance to clindamycin. This information is essential when considering bacterial strains for drug susceptibility screening, and as a result, MRSA142 was selected for further use as a test organism in an anti-MRSA drug discovery program. We isolated 4 marine-associated bacterial strains from several marine sponges and sediment; three of the strains showed anti-MRSA activity (Table 3 and Figure 4), including S6.2, which was isolated from a red Demospongia sponge, *Mycale grandis* (No. 1, Figure 2), and SK3, which was isolated from sediment. Normally, sponges are rich sources of diverse associated microorganisms, some of which produce antimicrobial metabolites, while sediment contains mainly antibiotic-producing actinobacteria[15]. However, this also depends on geographic variation and cultured media, even for the same species and hosts[15-17]. For example, 1234 bacterial strains were isolated from sponges in South Australian marine environments, of which 21% showed antimicrobial activity against MRSA[18]. A total of 460 strains were isolated from 18 sponges in Vietnam water, of which 90 strains exhibited antimicrobial activity, and in particular, 21 strains exhibited activity against *S. aureus*[19]. Actinobacteria are well distributed in marine sediment and show antimicrobial activity against several drug-resistant pathogens. According to this work, 92 actinobacteria, mainly *Streptomyces* spp., were isolated from Philippine marine sediment and exhibited anti-multidrug-resistant *S. aureus* activity[20], while 6 actinobacterial strains isolated from Nicobar Island, the Andaman Sea, also showed anti-*S. aureus* activity[21]. The above data indicate the high diversity of antimicrobial compounds produced by associated marine bacteria; accordingly, intensive surveys and screening for their antimicrobial activity should be performed on bacteria in various regions. Assessment of the anti-*staphylococcal* activity of the extracts from marine-associated bacteria showed that strains SK3 and S6.2 created the largest zone of inhibition (Figure 4 and Table 3).

Various sponges have been reported as sources of anti-MRSA compound-producing bacteria, including *Haliclona* sp., *Axinella* sp., *Dysidea* sp., *Epipolasis* sp., *Neopretosia* sp., and *Mycale* sp.[22]. In this study, we isolated an unidentified bacterium. The extract (coding as 6.2) from this bacterium exhibited anti-MRSA activity with a MIC value of 0.156 mg/mL. Previous studies have shown that the genus Mycale hosts a diverse array of associated bacteria, particularly *Bacillus* sp., *Vibrio* spp., *Streptomyces* sp., *Cobetia* sp., *Pseudomonas*, and *Nocardiopsis* sp.[23]. Some of these bacteria produce various classes of anti-MRSA compounds, including brominated biphenyldiols, peptides, terpenoids, alkaloids, and molecules containing five-membered lactone rings[24]. For instance, a novel linear peptide, bogorol A, demonstrates high anti-MRSA potency with an MIC value of 2.5 μg/mL, while 3,3′,5,5′-tetrabromo-2,2′-biphenyldiol ex-

hibits MIC values ranging from <0.25 to 2 mg/mL.[25-26]. Additional examples have been summarized by Liang et al. (2023)[23]. In this work, we analyzed the preliminary data of the active extract. However, the pure active compounds responsible for the observed anti-MRSA activity have not yet been identified.

HPLC was primarily used for the analysis of active components in the extracts (Figure 6). The HPLC chromatograms revealed that the active constituents contained in the S6.2 extract were located at retention times (Rt) of 25.583 and 29.023 min, while SK3 showed only one peak at 29.18 min. Other peaks (at Rt 0 - 20.703 min) in both the S6.2 and SK3 extracts were components of the medium (chromatogram of pure medium was used as a reference), which did not exhibit activity against MRSA. However, the type of active compounds still remains unidentified until they are purified and characterized. The MIC and MBC values (Table 3) revealed that strain SK3 contained the highest potency of anti-MRSA constituents, which was observed as a peak at Rt 29.187 min and probably a very minor compound at Rt 29.597 min on HPLC chromatogram. SK3 was accordingly selected for further study. SK3 is a gram-negative bacterial strain that formed a circular, shiny colony on marine agar (Figures 3 and 7). It was cultured in 9 different solid media for one week, including marine agar (MA), MHA (Mueller-Hinton Agar), LB (Luria Bertani), and ISP No 2-7, to observe colony features and anti-MRSA activity potential. Its colony features and anti-MRSA activity potential differed according to the culture medium, as shown in Figure 7.

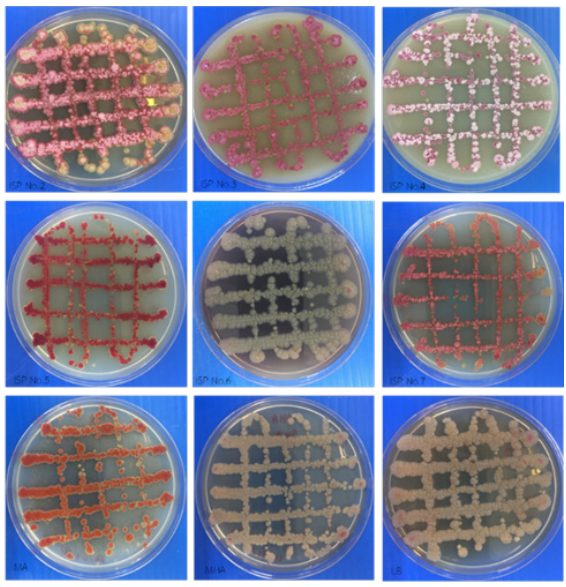


Figure 7. Colony feature of SK3 on different kind of solid media

The growth and potential of bioactive compound synthesis of marine actinobacteria varies on different culture media, as found in some previous reports. ISP no. 2 promoted a high degree of antimicrobial compound production in many marine actinomycetes (also found in SK3), but yeast extract peptone (YP) and starch yeast extract peptone (SYP) did not support active compound production[27,19]. Based on the principle that many marine bacteria produce secondary metabolites that are released into the environment, the potential for secondary metabolite production of SK3 was examined by the agar plug diffusion technique. A portion of the individual solid medium on which bacteria had grown was excavated and then transferred into drilled wells of MRSA discs. We found that SK3 produced the strongest anti-MRSA metabolites when cultured with marine agar (MA), ISP No. 2, 5 and 7 (Figure 7). This indicated that nutrient composition and concentration were related to the growth form of SK3 and also their secondary metabolite synthesis. Generally, appropriate concentrations of elemental ions, monosaccharides, peptone, and yeast extract are essential components for the growth of marine bacteria and antibacterial metabolite synthesis[27]. Sodium and potassium ions are involved in oxidative metabolism processes and biologically active metabolite production, while peptone and yeast extract are sources of organic carbon and monosaccharides[28]. Minor concentrations of amino acids such as L-asparagine and L-tyrosine are still essential cofactors for producing secondary metabolites and/or growth promoters[29-30], as found in ISP No. 5. It is worth noting that starch or complex carbohydrates contained in ISP No. 3 and 4 may have a negative effect on bioactive compound synthesis, as estimated by the inhibition zone.

STATEMENT OF ETHICS

This study does not require any ethical approval.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rachow K: Acquisition, analysis of data, and statistical analysis; Patchara P: Design project, data analysis, and drafting manuscript; Monthon L: Design project and critical review manuscript.

FUNDING SOURCES

The grant supported by Fundamental Fund (FF) and Ministry of Higher Education, Research and Innovation.

ACKNOWLEDGMENTS

We would like to thank Department of Marine Sciences, Rajamangala University of Technology Srivijaya and School of Allied Health Sciences, Walailak University for laboratory support.

REFERENCES

1. Hassanzadeh P, Hassanzadeh Y, Rezai E, Mostamedifar M. Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from HIV patients referring to HIV referral center, Shiraz, Iran, 2011-2012. Iran J Med Sci, 2015;40(6):526-530.

2. Fowler JR, Greenhill D, Schaffer AA, Thoder JJ, Ilyas AM. Evolving incidence of MRSA in urban hand infections. Orthopedics, 2013;36(6):796-800. Doi: 10.3928/01477447-20130523-27

3. Green BN, Johnson CD, Egan JT, Rosenthal M, Griffith EA, Evans MW. Methicillin-resistant *Staphylococcus aureus*: an overview for manual therapists. J Chiropr Med, 2012;11(1):64-76. Doi: 10.1016/j.jcm.2011.12.001

4. Nene KK, Sebastien K, Bowo-Ngandji A, Jane-Francis TKA, Hortense GK, Roland NN, et al. Methicillin-resistant *Staphylococcus aureus* carriage amongneonate mothers, healthcare workers, and environmental samples in neonatal intensive care units: a systematic review. Biomed Res Int, 2024;1-10. Doi: 10.1155/2024/5675786

5. Gonz´alez-Machado C, Alonso-Calleja C, Capita R. Prevalence and types of methicillin-resistant Staphylococcus aureus (MRSA) in meat and meat products from retail outlets and in samples of animal origin collected in farms, slaughterhouses and meat processing facilities. A review. Food Microbiol, 2024;11:1-9. Doi: 10.1016/j.fm.2024.104580

6. Mohamad Farook NA, Argimón S, Abdul Samat MN, Salleh SA, Sulaiman S, Tan TL, et al. Diversity and dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) genotypes in Southeast Asia. Trop med Infect, 2022;7(12):438. Doi: 10.3390/tropicalmed7120438

7. Bunsan S. The study of antimicrobial resistance (amr) and development of amr reporting guidelines at kap choeng hospital, surin province. Acad J Mahasarakham Prov Public Health Office, 2023;8(15):83-91.

8. Rahman S, Mumtaz S, Mufti AJ, Shah SH, Rahman M. Incidence of methicillin resistant *Staphylococcus aureus* in Peshawar. J Ayub Med Coll Abbottabad, 2011;23(1):99-101.

9. Stein M, Komerska J, Prizade M, Sheinberg B, Tasher D, Somekh E. Clindamycin resistance among Staphylococcus aureus strains in Israel: implications for empirical treatment of skin and soft tissue infections. Int J Infect Dis, 2016;46:18-21. Doi: 10.1016 /j.ijid.2016.02.016

10. Hooper, JNA, Van Soest, RWM. Systema Porifera. A guide to the classification of sponges. In: Hooper, JNA, Van Soest, RWM, Willenz, P, editors. Systema Porifera. Boston, MA: Springer; 2002. p. 1-7. Doi: 10.1007/978-1-4615-0747-5

11. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: a review. J Pharm Anal, 2016;6:71-79. Doi: 10.1016/j.jpha.2015.11.005

12. Amirkhiz MF, Rezaee MA, Hasani A, Aghazadeh M, Naghil B. SCCmec typing of methicillin-resistant Staphylococcus aureus: an eight year experience. Arch Pediatr Infect Dis, 2015;3(4):e30632. Doi: 10.5812/pedinfect.30632

13. Elshabrawy WO, Zaki ME, Kamel MF. Genetic and phenotypic study of methicillin-resistant *Staphylococcus aureus* among patients and health care workers in Mansoura University Hospital, Egypt. Iran J Microbiol, 2017;9(2):82-88.

14. Dewanjeea S, Gangopadhyayb M, Bhattacharyaa N, Khanraa R, Duaa TK. Bioautography and its scope in the field of natural product chemistry. J Pharm Anal, 2015;5(2):75-84. Doi: 10.1016/j.jpha.2014.06.002

15. Özcan K, Aksoy SÇ, Kalkan O, Uzel A, Hames-Kocabas EE, Bedir E. Diversity and antibiotic-producing potential of cultivable marine-derived actinomycetes from coastal sediments of Turkey. J Soils Sediments, 2013;13:1493-1501. Doi: 10.1007/s11368-013-0734-y

16. Remya KS, Beena P, Bijesh PV, Sheeba A. Formulation development, evaluation and comparative study of effects of super disintegrants in cefixime oral disintegrating tablets. J Young Pharm, 2010;2(3):1250-1255. Doi:10.4103/0975-1483.66794

17. Dalisay DS, Williams DE, Wang XL, Centko R, Chen J, Anderson RJ. Marine sediment-derive Streptomyces from British Columbia, Canada are a promising microbiota resource for the discovery of antimicrobial natural products. PLoS One, 2013;8(10):77078. Doi: 10.1371/journal.pone.0077078

18. Padmanaban VP, Verna P, Venkatabaskaran S, Keppayan T, Gopal D, Sekar AK, et al. Antimicrobial potential and taxonomic investigation of piezotolerant Streptomyces sp. NIOT-Ch-40 isolated from deep-sea sediment. World J Microbiol Biotechnol, 2017;33:27. Doi: 10.1007/s11274-016-2193-2

19. Anteneh YS, Yang Q, Brown MH, Franco CMM. Antimicrobial activities of marine sponge-associated bacteria. Microorganisms, 2021;9:171. Doi: 10.3390/ microorganisms9010171

20. Dat TTH, Cuc NTK, Cuong PV, Smidt H, Sipkema D. Diversity and antimicrobial activity of Vietnamese sponge-associated bacteria. Mar Drugs, 2021;19(7):353. Doi: 10.3390/md19070353

21. Tenebro CP, Trono DJVL, Vicera CB, Sabido CBEM, Jr JAY, Macaspac AJM, et al. Multiple strain analysis of Streptomyces species from Philippine marine sediments reveals intraspecies heterogeneity in antibiotic activities. Sci Rep, 2021;11:17544. Doi: 10.1038/s41598-021-96886-4

22. Indraningrat AAG, Smidt H, Sipkema D. Bioprospecting sponge-associated microbes for antimicrobial compounds. Mar Drugs, 2016;14:87. Doi: 10.3390/md14050087

23. Liang J, She J, Fu J, Wang J, Ye Y, Yang B, et al. Advances in natural products from the marine-sponge-associated microorganisms with antimicrobial activity in the last decade. Mar Drugs, 2023;21:236. Doi: 10.3390/md21040236

24. Isnansetyo A, Kamei Y. MC21-A, a bactericidal antibiotic produced by a new marine bacterium, pseudoalteromonas phenolica sp. nov. O-BC30T, against methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother, 2003;47(2):480-488. Doi: 10.1128/AAC.47.2.480-488.2003

25. Alahmari AN, Hassoubah SA, Alaidaroos BA. Sponges-associated marine bacteria as sources of antimicrobial compounds. Nov Res Microbiol J, 2022;6(5):1742-1767. Doi: 10.21608/nrmj.2022.267424

26. Devkar HU, Thakur NL, Kaur P. Marine-derived antimicrobial molecules from the sponges and their associated bacteria. Can J Microbiol, 2023;69:1-16. Doi: 10.1139/cjm-2022-0147

27. Poosarla A, Venkata RL, Krishna RM. Isolation of potent antibiotic producing Actinomycetes from marine sediments of Andaman and Nicobar Marine Islands. J Microbiol Antimicrob, 2023;5:6-12. Doi: 10.5897/JMA11.075

28. Claverías FP, Undabarrena A, González M, Seeger M, Cámara B. Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaíso bay, Chile. Front Microbiol, 2015;6:737. Doi: 10.3389/fmicb.2015.00737

29. Pyane WJ. Effects of sodium and potassium ions on growth and substrate penetration of a marine pseudomonad. J Bacteriol, 1974;15(1):25-34. Doi: 10.1128/jb.80.5.696-700.1960

30. Jafarzade M, Yahya NA, Shayesteh F, Usup G, Ahmad A. Influence of culture conditions and medium composition on the production of antibacterial compounds by marine Serratia sp. WPRA3. J Microbiol, 2023;51(3):373-379. Doi: 10.1007/s12275-013-2440-2

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6336

Investigating the potential effects of thymoquinone on cisplatin-induced nausea and vomiting

Elif GULBAHCE-MUTLU[1*], Ayse Nur YILDIZ[2], Serife ALPA[3], Fatma Nur TURKOGLU[3], Hatice Nur SEFLEK[4], Zeynep Nur AKYAR[2]

1 KTO Karatay University, Faculty of Medicine, Department of Medical Biology, Konya, Türkiye
2 NEU University, Faculty of Medicine, Konya, Türkiye
3 KTO Karatay University, Faculty of Medicine, Department of Anatomy, Konya, Türkiye
4 KTO Karatay University, Faculty of Medicine, Department of Histology and Embryology, Konya, Türkiye

ABSTRACT

Cisplatin is a powerful chemotherapy drug used to treat certain types of cancer. The effects of this drug, nausea and vomiting, can reduce patients' quality of life and make it difficult to continue treatment. Thymoquinone is known to act on the gastrointestinal system. However, its effect on nausea is not clear. This study investigated the effects of thymoquinone on cisplatin-induced nausea. Animals were divided into four groups (control, cisplatin, thymoquinone, and cisplatin+thymoquinone). Cisplatin was administered intraperitoneally and thymoquinone by gavage. The amount of food consumed by the animals was recorded. In addition, gastrointestinal tissues were examined by hematoxylin and eosin (H&E) staining. The data obtained suggest that cisplatin causes damage to the gastrointestinal tract. However, it is possible that thymoquinone may contribute to the healing of this damage. Therefore, the addition of thymoquinone to patients undergoing chemotherapy has the potential to increase the effectiveness of the treatment process.

Keywords: cisplatin, gastrointestinal system, pica, thymoquinone

* Corresponding author: Elif GÜLBAHÇE MUTLU
E-mail: elif.mutlu@karatay.edu.tr
ORCIDs:
Elif GULBAHÇE MUTLU: 0000-0003-2391-2152
Ayse Nur YILDIZ: 0009-0002-4403-1944
Şerife ALPA: 0000-0001-8665-3632
Fatma Nur TURKOĞLU: 0000-0002-4529-5813
Hatice Nur SEFLEK: 0000-0003-2969-2322
Zeynep Nur AKYAR: 0009-0007-1841-4817
(Received 18 Oct 2024, Accepted 11 Mar 2025)

INTRODUCTION

Cisplatin (cis-dichlorodiammineplatinum) is a heavy metal compound that has shown antineoplastic activity in clinical and preclinical studies[1]. It is used in the clinic as an effective chemotherapeutic agent in many cancer treatments[2]. However, acute kidney injury, gastrointestinal problems, bleeding and a reduced immune response to infection are among the most common side effects observed[3]. Chemotherapy-induced nausea and vomiting (CINV) can cause a variety of adverse physical effects and significantly reduce the quality of life of patients undergoing chemotherapy. Persistent nausea and vomiting associated with CINV leads to serious physical complications such as dehydration, anorexia and unwanted weight loss[4]. Antiemetic agents are widely used to relieve this discomfort. Dopamine D2, histamine H1, serotonin 5HT3 (serotonin receptor), tachykinin NK1 receptor antagonists and corticosteroids among these agents are used as antiemetic prophylaxis and therapeutic in clinic[5,6].

Nigella sativa L. (Black cumin), a member of the Ranunculaceae family, is a fragrant herbaceous plant that blooms between April and August with blue and green flowers and grows to a height of about 60 cm[7,8]. Black cumin seeds are rich and diverse in chemical components; they contain amino acids, protein, carbohydrate, mixed and essential oils[7].This plant has been used by patients for gastrointestinal disorders such as abdominal pain, diarrhoea and flatulence[9] as an anti-carcinogenic[10,11] anti-inflammatory[12] and antioxidant[13]. In addition, black cumin oil has been reported to have various pharmacological effects such as antihistamine[14], antioxytocic[15], liver protector[16] and immune enhancer[17].

Most of the pharmacological activity in black cumin seed has been associated with the 'quinone' component. Chopra et al. (1956) discovered that Thymoquinone (TQ) is the main active component of the essential oil in black cumin[18]. Later, Houghton et al. (1995) reported that thymoquinone (TQ) is the main component of the essential oil[19]. TQ has attracted considerable scientific interest due to its high biological activity and low systemic toxicity, making it a promising alternative to classical therapeutic drugs[20]. Recent studies on TQ have shown that it has a protective effect against several free radical-generating compounds such as doxorubicin and carcinogenesis caused by different chemical compounds, diabetic neuropathy and membrane lipid peroxidation, and has anti-inflammatory and analgesic effects[21,22,23,24].

The gastro-protective mechanisms of TQ are to inhibit proton pumps, acid secretion and neutrophil infiltration while increasing mucus secretion and nitric oxide products[25]. Black cumin essential oil and TQ have been shown to have gastro-protective effects in relation with the maintenance of the redox

state in the gastric mucosa[26,27,28].

In this study, in order to evaluate the effect of TQ on cisplatin-induced nausea and vomiting in rats, the amount of kaolin consumption and animal weights were evaluated by applying the pica method. Gastrointestinal system tissues were also examined histologically.

METHODOLOGY

This study was conducted at Necmettin Erbakan University KONÜDAM Experimental Medicine Research and Application Centre and KTO Karatay University Faculty of Medicine Histology/Pathology Laboratory. The study protocol was approved by Necmettin Erbakan University KONÜDAM Experimental Medicine Research and Application Centre Animal Experiments Ethics Committee (2021-040) and adult female *Wistar albino* rats weighing 180-220 g obtained from the same unit were used.

Rats were housed under standard laboratory conditions (22-23°C ambient temperature, 50% humidity and 12-hour light/dark cycle). Water and food were provided during the experiment. The rats were kept in standard cages until the day of application, and after the experimental groups were formed and the applications were performed, they were taken to individual cages, and the experimental procedure was carried out.

Preparation of kaolin

Kaolin was prepared according to a previously described method[29]. Kaolin (ZAG Kimya, Cas No: 1332-58-7) was mixed with acacia gum (Sigma-Aldrich, Cas No: 9000-01-5) in a ratio of 99:1. It was then mixed with distilled water to form a paste and allowed to dry at room temperature for 72 hours.

Experimental procedure

All animals were subjected to a 3-day adaptation period before the start of the experiment. During this period, animals were housed in separate cages to provide access to both normal food and kaolin and experimental groups were formed. A total of 28 rats were used, 7 animals in each group.

Group 1; Control: No treatment was given to the animals in this group.

Group 2; Cisplatin (CIS): In this group, cisplatin [Koçak Farma (50mg/100mL concentrated solution for infusion)] was administered intraperitoneally (i.p) at a dose of 6mg/kg[30].

Group 3; Thymoquinone (TQ): Thymoquinone (Sigma-Aldrich) at a dose of 1.5 mg/kg was administered to the animals in this group by gavage[31].

Group 4; Cisplatin + Thymoquinone (CIS + TQ): In this group, 6 mg/kg dose of cisplatin was administered intraperitoneally and then 1.5 mg/kg dose of Thymoquinone was given by gavage[30,31].

No irritation, restlessness or other adverse effects (e.g. respiratory distress, abnormal movement or catalepsy) were detected in rats following i.p. administration. The amount of kaolin consumption was noted 12-24 h after administration to the animals in accordance with experimental groups[32]. To measure kaolin and food intake, the remaining kaolin and food, including those spilled out of the containers, were collected and kaolin intake, food intake and body weight of the animals were noted on each experimental day. After administration, animals were decapitated, and gastrointestinal tract tissues were placed in 10% buffered formalin solution for histopathological evaluation.

Histopathological examination

After fixation in 10% formalin solution for 48 hours, the tissues were passed through graded alcohol (70, 80, 90, 100%) and xylol (Xylene I, II) series for one hour and routine tissue follow-up procedures were performed. Then, 3µm thick sections were taken from the paraffin blocked samples on slides using a rotary microtome (Leica DSC2). The sections were freed from paraffin. The sections were stained with hematoxylin and eosin (H&E). H&E-stained slides were examined under a light microscope (Olympus SC50) and images were taken.

Statistical analysis

Statistical interpretation of the results was performed using the SPSS 22.0 computer package program, and arithmetic means and standard errors of all parameters were calculated. “The Shapiro-Wilk test was performed to determine the homogeneity of the data and it was found that the data had a normal distribution.” One-way analysis of variance (ANOVA) test was used to determine the difference between groups, and Tukey’s test, one of the multiple comparison tests, was used to determine which group the differences originated from. Differences at the $p<0.05$ level was considered significant.

RESULTS and DISCUSSION

Animal weight and kaolin consumption results

According to statistical analyses of animal weights taken at the beginning and end of the experiment, there was no change in the weights of the control and thymoquinone groups. However, CIS and Animal weights decreased in the CIS+TQ group, and this decrease was the highest in the CIS group and significant differences were observed between the groups ($p\leq0.05$) (Figure 1).

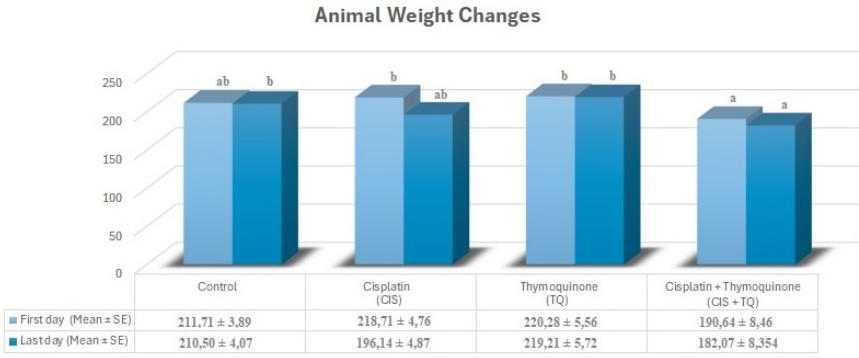


Figure 1. Animal weight changes (there was a significant difference between groups in the same column and carrying different letters [p<0.05]).

After application, the kaolin was left in the cages and the total amount of kaolin consumed was determined. According to the data obtained as a result of statistical analyses, it was determined that the highest kaolin consumption among the experimental groups was in the CIS group. The difference between the groups was found to be significant (p≤0.05) (Figure 2).

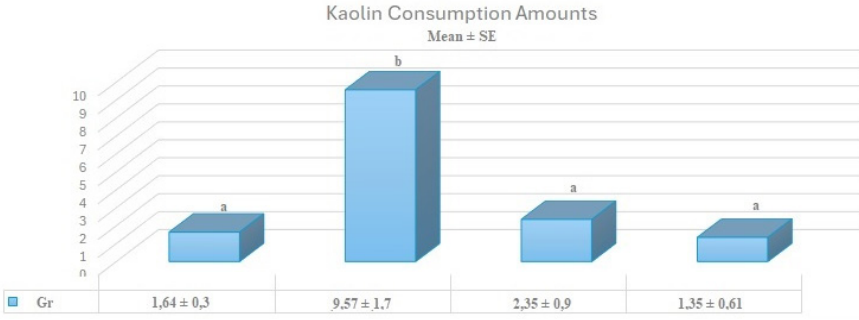


Figure 2. Kaolin consumption amounts (there was a significant difference between groups in the same column and carrying different letters [p<0.05]).

Histopathological results

The gastrointestinal tract tissues obtained after decapitation were histopathologically evaluated by H&E staining. In the control group and TQ group rats, the oesophagus showed normal histological structure; the mucosa, submucosa, which is the layer under the mucosa, and muscularis layer showed normal structure. In the CIS group, cisplatin caused damage in the oesophagus. Hyperkeratisation, degeneration and desquamation were observed in the mucosa. No significant difference was observed in the submucosa and muscularis layer. In the CIS+TQ group, no significant reduction in cisplatin-induced mucosal damage was observed (Figure 3).

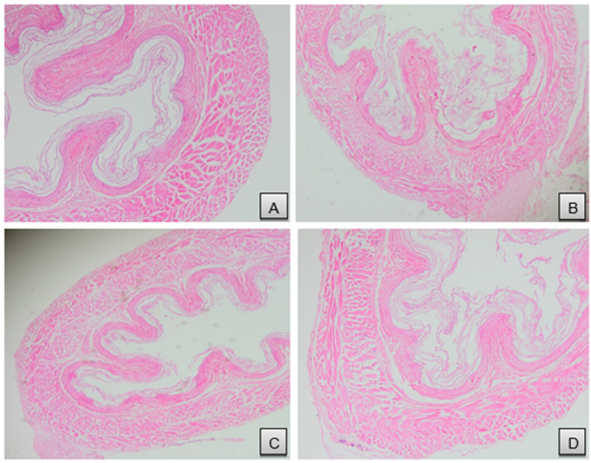


Figure 3. Esophageal histopathology x4. A: Control, B: CIS, C: TQ, D: CIS+TQ

When the histological structure of non-glandular anterior stomach was evaluated in control group rats, muscularis, submucosa and mucosa showed normal histological structure. Mucosa epithelium showed normal keratinisation. In rats given CIS, there was damage similar to the oesophagus structure. Hyperkeratosis, which is a thickening of the multilayered keratinized epithelial layer of the mucosa, was observed to be increased. Only in TQ group, keratinisation similar to the control group was observed. In CIS+TQ, there was no significant decrease in the hyperkeratosis caused by CIS (Figure 4).

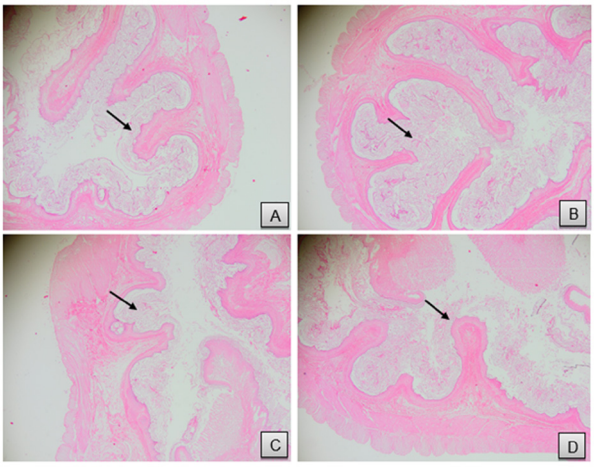


Figure 4. Histopathology of the stomach (non-glandular anterior stomach). Arrows point to squamous keratinized epithelium x4. A: Control, B: CIS, C: TQ, D: CIS+TQ H&E.

In control group rats, the stomach showed normal histological structure. The epithelium forming the mucosa of the fundic stomach and the gastric (fundic) glands in the lamina propria underneath showed normal histological structure. Submucosa and muscularis layer showed normal structure. The most prominent difference in the CIS group rats was cellular infiltration in the submucosa layer of the mucosa. There are also degenerations in the mucosal epithelial cells. The structure of the fundic glands shows a histological structure similar to the control group. TQ group showed normal histopathological structure. In the treatment group CIS+TQ, cellular infiltration and epithelial degeneration decreased (Figure 5).

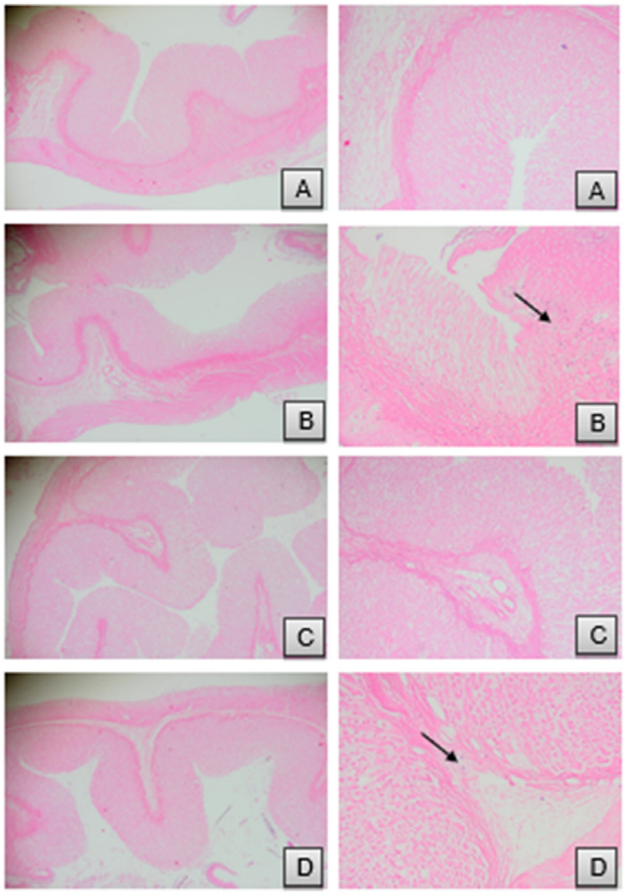


Figure 5. Histopathology of the stomach (fundus) x10. A: Control, B: CIS, C: TQ, D: CIS+TQ. (Arrows point to inflammatory cells). General image x4, close-up, H&E.

Small intestine of control rats showed normal histological structure. No histopathological changes were observed in the TQ group. Damage to the normal mucosa architecture was evident in rats given CIS. Degeneration and desquamation were observed at the ends of intestinal villi. Atrophic villi, wide cell spaces and cell atrophy were observed in the lamina propria. There was decreased fibrous content in the muscularis mucosa layer. In TQ+CIS group, thymoquinone decreased cisplatin-induced mucosal damage in the intestine, although it had no significant effect. In the muscularis mucosa, CIS-induced damage was significantly improved (Figure 6).

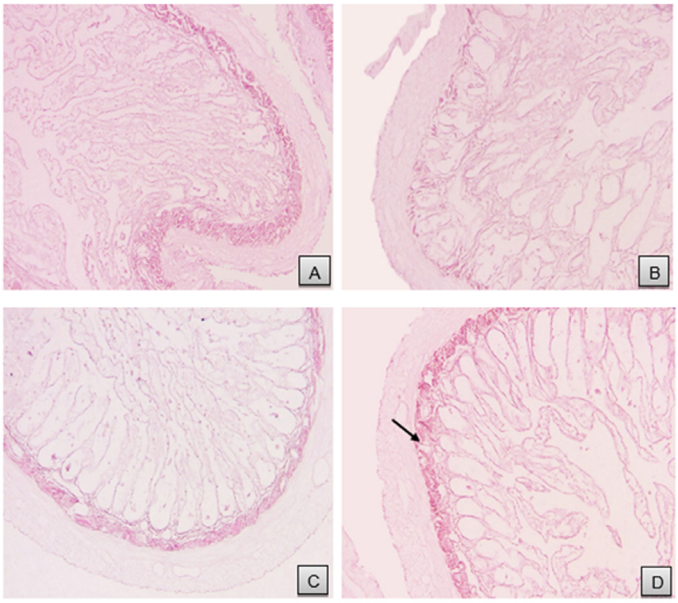


Figure 6. Small bowel histopathology x10. A: Control, B: CIS, C: TQ, D: CIS+TQ.

In control rats, the cecum showed normal histological structure. No adverse histopathological changes were observed in the TQ group. Cisplatin slightly damaged the cecal architecture. Thinning (decreased fibrous content) was observed in the muscularis externa layer. In the TQ+CIS group, mild damage to the cecal architecture continued, but the muscularis layer showed normal histological structure (Figure 7).

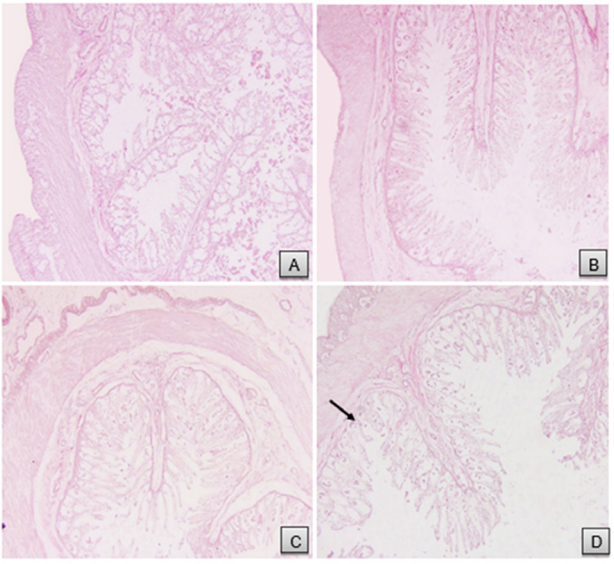


Figure 7. Histopathology of cecum x10. A: Control, B: CIS, C: TQ, D: CIS+TQ.

Histopathological evaluation revealed no severe degenerative changes in the architecture of the large intestine in the CIS group compared to the control group and the TQ group. This was the same in the CIS+TQ group (Figure 8).

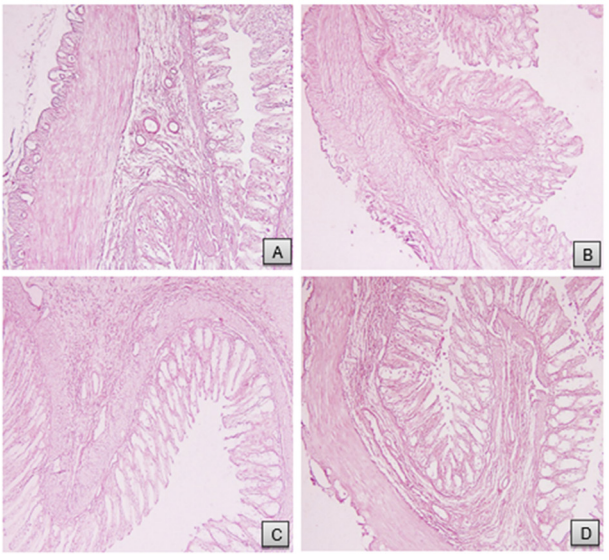


Figure 8. Large intestine histopathology x10. A: Control, B: CIS, C: TQ, D: CIS+TQ.

RESULTS and DISCUSSION

In this study, we observed that TQ reduced kaolin intake (pica) in cisplatin-treated rats and may partially contribute to gastrointestinal tract damage.

Various animal models have been used to evaluate emetic and antiemetic compounds[33,34,35]. Although these models have advantages such as cost and ease of animal handling, they also have limiting factors such as the absence of an emesis centre and the inability to demonstrate the vomiting reflex[36]. Although not through vomiting, the rat model responds to the effects of various stimuli that cause vomiting such as cisplatin, morphine, simulated motion sickness and radiation in a manner consistent with pica[37-39]. Cabezos et al. (2010) found that gastric motility was exacerbated in parallel with the increase in pica with chronic administration of cisplatin in mice. In addition, these researchers found that one week after the end of the treatment, no signs of gastric motility disorder remained, but basal kaolin intake was still higher than rats in control group[40]. Another study to investigate the effect of Xiao-Ban-Xia decoction (XBXD) on chemotherapy-induced nausea and vomiting (CINV) using rat pica model, parameters such as kaolin consumption, food intake and body weight were monitored after cisplatin administration and suggested that XBXD could be considered as a potential therapeutic agent in the treatment of CINV[41].

In this study, induction of pica in the first 24 hours following cisplatin (6 mg/kg, i.p.) in rats is consistent with previous studies using doses in the same range[37,40,41,42]. In addition, we concluded that the decrease in pica and simultaneous improvement in food intake in the group treated with CIS and then treated with TQ, and the reflection of this situation on animal weights showed the anti-nausea/antiemetic effect of TQ.

Low doses (≤3.5 mg/kg bwt) of CP treatment have been reported to cause villus atrophy but not affect crypt depth, thus reducing the villus-to-crypt ratio by approximately 47%. However, studies performed at higher doses (≥6 mg/kgwt) also revealed that CP caused disruption of mucosal glandular architecture, cryptablation, intense inflammatory cell infiltration in mucosal and submucosal layers, formation of crypt abscess, villus degeneration, decrease in villous density (rarefaction) and reduced villous height, enterocyte damage, nuclear crowding, cytoplasmic vacuolation[43-45]. Sathyanath et al. (2013) administered saponin and non-saponin obtained from the fractions of red ginseng before a single dose of intraperitoneal cisplatin (6 mg/kg) injection (-48, -24 and 0 h) and showed that it caused degenerative changes in the stomach (glandular region) and small intestine, mainly in the mucus-secreting cells and intestinal epithelium on the villi, with enlargement of intercellular spaces and disruption

of the epithelial structure[45]. The results of this study report that both red ginseng saponin and non-saponin improve feeding behavior against CP-induced pica in rats. Another group of investigators evaluated cisplatin-induced pathological changes in the GI tract using H&E staining and showed that Xiao-Ban-Xia-Tang decoction (XBXT), an antiemetic formula, can improve cisplatin-induced gastrointestinal tract damage and inflammatory response after 72 hours of model creation[46]. In parallel with the above-mentioned studies, in this study, we showed that cisplatin caused inflammatory damage in the GI tract, but TQ administration helped to ameliorate this damage.

In conclusion, this is the first study in which the effects of TQ on nausea and vomiting were investigated by pica method and histopathological examination of the gastrointestinal tract. The obtained findings contribute to the elucidation of the comprehensive mechanisms of TQ effect in CIS-induced pica, but further investigations are needed.

STATEMENT OF ETHICS

The study protocol was approved by Konya Necmettin Erbakan University Experimental Medicine Research and Application Center Laboratory Animals Ethics Board (No: 2021-040).

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Motivation / Concept: Elif Gulbahce-Mutlu, Ayse Nur Yildiz,

Design: Elif Gulbahce-Mutlu, Ayse Nur Yildiz,

Control/Supervision: Elif Gulbahce-Mutlu, Serife Alpa,

Data Collection and/or Processing: Elif Gulbahce-Mutlu, Ayse Nur Yildiz, Serife Alpa, Fatma Nur Türkoglu, Hatice Nur Seflek,

Analysis and/or Interpretation: Elif Gulbahce-Mutlu, Serife Alpa, Hatice Nur Seflek,

Literature Review: Elif Gulbahce-Mutlu, Ayse Nur Yildiz, Fatma Nur Türkoglu, Zeynep Nur Akyar,

Writing the Article: Elif Gulbahce-Mutlu, Serife Alpa, Fatma Nur Türkoglu, Hatice Nur Seflek,

Critical Review: Elif Gulbahce-Mutlu, Ayse Nur Yildiz, Serife Alpa, Fatma Nur Türkoglu, Hatice Nur Seflek, Zeynep Nur Akyar.

FUNDING SOURCES

This study was supported by Scientific and Technological Research Council of Türkiye [TUBITAK 2209-A; 1919B012101997] and Scientific Research Projects Coordinatorship of KTO Karatay University.

ACKNOWLEDGMENTS

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Scientific and Technological Research Council of Türkiye [TUBITAK 2209-A; 1919B012101997] and Scientific Research Projects Coordinatorship of KTO Karatay University.

REFERENCES

1. Ghosh S. Cisplatin: the first metal based anticancer drug. Bioorg Chem, 2019;88:102925. Doi: 10.1016/j.bioorg.2019.102925

2. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol, 2014;740:364-378. Doi: 10.1016/j.ejphar.2014.07.025

3. Romani, AM. Cisplatin in cancer treatment. Biochem. Pharmacol, 2022;206:115323. Doi: 10.1016/j.bcp.2022.115323

4. Cope, D. G. Clinical updates in nausea and vomiting. Semin Oncol Nurs, 2022;38(1):151249. Doi: 10.1016/j.soncn.2022.151249

5. Hawsawi ZA, Ali BA, Bamosa AO. Effect of *Nigella sativa* (black seed) and thymoquinone on blood glucose in albino rats. Ann Saudi Med, 2001;21(3-4):242-244. Doi: 10.5144/0256-4947.2001.242

6. El-Mahmoudy A, Shimizu Y, Shiina T, Matsuyama H, El-Sayed M, Takewaki T. Successful abrogation by thymoquinone against induction of diabetes mellitus with streptozotocin via nitric oxide inhibitory mechanism. Int Immunopharmacol, 2005;5(1):195-207. Doi: 10.1016/j.intimp.2004.09.00

7. Gun M. Holly Seed: *Nigella sativa*. Some knowledge corresponding to Nigella sativa's therapy ailment. Lokman Hekim Journal, 2012;2(1):43-46.

8. Dalli M, Bekkouch O, Azizi SE, Azghar A, Gseyra N, Kim B. *Nigella sativa* L. phytochemistry and pharmacological activities: a review (2019–2021). Biomolecules, 2021;12(1):20. Doi: 10.3390/biom12010020

9. Zakir F, Mishra H, Azharuddin M, Mirza MA, Aggarwal G, Iqbal Z. Gastrointestinal abnormalities and Nigella sativa: a narrative review of preclinical and clinical studies. In: Khan A, Rehman M, editors. Black seeds (Nigella sativa). Pharmacological and therapeutic applications. 2022. p. 355-386. Doi: 10.1016/B978-0-12-824462-3.00003-2

10. Habib MA, Chowdhury AI, Afroze M, Rahman T. Effects of Black cumin (Nigella sativa L.) on patients with cancer and tumor: a systematic review. J Curr Biomed Rep, 2021;2(1):38-43. Doi: 10.18502/jcbior.v2i1.9511

11. Baig WA, Alwosaibai K, Al-Jubran KM, Chaudhry TM, Al-Dowish N, Alsaffar F, et al. Synergistic anti-cancer effects of *Nigella sativa* seed oil and conventional cytotoxic agent against human breast cancer. Drug Metab Pers Ther, 2022;37(3):315-321. Doi: 10.1515/dmpt-2021-0053

12. Ahmad MF, Ahmad FA, Ashraf SA, Saad HH, Wahab S, Khan MI, et al. An updated knowledge of Black seed (*Nigella sativa* Linn.): review of phytochemical constituents and pharmacological properties. J Herb Med, 2021;25:100404. Doi: 10.1016/j.hermed.2020.100404

13. Alshwyeh HA, Aldosary SK, Ilowefah MA, Shahzad R, Shehzad A, Bilal S, et al. Biological potentials and phytochemical constituents of raw and roasted *Nigella arvensis* and *Nigella sativa*. Molecules, 2022;27(2):550. Doi: 10.3390/molecules27020550

14. Nyemb JN, Shaheen H, Wasef L, Nyamota R, Segueni N, Batiha GE. Black cumin: a review of its pharmacological effects and its main active constituent. Phcog Rev, 2022;16(32):107-125. Doi: 10.5530/phrev.2022.16.16

15. Hosni R, Haffez H, Elkordy, H. Common applications of black cumin Seed (*Nigella sativa*) oil in folk medicine. J Adv Pharm Res, 2023;7(1):1-14. Doi: 10.21608/aprh.2022.166251.119

16. Daba MH, Abdel-Rahman MS. Hepatoprotective activity of thymoquinone in isolated rat hepatocytes. Toxicol Lett, 1998;95(1):23-29. Doi: 10.1016/S0378-4274(98)00012-5

17. Talbott SM, Talbott JA. Effect of thymoquin black cumin seed oil as a natural immune modulator of upper-respiratory tract complaints and psychological mood state. Food Sci Nutri Res, 2022;5(1):1-6.

18. Chopra RN, Chopra SL, Chopra IC. Glossary of Indian medicinal plant. New Delhi, India: CSIR. 1956.

19. Houghton PJ, Zarka R, de las Heras B, Hoult JRS. Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. Planta Med, 1995;61(1):33-36. Doi: 10.1055/s-2006-957994

20. Güzelsoy P, Aydın S, Başaran N. Potential effects of thymoquinone the active constituent of black seed (Nigella sativa L.) on human health. J Lit Pharm Sci, 2018;7(2):118-135. Doi: 10.5336/pharmsci.2018-59816

21. Nagi MN, Mansour MA. Protective effect of thymoquinone against doxorubicin–induced cardiotoxicity in rats: a possible mechanism of protection. Pharmacol Res, 2000;41(3):283-289. Doi: 10.1006/phrs.1999.058

22. Phua CYH, Teoh ZL, Goh BH, Yap WH, Tang YQ. Triangulating the pharmacological properties of thymoquinone in regulating reactive oxygen species, inflammation, and cancer: therapeutic applications and mechanistic pathways. Life Sci, 2021;287:120120. Doi: 10.1016/j.lfs.2021.120120

23. Tabassum S, Rosli N, Ichwan SJA, Mishra P. Thymoquinone and its pharmacological perspective: a review. Pharmacol Res - Mod Chin Med, 2021;1:100020. Doi: 10.1016/j.prmcm.2021.100020

24. Perera, WPRT, Liyanage, JA, Dissanayake, KGC, Chandrasiri, WAL, Gunathilaka, H. A review on pharmacological activities and antimicrobial properties of *Nigella sativa* and isolated Thymoquinone. J Med Plants Stud, 2021;9(3):118-122. Doi: 10.22271/plants.2021.v9.i3b.1277

25. Magdy MA, Hanan el-A, Nabila el-M. Thymoquinone: novel gastroprotective mechanisms. Eur J Pharmacol, 2012;697(1-3):126-131. Doi: 10.1016/j.ejphar.2012.09.042

26. El-Abhar HS, Abdallah DM, Saleh S. Gastroprotective activity of *Nigella sativa* oil and its constituent, thymoquinone, against gastric mucosal injury induced by ischaemia/reperfusion in rats. J Ethnopharmacol, 2003;84(2-3):251-258. Doi: 10.1016/S0378-8741(02)00324-0

27. Abubakar HB, Falaki FA, Suleiman HI, Sufyan AJ, Jibril MM, et al. Proximate and elemental composition of black seed and jujube as formulation for the treatment of peptic ulcer. Environ Bioremediat Toxicol, 2023;6(1):19-23. Doi: 10.54987/jebat.v6i1.810

28. Danish I, Alam K. A Review on *Nigella sativa*'s medical potential: a miracle plant. J Newgen Res Pharm Healthc, 2024;2(2):93-115. Doi: 10.61554/ijnrph.v2i2.2024.104

29. Aung HH, Dey L, Mehendale S, Xie JT, Wu JA, Yuan CS. *Scutellaria baicalensis* extract decreases cisplatin-induced pica in rats. Cancer Chemother Pharmacol, 2003;52:453-458. Doi: 10.1007/s00280-003-0694-9

30. Brierley DI, Harman JR, Giallourou N, Leishman E, Roashan AE, Mellows BAD, et al. Chemotherapy-induced cachexia dysregulates hypothalamic and systemic lipoamines and is attenuated by cannabigerol. J Cachexia Sarcopenia Muscle, 2019;10(4):844-859. Doi: 10.1002/jcsm.12426

31. Shahid F, Farooqui Z, Abidi S, Parwez I, Khan F. Oral administration of thymoquinone mitigates the effect of cisplatin on brush border membrane enzymes, energy metabolism and antioxidant system in rat intestine. Biomed Pharmacother, 2017;94:1111-1120. Doi: 10.1016/j.biopha.2017.08.044

32. Tohei A, Kojima S, Ikeda M, Hokao R, Shinoda M. Effects of cyclophosphamide on the kaolin consumption (pica behavior) in five strains of adult male rats. J Vet Med Sci, 2011;73(7):901-906. Doi: 10.1292/jvms.10-0433

33. Zhong W, Shahbaz O, Teskey G, Beever A, Kachour N, Venketaraman V, Darmani NA. Mechanisms of nausea and vomiting: current knowledge and recent advances in intracellular emetic signaling systems. Int J Mol Sci, 2021;22(11):5797. Doi: 10.3390/ijms22115797

34. Nallamadan J, Kalusalingam A, Natarajan C, Krishnan M, Pillai NP, Ming LC, et al. Evaluation of anti-emetic activity of Syzygium aromaticum extracts in chick and rat models of emesis. J Pharm Res, 2024;28(4):1231-1243. Doi: 10.29228/jrp.804

35. Hasan R, Alshammari A, Albekairi NA, Bhuia MS, Afroz M, Chowdhury R, et al. Antiemetic activity of abietic acid possibly through the $5HT_3$ and muscarinic receptors interaction pathways. Sci Rep, 2024;14(1):6642. Doi: 10.1038/s41598-024-57173-0

36. Mehendale S, Aung H, Wang A, Yin JJ, Wang CZ, Xie JT, et al. American ginseng berry extract and ginsenoside Re attenuate cisplatin-induced kaolin intake in rats. Cancer Chemother Pharmacol, 2005;56:63-69. Doi: 10.1007/s00280-004-0956-1

37. Takeda N, Hasegawa S, Morita M, Matsunaga T. Pica in rats is analogous to emesis: an animal model in emesis research. Pharmacol Biochem Behav, 1993;45(4):817-821. Doi: 10.1016/0091-3057(93)90126-e

38. Morita M, Takeda N, Kubo T, Yamatodani A, Wada H, Matsunaga T. Effects of anti-motion sickness drugs on motion sickness in rats. ORL J Otorhinolaryngol Relat Spec, 1988;50(5):330-333. Doi: 10.1159/000276008

39. Yamamoto K, Takeda N, Yamatodani A. Establishment of an animal model for radiation-induced vomiting in rats using pica. J Radiat Res, 2002;43(2):135-141. Doi: 10.1269/jrr.43.135

40. Cabezos PA, Vera G, Martín-Fontelles MI, Fernández-Pujol R, Abalo R. Cisplatin-induced gastrointestinal dysmotility is aggravated after chronic administration in the rat. Comparison with pica. Neurogastroenterol Motil, 2010;22(7):797-e225. Doi: 10.1111/j.1365-2982.2010.01483

41. Zhao Y, Han J, Hu W, Da, Y, Wu X, Liao X, et al. Xiao-Ban-Xia decoction mitigates cisplatin-induced emesis via restoring PINK1/Parkin mediated mitophagy deficiency in a rat pica model. J Ethnopharmacol, 2024;318:116882. Doi: 10.1016/j.jep.2023.116882

42. Saeki M, Sakai M, Saito R, Kubota H, Ariumi H, Takano Y, et al. Effects of HSP-117, a novel tachykinin NK1-receptor antagonist, on cisplatin-induced pica as a new evaluation of delayed emesis in rats. Jpn J Pharmacol, 2001;86(3):359-362. Doi: 10.1254/jjp.86.359

43. Miyamoto Y, Miyamoto M. Immunohistochemical localizations of secretin, cholecystokinin, and somatostatin in the rat small intestine after acute cisplatin treatment. Exp Mol Pathol, 2004;77(3):238-245. Doi: 10.1016/j.yexmp.2004.05.007

44. Liu YL, Malik N, Sanger GJ, Friedman MI, Andrews PL. Pica—a model of nausea? Species differences in response to cisplatin. Physiol Behav, 2005;85(3):271-277. Doi: 10.1016/j.physbeh.2005.04.009

45. Sathyanath R, Hanumantha Rao BR, Kim HG, Cho JH, Son CG. Saponin and non-saponin fractions of red ginseng ameliorate cisplatin-induced pica in rats. Pharm Biol, 2013;51(8):1052-1060. Doi: 10.3109/13880209.2013.775660

46. Naqshbandi A, Rizwan S, Khan MW, Khan F. Dietary flaxseed oil supplementation ameliorates the effect of cisplatin on brush border membrane enzymes and antioxidant system in rat intestine. Hum Exp Toxicol, 2012;32(4):385-394. Doi: 10.1177/0960327112438929

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6337

Anti-microbial and anti-oxidant effects of *Campanula involucrata* Aucher ex A.DC. and *Nepeta menthoides* Boiss & Buhse from Iran

Solmaz ASNAASHARI[1], Ailin KHABIRI[2], Fariba HESHMATI-AFSHAR[3], Somaieh SOLTANI[4], Somayeh HALLAJ-NEZHADI[5*]

1 Tabriz University of Medical Sciences, Biotechnology Research Center, Tabriz, Iran
2 Tabriz University of Medical Sciences, Student Research Committee, Tabriz, Iran
3 Tabriz University of Medical Sciences, Faculty of Pharmacy, Department of Pharmacognosy, Tabriz, Iran
4 Tabriz University of Medical Sciences, Faculty of Pharmacy,Tabriz, Iran
5 Tabriz University of Medical Sciences, Drug Applied Research Center & Faculty of Pharmacy, Tabriz, Iran

ABSTRACT

Considering the global efforts against anti-microbial drug resistance and the need to find new natural sources with anti-microbial effects, the anti-microbial and anti-oxidant activities of two Iranian plants, *Campanula involucrata* and *Nepeta menthoides*, were evaluated. Aerial parts of both species were extracted using a Soxhlet apparatus and three solvents with different polarities (n-hexane, methylene chloride, and methanol), respectively. The methanol extracts of both species as the most potent parts were fractionalized using a solid-phase extraction (SPE) method. The anti-microbial activities were determined against two Gram-positive (*Staphylococcus aureus, Bacillus subtilis*), four Gram-negative bacteria (*Proteus morganii, Escherichia coli, Shigella flexneri, Salmonella typhi*), and a fungus (*Candida albicans*) species by disc diffusion method. Then, the extracts or fractions with the most potent anti-microbial activities were selected for evaluating their MIC (Minimum Inhibition Concentration). Finally, anti-oxidant potency and total phenol contents of men-

* Corresponding author: Somayeh HALLAJ-NEZHADI
E-mail: hallajnezhadis@tbzmed.ac.ir
ORCIDs:
Solmaz ASNAASHARI: 0000-0001-6187-309X
Ailin KHABIRI: 0009-0000-6808-6283
Fariba HESHMATI-AFSHAR: 0000-0002-1382-6106
Somaieh SOLTANI: 0000-0002-0102-2960
Somayeh HALLAJ-NEZHADI: 0000-0003-4730-205X
(Received 9 Aug 2023, Accepted 14 Feb 2024)

tioned extracts and fractions were evaluated using DPPH and Folin-Ciocalteu reagents. The results demonstrated significant anti-bacterial activities, especially in methanolic extracts of both species and 40% and 10% methanol/water fractions in *C. involucrata* and *N. menthoides*, respectively. Moreover, anti-oxidant activities and total phenol contents of different extracts confirmed the presence of bioactive ingredients in the methanolic extracts and their relevant fractions. Additional studies are necessary to isolate and characterize bioactive compounds and their *in vivo* anti-microbial properties.

Keywords: antibiotic, anti-oxidant activity, *Campanula*, *Nepeta*

INTRODUCTION

Antibiotic resistance is one of the most important global challenges, which means the ability of microorganisms to thrive and tolerate antibiotics[1]. The spread of various infectious diseases and indiscriminate usage of a wide range of anti-microbial drugs leads to the efforts to encourage people to rational use of antibiotics to decrease the volume of consumption and reduce resistance rates[2]. Antibiotic resistance is one of the main topics with a high priority for WHO (World Health Organization). In 2015, the World Health Assembly adopted the global action plan for the antibiotic resistance problem with five strategic purposes, which include: 1) Improving awareness, 2) Supporting the research, 3) Decreasing the incidence of infection, 4) Optimizing the antibiotic uses, and 5) Development of the economic case for maintainable investment in new drugs, diagnostics, and other interventions for the needs of all countries[3]. Although about 50 years have passed since the discovery of antibiotics in the golden era, antibiotic resistance is still one of the most important issues in developed and developing countries[4,5]. According to previous studies, more than 80% of the prescribed antibiotics for upper respiratory infections are unsuitable and unnecessary, with harmful consequences such as antibiotic resistance, which causes noteworthy morbidity and mortality and imposes a massive global economic burden[1,6]. After the large-scale production of penicillin during World War II, the need to produce and discover herbal medicines increased, and the pharmaceutical companies focused their efforts on finding and producing new antibiotics. Although the use of natural products reduced with the development of chemistry in pharmaceutical industries later, the use of various drugs with natural sources for different health problems is still popular among people. Therefore, for many pharmaceutical companies which continue to natural products discovery research, the overall process for finding and developing novel natural compounds has not changed[7]. Along with the challenges of discovering

antibiotics with natural sources, we selected two Iranian species (*Campanula involucrata* Aucher ex A.DC. and *Nepeta menthoides* Boiss & Buhse) and evaluated possible anti-bacterial and antifungal as well as anti-oxidant activities. Moreover, the total phenol content of active extract of both species was assessed. The genus *Campanula* (Campanulaceae) has 44 species of annual and perennial plants in Iran[8]. The distribution of the plants of this genus is generally in Asia, Europe, and North and Northwest Africa[9,10]. Previous literature reviews have demonstrated different biological and pharmacological activities of the plants of *Campanula* genus, including, anti-oxidant, anti-microbial, anti-inflammatory, antidiabetic, anti- nociceptive, wound healing, and cytotoxic effects[9,11,12]. According to phytochemical studies, these plants are a rich source of flavonoids (especially anthocyanins) and saponin structures[9,11]. Furthermore, the presence of alkaloids, cardiac glycosides, sterols, triterpenoids, and tannins was confirmed in the different species of this genus[13]. The second evaluated species, *Nepeta menthoides*, (Labiatae) is one of 75 identified species of the *Nepeta* genus of Iran[14]. The plants of this genus are distributed in Asia, Europe, and Africa and are used widely in traditional medicine[15]. A wide range of effects, such as anti-asthmatic, anti-spasmodic, anti-septic, antitussive, astringent, blood depurative, diuretic, diaphoretic, emmenagogue, febrifuge, lowering blood pressure, and sedative properties have been reported from the various species of this genus[15,16]. Furthermore, monoterpenoids (nepetalactones and iridoids), sesquiterpenoids, diterpenoids, triterpenoids, and flavonoids were identified as the responsible structural groups of these plants[15].

METHODOLOGY

Plant material

The aerial parts of *C. involucrata* and *N. menthoides* were collected respectively from Goy Zangi Mountain (2800 meters above the sea) and Sahand mountains (3457 meters above the sea) in East Azarbaijan province in Iran. The identity of the studied species has been approved in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. Extraction and fractionation fifty grams of dried and powdered aerial parts of both species were extracted using Soxhlet apparatus with n-Hexane, methylene chloride, and methanol (500mL each, Caledon Company, Canada). The obtained 6 extracts were concentrated separately using a rotary evaporator (Heidolph, Germany) at 45°C. Then 2 grams of methanol extract was weighed and subjected to solid-phase extraction (SPE) using a C18 Sep-Pak cartridge (Waters, USA), with a step gradient of MeOH/water mixture elution (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). All these fractions were dried using a rotary evaporator at the temperature of 45°C[17].

Anti-microbial assay

Microbial strains

Examined microorganisms included two species of Gram-positive bacteria (*Staphylococcus aureus* PTCC 1112, *Bacillus subtilis* PTCC 1715), four strains of Gram-negative species (*Proteus morganii* PTCC 1078, *Escherichia coli* PTCC 1533, *Shigella flexneri* PTCC 1234, *Salmonella typhi* PTCC1230) and a fungus, *Candida albicans* (PTCC 5027) which were purchased in lyophilized culture from the Iranian bacterial collection center.

Disc diffusion test

The Agar disc diffusion method was used to assess anti-microbial effects as described by Bauer et al.[18]. For the experiments, an overnight culture of microorganisms in Mueller–Hinton broth was used to provide a suspension with turbidity equivalent to the 0.5 McFarland tube (1.5×10^8 bacteria/ml). Briefly, the plates containing Mueller–Hinton agar were inoculated with one of the microorganisms by spreading microbial suspension onto the surface of the medium with a sterile cotton swab. Then, paper discs (6 mm in diameter) were placed on the surface of the inoculated agar. The 50% DMSO and the standard disc of Amikacin were used as negative and positive controls, respectively. The extracts were dissolved in 50% DMSO to a final concentration of 100 mg/mL, then 50 µL of different extracts solutions were added to placed discs on the culture medium, and then they were incubated in a refrigerator for 30 minutes to allow the anti-microbial agents diffuse in the agar. Then, the plates were incubated for 24 hours at 37°C. The anti-microbial effects of extracts were determined by measuring the diameter of inhibition zones (DIZ) around the sterile discs. The experiments were repeated at least three times, and then the mean DIZ was calculated[19,20]. The sensitivity was classified as follows based on the DIZ: not sensitive (DIZ≤8 mm), sensitive (DIZ=9–14 mm), very sensitive (DIZ=15–19 mm), and extremely sensitive (DIZ≥20 mm)[21-23].

Minimum Inhibitory Concentration (MIC)

Extracts or fractions with the most potent anti-bacterial activities were selected to evaluate their MIC value. The MIC was determined by the macro-dilution method with minor modifications to the guidelines of the Clinical and Laboratory Standards Institute[24]. Serial two-fold dilutions of the extracts were prepared in a Mueller–Hinton broth medium in tubes, and then, an equal volume of the bacterial suspension in Mueller–Hinton broth was added to each dilution to result in a final cell density of around 5×10^6 CFU/mL. After incubation at 35°C for 18 hours, the concentration of anti-microbial contained in the first

clear tube is read as the MIC. A tube containing DMSO, culture medium, and the bacterial suspension was utilized as a positive control, and a tube containing extracts, DMSO, and culture medium was used as a negative control. Then, the tubes were incubated at 37°C for 24h.

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay

Anti-oxidant activities of extracts and fractions with different polarities of *C. involucrata* and *N. menthoides* aerial parts were evaluated using the DPPH reagent (Sigma Aldrich, Germany). For preparing the DPPH reagent, 4 mg of DPPH powder was dissolved in 50 mL methanol or chloroform using ultrasonic bath equipment. Methanol and chloroform were used for polar and non-polar extracts, respectively. Then, the stock solution of extracts was prepared at a concentration of 1 mg/mL, and serial dilutions were made in 10 different concentrations in methanol or chloroform. In the next step, 2 mL of diluted solutions of extracts were mixed with 2 mL of DPPH reagent and were allowed to accrue reactions in 30 minutes. Finally, the UV-Vis absorbance of samples was recorded at 517 nm, and the percentage of reduction capacity of DPPH was calculated according to:

$$Reduction\ capacity\ (\%) = (absorbance_{Blank} - absorbance_{sample}) / absorbance_{Blank}$$

The blank solution contained all substances except the extract and standard compound. The final results were reported as RC50 (Reduction Capacity 50%), defined as the extract concentration providing 50% loss of DPPH activity. All tests were repeated three times, and a similar method was performed for quercetin as the positive control[20].

Total phenol content assay (TPC)

The phenolic contents of extracts were measured using the Folin-Ciocalteu reagent (Merck, Germany). The samples were dissolved in the acetone 60% solution to obtain a 5 mg/mL concentration. Then 1 mL of these solutions were mixed with 200 µL Folin-Ciocalteu reagent (1:1 mixed with water) and 1 mL of 2% Na_2CO_3 and incubated at room temperature for 30 minutes. The absorbance of the samples was read at 750 nm using spectrophotometer equipment (Pharmacia Biotech, England). The same procedure was performed for different concentrations of gallic acid as the standard compound, and the sample without any extract was used as blank. The measurements were done in triplicate[25].

RESULTS and DISCUSSION

In the present research, anti-bacterial activities of *C. involucrata* and *N. menthoides* aerial parts extracts and their fractions of the most potent extracts were studied against two Gram-positive, four Gram-negative bacteria, and a fungus. The results are separately represented for both plant species in Table 1 to Table 4.

Table 1. Anti-bacterial activities (DIZs) of *C. involucrata* extracts and methanolic fractions

		DIZ (mm)									
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**	**Total extract**	**Amikacin**
Proteus morganii	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	25
Staphylococcus aureus	26	29	29	10	21	28	11	ND	ND	29	28
Escherichia coli	ND	ND	27	ND	ND	16	ND	ND	ND	27	29
Bacillus subtilis	10	16	31	ND	ND	18	ND	ND	ND	31	28
Shigella flexneri	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22
Salmonella typhi	ND	18	31	ND	ND	14	ND	ND	ND	31	26
Candida albicans	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND

*Not determined

Table 2. Anti-bacterial activities (DIZs) of *N. menthoides* extracts and methanolic fractions

	DIZ (mm)										
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**	**Total extract**	**Amikacin**
Proteus morganii	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	21
Staphylococcus aureus	21	40	32	27	21	22	20	19	19	32	23
Escherichia coli	ND	8	18	10	ND	ND	ND	ND	ND	18	26
Bacillus subtilis	19	22	31	25	ND	ND	ND	ND	14	31	22
Shigella flexneri	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17
Salmonella typhi	20	29	34	18	ND	ND	ND	ND	13	34	17
Candida albicans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Not determined

Table 3. Minimum inhibitory concentration (MIC) values of *C. involucrata* extracts and methanolic fractions

Microorganism	MIC (µg/mL)								
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**
Staphylococcus aureus	ND*	125	31.25	250	125	62.5	250	ND	ND
Escherichia coli	ND	500	125	ND	ND	500	ND	ND	ND
Bacillus subtilis	ND	125	62.5	ND	ND	250	ND	ND	ND
Salmonella typhi	ND	500	250	ND	ND	500	ND	ND	ND

*Not determined

Table 4. Minimum inhibitory concentration (MIC) values of *N. menthoides* extracts and methanolic fractions

Microorganism			MIC (µg/mL)						
Bacterial strain	**n- Hexane**	**Methylene Chloride**	**Methanolic extract**	**Fr. 10%**	**Fr. 20%**	**Fr. 40%**	**Fr. 60%**	**Fr. 80%**	**Fr. 100%**
Staphylococcus aureus	ND*	62.5	15.625	31.25	250	250	250	250	250
Escherichia coli	ND	500	62.5	250	ND	ND	ND	ND	ND
Bacillus subtilis	ND	62.5	31.25	62.5	ND	ND	ND	ND	125
Salmonella typhi	ND	125	62.5	250	ND	ND	ND	ND	250

*Not determined

Moreover, the free radical scavenging capacity, total phenol contents of the extracts, and the most potent extract fractions are shown in Tables 5 and 6.

Table 5. Anti-oxidant activities (RC50: mg/mL) of *C. involucrata* and *N. menthoides* extracts and fractions

Plants	n- Hexane	Methylene Chloride	Methanolic extract	Fr. 10%	Fr. 20%	Fr. 40%	Fr. 60%	Fr. 80%	Fr. 100%
C. involucrate	ND*	ND	167 ± 0.0014	ND	149 ± 0.0014	46 ± 0.0007	ND	ND	ND
N. menthoides	ND	ND	178 ± 0.0014	23 ± 0.0007	156 ± 0.0021	256 ± 0.0071	ND	ND	ND
Quercetin	3.9 ± 0.002								

*Not determined

Table 6. Total phenol contents (mg GAE/g) o*f C. involucrata* and *N. menthoides* extracts and fractions

Plants	n- Hexane	Methylene Chloride	Methanolic extract	Fr. 10%	Fr. 20%	Fr. 40%	Fr. 60%	Fr. 80%	Fr. 100%
C. involucrate	-	-	154	137	172	0.461	88	119	11
N. menthoides	-	-	161	180	156	199	58	98	83

The DIZs of three different extracts of *C. involucrata* were studied on the seven microbial species and the results showed that the methanol extract possessed significant anti-bacterial effects against *S. aureus*, *B. subtilis, S. typhi* and, *E. coli* (extremely sensitive (DIZ≥20 mm)). The observed anti-bacterial activities were more potent than the anti-bacterial activity of Amikacin (as a positive standard) in all cases except *E. coli*. Also, among the fractions of methanol extract, the 40% fraction was the most potent but weaker than methanolic extract (Table 1). Moreover, the presented MIC values of extracts and fractions in Table 3 confirmed that the methanol extract was the most active part toward S. aureus (MIC=31.25 µg/ml). The obtained results may be attributed to the synergistic effects of the available compounds in the methanol extract[26]. Several studies have shown notable pharmacological activities of various species of the *Campanula* genus. According to a published study in 2011 in Turkey, whole plant essential oil of *Campanula olympica* demonstrated moderate anti-bacterial and antifungal activities against *Escherichia coli, Yersinia pseudotuberculosis, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus, Mycobacterium smegmatis,* and *Candida albicans*[27]. Based on the other research, the volatile oil of *Campanula portenschlagiana*, an endemic species to Croatia, revealed moderate to potent anti-bacterial activities against tested Gram-positive species (*Enterococcus faecalis, Staphylococcus*

aureus, Clostridium perfringens, Listeria monocytogenes, and *Bacillus cereus*) with MIC values of 62.5 to 125 µg/mL. Moreover, this oil had stronger activities against Gram-negative species, including *Escherichia coli, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*, with MIC values of 7.8 to 62.5 µg/ml[28]. The phytochemical evaluation indicated that diterpene alcohols, the essential oil's major constituents, could be the responsible ingredients in different biological effects[28]. The same study reported that the aqueous extract of *Campanula portenschlagiana* with a total phenol content of 40.6 mg GAE/g showed lower anti-microbial activities than essential oil. The MIC values of the aqueous extract were 125 to 500 µg/ml against Gram-positive and 125 to 250 µg/ml for Gram-negative[28].

In another study, ethanol and methanol extracts *of Campanula glomerata* L. showed potent anti-bacterial activities against *Streptococcus pyogenes* and *Klebsiella pneumonias*, respectively. Moreover, different extracts of *Campanula olympica* Boiss. demonstrated significant anti-bacterial activities toward *Streptococcus pyogenes, Klebsiella pneumonias*, and *Escherichia coli*[29]. In a recent study, the dichloromethane extracts of leaves of *Campanula retrorsa* showed a moderate anti-microbial effect on *Acinetobacter baumanii* and *Candida albicans*[30]. There are limited available data on the *C. involucrata* biological, pharmacological and phytochemical properties. Hashemi and Zarei reported the tyrosinase inhibitory activity of *C. involucrata* species from Kurdistan, Iran. According to their results, 50% inhibition capacity (IC50) of n-hexane extract was 0.575 µg/mL and it indicated a significant inhibition value (75.62%, 75.45% and, 62.26%) at concentration of 1 µg/mL. Therefore, it can be a useful natural source for suppressing unpleasant hyperpigmentation in human skin[31]. Furthermore, the methanol extract of *C. involucrata* exhibited a significant anti-oxidant effect and inhibition (>60%) against the alpha-glucosidase enzyme, where the inhibition capacity (IC50) was 0.02 mg/mL. Consequently, it may be able to prevent the development of diabetic symptoms[32,33]. The phenolic compounds, especially flavonoids, have been isolated in abundance from the methanol extract of *Campanula pyramidalis* and *Campanula alata* species[34,35]. Moreover, the presence of anthocyanin structures from *Campanula medium* petals were reported previously[36]. Therefore, the significant anti-microbial activity of the methanolic extract of the investigated plant could be relate to the presence of flavonoid compounds including anthocyanin compounds. According to Tables 2 and 4, the second tested species, *N. menthoides*, was more potent than *C. involucrata* in most cases. Among the triple extracts of *N. menthoides*, the methanol extract had the most potent anti-bacterial effects. The DIZs of methanol extract on *S. aureus, B.*

subtilis, and *S. typhi* (extremely sensitive [DIZ≥20 mm]) were nearly 1.5 times greater than those of Amikacin. Also, the 10% fraction of methanol extract was the most potent relative to the other fractions. In the case of *N. menthoides*, as in *C. involucrata* species, the anti-bacterial power of the methanolic extract was higher than its isolated fractions. According to previous studies, the essential oil of *Nepeta crispa* showed important anti-bacterial activities against all the tested seven Gram-positive and Gram-negative bacteria and four fungi[16]. The main constituents were found to be 1,8-cineol (47.9%) and 4aα,7α,7a-nepetalactone (20.3%). The other research by Kahkeshani et al. revealed that, unlike the essential oils, 1,8-cineole showed no inhibition on fungi specially *Aspergillus* species, but it was more potent than the essential oil against Gram-negative species[37]. Studies on the different parts of the other species, *Nepeta persica*, indicated that the anti-bacterial effects of the essential oils might be because of their great content of nepetalactone isomers[38]. Further studies showed that the 50% methanolic extract of *N. menthoides* had significant inhibitory effects against the Gram-positive bacterial strains, and there was a direct relationship between total flavonoid contents of *N. menthoides* extracts and anti-bacterial activities. Additionally, *N. menthoides* was introduced as a good source of natural bioactive structures[14].

There are several studies about the anti-bacterial and antifungal effects of different species of *Nepeta*. In a recently published review article, traditional uses and pharmacological effects, as well as phytochemical properties of the plants of *Nepeta* genus were described. The authors have frequently mentioned the anti-microbial activities of various species of the *Nepeta* genus[39]. Moreover, new details about these effects of various species in different areas are being updated. For example, methanol extract of *Nepeta juncea* leaves showed high biological effects such as anti-microbial activity[40]. Among different extracts of *Nepeta cataria*, the maximum inhibition percentage toward examined bacteria species was 250-1000 µg/mL; and methanol and ethanol-based extracts were the potent parts[41]. Primary phytochemical analysis of this species confirmed the presence of phenolic compounds, tannins, flavonoids, cardiac glycosides, terpenoids, anthraquinones, and alkaloids in the extracts[41]. Generally, based on the literature, most of these compounds have demonstrated significant anti-microbial activities[42-47]. The other biological properties studied in this research were the measurement of anti-oxidant activities of different extracts and fractions of two mentioned plant species. Based on anti-oxidant results in Table 5, the methanolic extract of *C. involucrata* and its 40% fraction and the methanolic extract of *N. menthoides* and its 10% fraction were the most potent parts. Moreover, the evaluation of the results of phenolic contents

(Table 6) established that only methanolic extracts possessed acceptable contents of active natural phenolic compounds with TPC values of 154 mg GAE/g (*C. involucrata*) and 161 mg GAE/g (*N. menthoides*). Similar outcomes were reported previously in different species of *Campanula* and *Nepeta* species[13,30,48,49]. This evidence indicates the presence of numerous active natural compounds in various species of both plant genera that can be important for future pharmacological, biological, and clinical studies.

STATEMENT OF ETHICS

There are no ethical issues with human or animal subjects.

CONFLICT OF INTEREST STATEMENT

Nothing to declare.

AUTHOR CONTRIBUTIONS

Design: Fariba Heshmati-Afshar and Somayeh Hallaj-Nezhadi

Acquisition of data: Ailin Khabiri and Somaieh Soltani

Analysis of data: Solmaz Asnaashari and Somayeh Hallaj-Nezhadi

Drafting of the manuscript: Solmaz Asnaashari and Somayeh Hallaj-Nezhadi.

FUNDING SOURCES

This research received no grant from any funding source.

ACKNOWLEDGMENTS

The presented data in this manuscript were obtained from a Pharm.D. thesis (No. 29) registered by Tabriz University of Medical Sciences.

REFERENCES

1. Kurauchi A, Struchiner CJ, Wilder-Smith A, Massad E. Modelling the effect of a dengue vaccine on reducing the evolution of resistance against antibiotic due to misuse in dengue cases. Theor Biol Med Model, 2020;17(1):1-7. Doi: 10.1186/s12976-020-00125-8

2. Carey B, Cryan B. Antibiotic misuse in the community--a contributor to resistance? Ir J Med, 2003;96(2):43-4,46.

3. Mendelson M, Matsoso MP. The World Health Organization global action plan for antimicrobial resistance. SAMJ, 2015;105(5):325. Doi: 10.7196/SAMJ.9644

4. Moges F, Endris M, Mulu A, Tessema B, Belyhun Y, Shiferaw Y, et al. The growing challenges of antibacterial drug resistance in Ethiopia. J Global Antimicrob Resist, 2014;2(3):148-154. Doi: 10.1016/j.jgar.2014.02.004

5. Hoffman PS. Antibacterial discovery: 21st century challenges. Antibiotics, 2020;9(5):213. Doi: 10.3390/antibiotics9050213

6. Razzaque MS. Implementation of antimicrobial stewardship to reduce antimicrobial drug resistance. Expert Rev Anti-infect Ther, 2020;19(5):559-562. Doi: 10.1080/14787210.2021.1840977

7. Baker DD, Chu M, Oza U, Rajgarhia V. The value of natural products to future pharmaceutical discovery. Nat Prod Rep, 2007;24(6):1225-1244. Doi: 10.1039/b602241n

8. Aghabeigi F, Assadi M. The genus Campanula (Campanulaceae) in Iran. Edinb J Bot, 2008;65(3):375-385. Doi: 10.1017/S0960428608004848

9. Alhage J, Elbitar H, Taha S, Benvegnu T. In vitro assessment of antioxidant, antimicrobial, cytotoxic, anti-inflammatory, and antidiabetic activities of Campanula retrorsa crude extracts. Pharmacogn Res, 2018;10(4):397-403. Doi: 10.4103/pr.pr_73_18

10. Mozaffarian V. Identification of medicinal and aromatic plants of Iran. 3rd ed. Iran, Tehran; Farhang Moaser; 2012.

11. Park SH, Sim YB, Lim SS, Kim JK, Lee JK, Suh HW. Antinociception effect and mechanisms of *Campanula punctata* extract in the mouse. Korean J Physiol Pharmacology, 2018;14(5):288-289. Doi: 10.4196/kjpp.2010.14.5.285

12. Suntar I, Akkol EK, Gonenc TM, Erdogan TF, Keles H, Kivçak B. Scientific assessment of the anti-inflammatory and wound healing potential of *Campanula lyrata* subsp. *lyrata*, a Turkish folk remedy. Turk J Pharm Sci, 2015;12(2):157-168. Doi: 10.5505/tjps.2015.58066

13. Moosavi SR, Ardekani MRS, Vazirian M, Lamardi SNS. Campanula latifola, giant bellflower: ethno-botany, phytochemical and antioxidant evaluation. Trad Integr Med, 2018;3(3):113-119.

14. Ghandchi S, Jamzad M. Total flavonoids contents and antibacterial activity of the extracts of two Labiateae species: *Nepeta menthoides* and *Thymus trautvetteri*. J Med Plants Prod, 2015;4(1):77-82. Doi: 10.22092/jmpb.2015.108894

15. Formisano C, Rigano D, Senatore F. Chemical constituents and biological activities of *Nepeta* species. Chem Biodiversity, 2011;8(10):1783-1818. Doi: 10.1002/cbdv.201000191

16. Sonboli A, Gholipour A, Yousefzadi M, Mojarrad M. Antibacterial activity and composition of the essential oil of *Nepeta menthoides* from Iran. Nat Prod Commun, 2009;4(2):283-286. Doi: 10.1177/1934578X09004002

17. Delazar A, Asnaashari S. Two iridoid structures from *Eremostachys macrophylla* Montbr. & Auch. rhizomes. J Rep Pharm Sci, 2018;7(2):221-226.

18. Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 1966;45(4):493-496. Doi: 10.1093/ajcp/45.4_ts.493

19. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: a review. J Pharm Anal, 2016;6(2):71-79. Doi: 10.1016/j.jpha.2015.11.005

20. Asgharian P, Delazar A, Lotfipour F, Asnaashari S. Bioactive properties of *Eremostachys macrophylla* Montbr. & Auch. rhizomes growing in Iran. Pharm Sci, 2017;23(3):238-243.

21. Djabou N, Lorenzi V, Guinoiseau E, Andreani S, Giuliani M-C, Desjobert J-M, et al. Phytochemical composition of Corsican *Teucrium* essential oils and antibacterial activity against foodborne or toxi-infectious pathogens. Food Control, 2013;30(1):354-363. Doi: 10.1016/j.foodcont.2012.06.025

22. Li Z-H, Cai M, Liu Y-S, Sun P-L, Luo S-L. Antibacterial activity and mechanisms of essential oil from *Citrus medica L.* var. *sarcodactylis*. Molecules, 2019;24(8):1577. Doi: 10.3390/molecules24081577

23. Ponce A, Fritz R, Del Valle C, Roura S. Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. LWT-Food Sci Technol, 2003;36(7):679-684. Doi: 10.1016/S0023-6438(03)00088-4

24. Wikler MA. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. CLSI (NCCLS), 2006;26:M7-A.

25. Asnaashari S, Delazar A, Asgharian P, Lotfipour F, Moghaddam SB, Afshar FH. *In-vitro* bioactivity and phytochemical screening of extracts from rhizomes of *Eremostachys azerbaijanica* Rech.f. growing in Iran. Iran J Pharm Research, 2017;16(1):306-314.

26. Tafesh A, Najami N, Jadoun J, Halahlih F, Riepl H, Azaizeh H. Synergistic antibacterial effects of polyphenolic compounds from olive mill wastewater. Evid Based Complementary Altern Med, 2011(1):1-9. Doi: 10.1155/2011/431021

27. Tosun G, Kahriman N, Coskuncelebi K, Genc H, Karaoglu S, Yayli N. Chemical composition and biological activity of the essential oil of *Campanula olympica* Boiss. Asian J Chem, 2011;23(6):2389-2391.

28. Politeo O, Skocibusic M, Burcul F, Maravic A, Carev I, Ruscic M, et al. *Campanula portenschlagiana* Roem. et Schult.: chemical and antimicrobial activities. Chem Biodiversity, 2013;10(6):1072-1080. Doi: 10.1002/cbdv.201200094

29. Usta C, Yildirim AB, Turker AU. Antibacterial and antitumour activities of some plants grown in Turkey. Biotechnol Biotechnol Equip, 2014;28(2):306-315. Doi: 10.1080/13102818.2014.909708

30. Alhage J, Elbitar H, Taha S, Benvegnu T. In vitro assessment of antioxidant, antimicrobial, cytotoxic, anti-inflammatory, and antidiabetic activities of Campanula retrorsa crude extracts. Pharmacogn Res, 2018;10(4):397-403. Doi: 10.4103/pr.pr_73_18

31. Hashemi F, Zarei MA. Tyrosinase inhibitory activity within hexane extract of ten screened plants from Kurdistan province of Iran. Int J Adv Biol Biomed Res, 2014;2(11):2795-2799.

32. Zarei MA, Tahazadeh H. Alpha-glucosidase inhibitory activity in methanol extract of some plants from Kurdistan province. J Med Plants, 2020;18(72):227-235. Doi: 10.29252/jmp.4.72.S12.227

33. Zarei MA, Almasi H. α-glucosidase inhibition activity and antioxidant properties of aerial parts methanol extract from *Silene ampullata* Bioss and *Campanula involucrata* Auch. ex Dc. J Plant Res, 2021;34(1):16-27.

34. Janković IB, Drobac MM, Lakušić DV. Compounds of the methanolic leaf extract as chemotaxonomic markers for the *Campanula pyramidalis* complex (Campanulaceae). Acta Bot Croat, 2014;73(2):481-490. Doi: 10.2478/botcro-2014-0013

35. Touafek O, Kabouche Z, Brouard I, Barrera Bermejo J. Flavonoids of *Campanula alata* and their antioxidant activity. Chem Nat Compd, 2011;46(6):968-970. Doi: 10.1007/s10600-011-9799-2

36. Miyahara T, Tani T, Takahashi M, Nishizaki Y, Ozeki Y, Sasaki N. Isolation of anthocyanin-7 O-glucosyltransferase from Canterbury bells (*Campanula medium*). Plant Biotechnol, 2014;31(5):555-559. Doi: 10.5511/plantbiotechnology.14.0908a

37. Kahkeshani N, Hadjiakhoondi A, Navidpour L, Akbarzadeh T, Safavi M, Karimpour-Razkenari E, et al. Chemodiversity of *Nepeta menthoides* Boiss. & Bohse. essential oil from Iran and antimicrobial, acetylcholinesterase inhibitory and cytotoxic properties of 1, 8-cineole chemotype. Nat Prod Res, 2018;32(22):2745-2748. Doi: 10.1080/14786419.2017.1378202

38. Shafaghat A, Oji K. Nepetalactone content and antibacterial activity of the essential oils from different parts of *Nepeta persica*. Nat Prod Commun, 2010;5(4):625-628. Doi: 10.1177/1934578X1000500427

39. Sharma A, Cooper R, Bhardwaj G, Cannoo DS. The genus *Nepeta*: traditional uses, phytochemicals and pharmacological properties. J Ethnopharmacol, 2021;268(1):1-25. Doi: 10.1016/j.jep.2020.113679

40. Sharifi-Rad M, Epifano F, Fiorito S, Álvarez-Suarez JM. Phytochemical analysis and biological investigation of *Nepeta juncea* Benth. different extracts. Plants, 2020;9(5):1-17. Doi: 10.3390/plants9050646

41. Scalbert A. Antimicrobial properties of tannins. Phytochemistry, 1991;30(12):3875-3883. Doi: 10.1016/0031-9422(91)83426-L

42. Xie Y, Yang W, Tang F, Chen X, Ren L. Antibacterial activities of flavonoids: structure-activity relationship and mechanism. Curr Med Chem, 2015;22(1):132-149.

43. Arora DS, Sood H. In vitro antimicrobial potential of extracts and phytoconstituents from *Gymnema sylvestre* R. Br. leaves and their biosafety evaluation. AMB express, 2017;7(1):115. Doi: 10.1186/s13568-017-0416-z

44. Comini L, Montoya SN, Páez P, Argüello GA, Albesa I, Cabrera J. Antibacterial activity of anthraquinone derivatives from *Heterophyllaea pustulata* (Rubiaceae). J Photochem Photobiol B: Biol, 2011;102(2):108-114. Doi: 10.1016/j.jphotobiol.2010.09.009

45. Guimarães AC, Meireles LM, Lemos MF, Guimarães MCC, Endringer DC, Fronza M, et al. Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules, 2019;24(13):2471. Doi: https://doi.org/10.3390/molecules24132471

46. Cushnie TT, Cushnie B, Lamb AJ. Alkaloids: an overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents, 2014;44(5):377-386. Doi: 10.1016/j.ijantimicag.2014.06.001

47. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in Middle Eastern plants. Front Microbiol, 2019;10:1-28. Doi: 10.3389/fmicb.2019.00911

48. Dumlu M, Gurkan E, Tuzlaci E. Chemical composition and antioxidant activity of *Campanula alliariifolia*. Nat Prod Res, 2008;22(6):477-482. Doi: 10.1080/14786410701640429

49. Süntar I, Nabavi SM, Barreca D, Fischer N, Efferth T. Pharmacological and chemical features of *Nepeta* L. genus: its importance as a therapeutic agent. Phytother Res, 2018;32(2):185-198. Doi: 10.1002/ptr.5946

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6338

Characterization of drug-related problems in gram-negative bloodstream infections with clinical pharmacist input - a prospective study

Selda AYDIN[1*], Rumeysa CAKMAK[2], Meyha SAHIN[1], Elif GUNER YENIAYDIN[2], Kıvanç KÖK[3], Mesut YILMAZ[1]

1 Istanbul Medipol University, School of Medicine, Department of Infectious Diseases and Clinical Microbiology, Istanbul, Türkiye

2 Istanbul Medipol University, Graduate School of Health Sciences, Department of Clinical Pharmacy, Istanbul, Türkiye

3 Istanbul Medipol University, Regenerative and Restorative Medicine Research Center (REMER), Research Institute for Health Sciences and Technologies (SABITA), Istanbul, Türkiye

ABSTRACT

Patients with Gram-negative bacilli bloodstream infections (GNB-BSIs) have a high mortality and morbidity rate. This can result in an increased length of hospitalizations and risk of polypharmacy. This study investigates drug-related problems (DRPs) and associated factors during antimicrobial treatment in GNB-BSI patients. The prospective observational study was conducted between April 2023 and April 2024 at a 970-bed tertiary care university hospital in Istanbul. The study included 150 adult patients with a mean age of 58 years, and 57.3% of patients were male. Multivariable logistic regression analysis highlighted significant associations between DRPs and the presence of comorbidities, the duration of the patient's hospitalization, time to adequate antimicrobial therapy and the number of prescribed medications per patient ($p<0.05$). In conclusion, this study underscores the significance of clinical pharmacists' collaboration with clinicians in the identification and assessment of drug-related problems (DRPs) within the clinical department.

* Corresponding author: Selda AYDIN
E-mail: seldaaydin@medipol.edu.tr
ORCIDs:
Selda AYDIN: 0000-0002-3131-442X
Rumeysa ÇAKMAK: 0000-0001-8930-741X
Meyha SAHIN: 0000-0003-4147-3587
Elif GUNER YENIAYDIN: 0009-0008-9079-492X
Kıvanç KÖK: 0000-0002-1502-1600
Mesut YILMAZ: 0000-0001-8022-7325
(Received 2 Jul 2024, Accepted 5 Nov 2024)

Keywords: bloodstream infections, gram-negative bacteria, drug-related problems, antibiotic stewardship, clinical pharmacist

INTRODUCTION

Gram-negative bacilli bloodstream infections (GNB-BSIs) are a major public health problem due to their high rates of morbidity and mortality[1]. Timely initiation of appropriate antibiotic therapy is critical in the treatment of BSIs. to decrease mortality and poor outcomes[2,3]. However, the increasing prevalence of antimicrobial resistance (AMR) poses a significant challenge to the treatment and management of GNB-BSIs[4,5].

The resistance crisis highlights the need for multidisciplinary antimicrobial stewardship (AMS) programs that require collaboration between healthcare professionals, including infectious disease specialists, clinical pharmacists and microbiologists, to monitor antibiotic use and resistance patterns and implement evidence-based guidelines[6].

Clinical pharmacists play a critical role in AMS programs and drug-related problems (DRPs) in infectious disease[7,8]. These problems can take many various forms, including inappropriate drug selection, dosing errors, drug interactions, and patient non-compliance, among others[9]. The overall use of antibiotics, associated costs, duration of treatment and infections caused by multi-drug resistant organisms have been reduced by the participation of clinical pharmacists. Furthermore, the use of appropriate antibiotics[10,11].

DRPs in bloodstream infections (BSIs) is a major concern as it leads to treatment failures and increases healthcare costs[12]. The pharmacist-led review of DRPs has become a pivotal strategy in the prevention and mitigation of drug-related harm[13].

The primary objective of this study was to examine the prevalence and types of DRPs in critically ill patients, specifically those in hematology-oncology and intensive care units, who developed GNB-BSI in our hospital. The secondary objective was to identify factors associated with an increased risk of DRPs.

METHODOLOGY

Study design, setting and population

We conducted a prospective study between April 2023 and April 2024 in Istanbul, Turkey. Our facility was a tertiary care university hospital with a 90-bed of intensive care units (ICU) and a 970-bed capacity comprehensive

hematology and oncology wards. On the other hand, hematopoietic stem cell transplantation and solid organ transplantation were also performed

This study was conducted with the participation of a clinical pharmacist. The clinical pharmacist had a daily ward round with the responsible physician and other healthcare professionals. She observed the clinical follow-up of the patients, the treatments they received, the DRPs that developed and the progression of DRPs.

Patients who met the following inclusion criteria were included in the study.

Inclusion criteria

Inpatients≥18 years of age,

Patients with GNB-BSI,

Patients in the hematology ward, oncology ward, solid organ transplantation ward, hematopoietic stem cell transplantation ward or ICU.

Exclusion criteria

Patients who refused to participate the study,

Patients were re-admitted during the data collection period,

Patients were discharged within 48 hours after the initial positive blood culture signal,

Patients that refused treatment or did not receive treatment,

Patients who died within 48 hours after the initial positive blood culture signal,

During the study period, GNB-BSIs were identified in 172 patients, but 150 patients were included in the final,

During the study period, GNB-BSIs were identified in 172 patients, but 150 patients were included in the final analysis. The reason for exclusion (n=22) was due to patients who died or were discharged within 48 hours after the first blood culture. Figure 1 shows the flowchart of the study design.

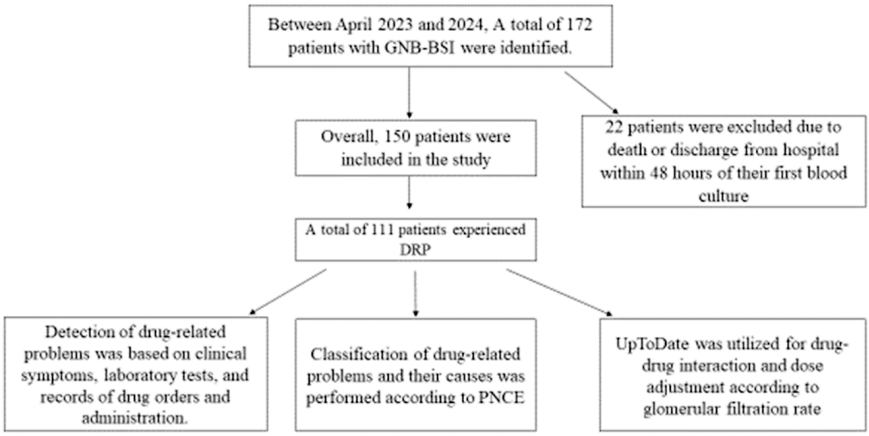


Figure 1. Flowchart of the study design

The study was approved by the Istanbul Medipol University Local Ethics Committee for non-interventional clinical research (E-10840098-772.02-803/31.01.2023).

Data collection

Gram stain was performed when there was a sign of microbial growth on the blood cultures. Monitoring of patients was begun with detection of a GNB. The follow-up of the patients continued throughout the treatment process of the bloodstream infection until discharge or death.

The patients' sociodemographic characteristics, comorbidities, Body Mass Index (BMI), medical history, the time interval between the onset of BSI and the initiation of appropriate antimicrobial therapy, antimicrobials used, length of hospital stay since the first positive blood culture (days), number of prescribed medications, profile bacteria that caused BSI, carbapenem resistant, and DRPs were recorded.

The Pharmaceutical Care Network Europe (PCNE) classification version 9.01 was used to determine of DRPs in the patients[14]. In the event of a life-threatening situation, the clinicians implemented the necessary interventions.

Researching of the PubMed database revealed a paucity of studies on the topic of DRP in adult patients with infectious diseases. The objective of the study was to ascertain the incidence of DRP and to identify associated risk factors.

Statistical analysis

Statistical analysis included both descriptive and inferential statistics. Frequency, proportion, mean and standard deviation (SD) were applied as descriptive statistics measures. Inferential statistics analysis was performed by using categorical data as input. To this end, the distribution of categorical variables was summarized by count and proportions with the purpose to perform comparison based on the DRP status (namely, the presence or absence of DRPs). In this regard, the chi-square test was performed, which was followed by univariate and multivariate logistic regression. The Chi-square test (χ^2) was applied to test difference in distribution of categorical variables between the two study groups (namely patients with DRPs and without DRPs). In order to assess the association between the variables in more detail and to gain complementary insights regarding the covariates of DRPs, crude odds ratios and adjusted odds rations were computed using univariate and multivariate logistic regression, respectively. The statistical significance of association between the studied independent variables and DRPs was tested using the multivariate logistic regression analysis method. To this end, significance of variables was summarized by single p value or multiple p values, depending on the number of categories. In case of more than one category, the overall p values were provided. For the evaluation of all inferential statistics results, the statistical significance level $p<0.05$ was considered as the cut-off threshold value for significance. The SPSS software (version 22) was utilized for statistical analysis.

RESULTS and DISCUSSION

A total of 150 patients with bloodstream infections were included in the study and in total 237 DRPs were identified in 74% of the patients. The median age of the patients was 58 years and 86% were male. The socio-demographic data of the patients are shown in Table 1.

Table 1. Characteristics of the patients with GNB-BSIs

Characteristics	Frequency n (%)	Mean (SD)
Gender Male Female	 86 (57.33) 64 (42.67)	
Age, years 18-45 (n, %) 46-65 (n, %) >65 (n, %)	 39 (26) 69 (46) 42 (28)	58 (16.28)
Body Mass Index (BMI) Weight (kg) Height (m)		25.93 (6.34) 70 (20) 1.65 (12.25)
Comorbidities 1 2 3 4 5	 61 (40.67) 44 (29.33) 20 (13.33) 14 (9.33) 7 (4.67)	2.13 (1.28)
The 10 most common comorbidities Hypertension Cancer Type 2 Diabetes Mellitus Leukemia Kidney failure Lymphoma Coronary Artery Disease Multiple Myeloma Chronic Heart Failure Liver failure	 45 (30) 43 (28.67) 40 (26.67) 27 (18) 27 (18) 17 (4.67) 11 (7.33) 11 (7.33) 10 (6.67) 7 (4.67)	
Length of hospital stay since the first positive blood culture, (days)		15 (34.24)
Number of prescribed medications		20 (7.41)
Total number of patients readmitted to hospital within 30 days	42 (28)	

Note: SD: standard deviation.

Immunosuppressed and critically ill patients accounted for 77% of the total patient population. The Table 2 presents the characteristics of the pathogens in the initial blood culture.

Table 2. Characteristics of the pathogens in the initial blood culture

	Frequency (n)	Percentage (%)
Gram-negative bacteria Carbapenem resistant	150 58	100 38.67
Escherichia coli Carbapenem resistant	66 9	44 14
Klebsiella spp. Carbapenem resistant	50 26	33.33 52
Pseudomonas spp. Carbapenem resistant	19 19	12.66 100
Enterobacter spp. Carbapenem resistant	6 2	4 33.33
Acinetobacter spp. Carbapenem resistant	1 1	0.66 100
Other Gram-negative bacteria Carbapenem resistant	8 1	5.3 13

The most common pathogens were *E. coli* 44% (n=66) and *K. pneumoniae* 33.3% (n=50). The frequency and percentage of antibiotics used are shown in Figure 2.

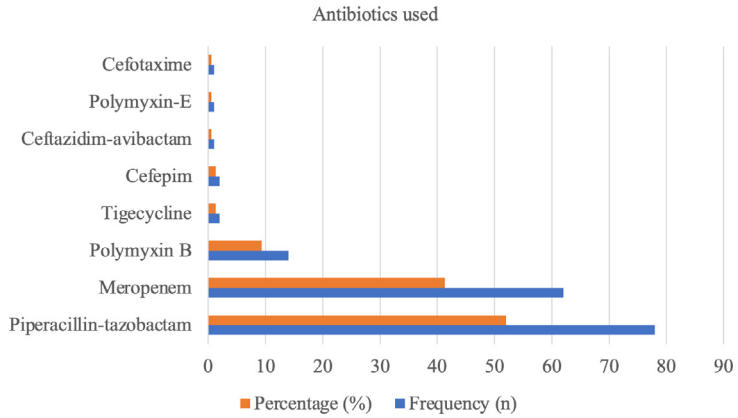


Figure 2. The frequency and percentages the antibiotics used.

The most commonly used antibiotics were piperacillin-tazobactam with 52%. Table 3 illustrates the time interval between initial appropriate antibacterial treatment and the onset of symptoms indicative of a bloodstream infection.

Table 3. The time interval between the onset of BSI and the initiation of appropriate antimicrobial therapy

Time to adequate antimicrobial therapy	Frequency (n)	Percentage (%)
<6 hour	80	53.33
7-24 hour	47	31.33
25-48 hour	17	11.33
>49 hour	6	4
TOTAL	150	100

It was observed that 85% of patients received appropriate antibacterial treatment within the first 24 hours after the onset of symptoms consistent with a bloodstream infection.

The primary types of DRPs were treatment effectiveness (P1) (33%), treatment safety (P2) (53%), and other issues (P3) (14%). The problems are shown in Figure 3, and the causes of DRPs are shown in Table 4.

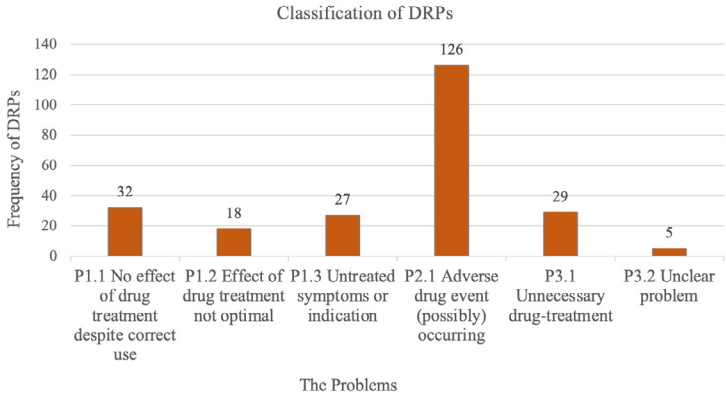


Figure 3. The problems, PCNE classification for drug-related problems V9.1

Table 4. The causes, PCNE classification for drug-related problems V9.1

Causes (including possible causes for potential problems)	**268**	
	Number	**%**
N1. Drug selection	115	42.91
N2. Drug form	0	-
N3. Dose selection	25	9.32
N4. Treatment duration	82	30.59
N5. Dispensing	-	-
N6. Drug use process	6	2.23
N7. Patient related	-	-
N8. Patient transfer related	19	7.01
N9. Other	21	7.83

The most common causes of DRPs were drug selection (42.91%), treatment duration (30.59%), and dose selection (9.3%). By the day-28, 13.3% (n=20) patients had died, 52.6% (n=79) patients had been discharged from the hospital, 2% (n=3) patients were still followed in the ICU, and 32% (n=48) patients were still followed in the wards.

Based on the estimates of the performed $\chi 2$ tests (in the form of $\chi 2$ statistics and p values), it is plausible to conclude that a significant difference ($p<0.05$) exists between patients with DRP and patients without DRP with respect to the following categorical variables: age ($p=0.043$), presence of comorbidity ($p=0.002$), length of stay at hospital ($p=0.024$), time to adequate antimicrobial therapy ($p=0.012$) and number of prescribed medications per patient ($p<0.001$) (Table 5).

Table 5. Chi-square test results for difference in distribution of variables between patients with DRPs and without DRPs

Variables	Category	DRPs Yes n (%)	DRPs No n (%)	χ^2 statistic	χ^2 p-value
Sex	Female	44 (68.75)	20 (31.25)	1.60	0.206
	Male	67 (77.90)	19 (22.10)		
Age (years)	18-45	23 (58,97)	16 (41)	6.28	0.043*
	46-65	54 (78,26)	15 (21,74)		
	>65	34 (80,95)	8 (19)		
Presence of comorbidity	No	42 (61.76)	26 (38.24)	9.68	0.002*
	Yes	69 (84.15)	13 (15.85)		
Length of stay at hospital (days)	<7	37 (63.79)	21 (36.21)	5.12	0.024*
	≥7	74 (80.43)	18 (19.57)		
Time to adequate antimicrobial therapy (hours)	<12	60 (66.67)	30 (33.33)	6.29	0.012*
	≥12	51 (0.85)	9 (0.15)		
Number of prescribed medications per patient	7-14	19 (55.88)	15 (44.12)	23.0	< .001*
	15-19	23 (57.50)	17 (42.50)		
	20-29	51 (92.73)	4 (7.27)		
	>30	18 (85.71)	3 (14.29)		
Carbapenem resistance	No	63 (68.48)	29 (31.52)	3.77	0.052
	Yes	48 (82.76)	10 (17.24)		

DRPs: drug related problems; * indicates significance at p<0.05.

Furthermore, the results of univariate and multivariate logistic regression analyses, which were performed as a further step to assess associations between the studied variables, are shown in the Table 6.

Table 6. Bivariate and Multivariate logistic regression analysis result of DRPs covariates among patients

Variables	Category	DRPs Yes n (%)	DRPs No n (%)	COR (95% CI)	AOR (95% CI)	p-value	Overall p-value
Sex	Female	44 (68.75)	20 (31.25)	1	1		
	Male	67 (77.90)	19 (22.10)	1.603 (0.769-3.340)	1.421 (0.563-3.586)	0.457	
Age (years)	18-45	23 (58.97)	16 (41)	1	1		0.466
	46-65	54 (78.26)	15 (21.74)	2.504 (1.063-5.900)	1.953 (0.670-5.688)	0.220	
	>65	34 (80.95)	8 (19)	2.957 (1.087-8.038)	1.631 (0.462-5.758)	0.448	
Presence of comorbidity	No	42 (61.76)	26 (38.24)	1	1		
	Yes	69 (84.15)	13 (15.85)	3.286 (1.524-7.085)	2.877 (1.122-7.375)	0.028*	
Length of stay at hospital (days)	<7	37 (63.79)	21 (36.21)	1	1		
	≥7	74 (80.43)	18 (19.57)	2.333 (1.110-4.905)	2.770 (1.041-7.372)	0.041*	
Time to adequate antimicrobial therapy (hours)	<12	60 (66.67)	30 (33.33)	1	1		
	≥12	51 (0.85)	9 (0.15)	2.833 (1.232-6.519)	4.993 (1.586-15.716)	0.006*	

Number of prescribed medications per patient	7-14	19 (55.88)	15 (44.12)	1	1		0.000*
	15-19	23 (57.50)	17 (42.50)	1.068 (0.425-2.687)	1.260 (0.394-4.036)	0.697	
	20-29	51 (92.73)	4 (7.27)	10.066 (2.965-34.173)	14.379 (3.427-60.343)	0.000*	
	>30	18 (85.71)	3 (14.29)	4.737 (1.171-19.155)	6.974 (1.330-36.567)	0.022*	
Carbapenem resistance	No	63 (68.48)	29 (31.52)	1	1	1	0.951
	Yes	48 (82.76)	10 (17.24)	2.210 (0.982-4.971)	0.969 (0.351-2.673)	0.951	

DRPs: drug related problems; AOR: adjusted odds ratio; CI: confidence interval; COR: crude odds ratio.; * indicates significance at $p<0.05$.

Here, univariate logistic regression provided crude (unadjusted) odd rations, while multivariate logistic regression analyses computed adjusted odd ratios and p values. To this end, multivariate regression analysis delineated statistically significant association between DRPs (the dependent variable) and the following covariates (independent variables): presence of comorbidity ($p=0.028$), length of stay at hospital ($p=0.041$), time to adequate antimicrobial therapy ($p=0.006$) and number of prescribed medications per patient ($p<0.001$). Overall, the significant associations between these four variables and the mentioned DRP status were detected by both the $\chi 2$ tests and the multivariate logistic regression. Consequently, the $\chi 2$ test estimates and the observed odds ratios altogether suggest that presence of comorbidity, increase in length of stay at hospital, increase in time to adequate antimicrobial therapy and increase in number of prescribed medications per patient are potential risk factors of DRPs.

The prevalence and types of DRPs

The DRPs was detected in nearly three-quarters of our patients, and the most common antimicrobial treatment was piperacillin-tazobactam. The incidence of DRPs ranges from 8.54% to 99.16% (12). A single-center study has reported antibiotic-associated DRPs 87.3% in patients with community-acquired pneumonia[15]. On the other hand, in a prospective study on patients with various systemic bacterial infections, the rate of DRPs was found 71.51%[16]. In addition, two studies among patients with COVID-19 in showed that 65.3% and 33.2% of patients had at least one drug related problem, respectively[17,18]. DRPs have been reported within a wide range in the literature, and the rate of DRPs in our

study was considered high compared to previous reports. The wide range of DRPs is thought to be due to differences in the way the trials were conducted, the characteristics of the patients included, and the follow-up practices of the centers. With all that, our study is thought to reflect more reliable data because of its prospective design.

The most commonly observed DRPs in previous studies were treatment safety and treatment efficacy, ranging from 29.9% to 77.18% and 18.44% to 47.9%, respectively, similar to our study[16,19,20]. The leading causes of these DRP types were often related to drug selection and dose selection[21,22]. Our results were partially in contrast with these findings since we identified drug selection and treatment duration as the most common causes for DRPs. The duration of antibiotic use was extended in our study. The effect of appropriate dose and duration of antibiotic use on clinical outcomes, adverse reaction of antibiotics and AMR is well known[2,4]. However, our study contributed to the literature by presenting striking data emphasizing the association of inappropriate doses and durations of antibiotic use with DRPs. This results also highlight the critical role of a clinical pharmacist, even for a single parameter such as treatment duration.

Risk factors for DRPs

Our study underscores comorbidities, length of hospital stay, time to adequate antimicrobial therapy and increased number of drugs prescribed per patient as potential risk factors for DRPs.

We revealed that the DRPs was higher in patients with comorbidities, similar to previous studies[23-25]. Another factor that associated with development of DRPs was delays in initiating appropriate antimicrobial therapy in our study. This delay is strongly associated with worse clinical outcomes, including an increased risk of progression to organ failure[2] that causes more intervention and using a larger number of medications. Existing literature has shown that early and targeted antimicrobial therapy significantly reduces both complications and mortality in patients with BSIs[26,27]. And the third one was length of hospital stay that increases the risk of DRPs in our study which consistent with literature data[16,25]. The findings of the present study suggest an association between DRPs and a number of factors, including the presence of comorbidities, delays in the initiation of appropriate treatment and prolonged hospitalization. DRPs were thought to be associated with many factors, when all findings of our study were taken into consideration; the presence of comorbidities, delays in appropriate treatment and prolonged hospitalization. All of these factors are thought to be associated with polypharmacy. In conclusion, the factors that di-

rectly or indirectly influence polypharmacy should be considered significant in the context of DRP and the management of polypharmacy should be improved.

The increasing risk of drug interactions and adverse events by the polypharmacy is well-known[16,24]. We have also shown statistically significant association between development of DRPs and high number of the prescribed medications per patient. Our findings are compatible with the previous study that reported higher rates of DRPs in patients undergoing polypharmacy[28]. These results from our study have highlighted the importance of collaboration between the clinical pharmacologist and clinicians. The EUROBACT-2 study found that infrequent consultations with clinical pharmacists were associated with higher mortality rates, underscoring their critical role in optimizing antimicrobial therapy for hospital-acquired BSIs[29]. Clinical pharmacists improve patient outcomes through medication counseling, adherence support, and follow-up care, reducing adverse drug reactions and medication errors. Despite higher initial costs associated with their interventions, the overall economic impact is positive when considering the savings from avoided adverse drug events[30,31].

In our study, the relationship between DRPs and carbapenem resistance was not statistically significant in the Chi-square test and multivariate analysis. This hints that the actual effect could be context-dependent and much complex, making it more difficult to detect. The elaborate and comprehensive investigation with larger sample size is needed to clarify the impact of carbapenem resistance.

In this regard, this study emphasis for the importance of clinical pharmacists in the detection and evaluation of DRPs and in the management of antimicrobial therapy for GNB-BSIs in collaboration with infectious disease specialists in GNB-BSIs.

The large number of our cases and the prospective design of our study increase the reliability of our study. On the other hand, our single-center data, which reflects the clinical practice of our center, is a limitation of our study.

STATEMENT OF ETHICS

The study was approved by the Istanbul Medipol University Local Ethics Committee for non-interventional clinical research (E-10840098-772.02-803/31.01.2023).

CONFLICT OF INTEREST STATEMENT

The authors affirm that the research was carried out without any affiliations or financial associations that could be perceived as a possible conflict of interest.

AUTHOR CONTRIBUTIONS

Concept – Selda Aydin, Rumeysa Cakmak, Meyha Sahin, Mesut Yilmaz (authors contributed equally); Design – Selda Aydin, Rumeysa Cakmak, Mesut Yilmaz (authors contributed equally); Data Collection and Processing – Rumeysa Cakmak, Meyha Sahin, Elif Güner Yeniaydin; Statistical Analysis and Interpretation – Kıvanç Kök; Literature Search – Selda Aydin, Rumeysa Cakmak; Drafting of the Manuscript – Rumeysa Cakmak, Meyha Sahin; Critical Revision of the Manuscript – Selda Aydin, Rumeysa Cakmak, Kıvanç Kök, Mesut Yilmaz.

FUNDING SOURCES

This study was not funded by any organization.

ACKNOWLEDGMENTS

The authors of this study are grateful to Istanbul Medipol University for providing opportunities and support.

REFERENCES

1. Xu S, Song Z, Han F, Zhang C. Effect of appropriate empirical antimicrobial therapy on mortality of patients with Gram-negative bloodstream infections: a retrospective cohort study. BMC Infect Dis, 2023;23(344). Doi: 10.1186/s12879-023-08329-2

2. Van Heuverswyn J, Valik JK, Desirée van der Werff S, Hedberg P, Giske C, Nauclér P. Association between time to appropriate antimicrobial treatment and 30-day mortality in patients with bloodstream infections: a retrospective cohort study. Clin Infect Dis, 2023;76(3):469-478. Doi: 10.1093/cid/ciac727

3. Lee CC, Chen PL, Ho CY, Hong MY, Hung YP, Ko WC. Prompt antimicrobial therapy and source control on survival and defervescence of adults with bacteraemia in the emergency department: the faster, the better. Crit Care, 2024;1:28(1). Doi: 10.1186/s13054-024-04963-7

4. Waters J, Shorr AF. Bloodstream infection and Gram-negative resistance: the role for newer antibiotics. Antibiotics, 2023;12(6):977. Doi: 10.3390/antibiotics12060977

5. Holmes CL, Anderson MT, Mobley HLT, Bachman MA. Pathogenesis of Gram-negative bacteremia. Clin Microbiol Rev, 2021;34(2):e00234-20. Doi: 10.1128/cmr.00234-20

6. Khadse SN, Ugemuge S, Singh C. Impact of antimicrobial stewardship on reducing antimicrobial resistance. Cureus, 2023;15(12):e49935. Doi: 10.7759/cureus.49935

7. Cheng J, Dang CD, Li X, Wang JJ, Huang X, Li Y, et al. The participation of clinical pharmacists in the treatment of patients with central nervous system infection can improve the effectiveness and appropriateness of anti-infective treatments: a retrospective cohort study. Front Pharmacol, 2023;14:1226333. Doi: 10.3389/fphar.2023.1226333

8. Dighriri IM, Alnomci BA, Aljahdali MM, Althagafi HS, Almatrafi RM, Altwairqi WG, et al. The role of clinical pharmacists in Antimicrobial Stewardship Programs (ASPs): a systematic review. Cureus, 2023;15(12). Doi: 10.7759/cureus.50151

9. Ayele Y, Tesfaye ZT. Drug-related problems in Ethiopian public healthcare settings: systematic review and meta-analysis. SAGE Open Med, 2021;9. Doi: 10.1177/20503121211009728

10. Xia H, Li J, Yang X, Zeng Y, Shi L, Li X, et al. Impacts of pharmacist-led multifaceted antimicrobial stewardship on antibiotic use and clinical outcomes in urology department of a tertiary hospital in Guangzhou, China: an interrupted time series study. J Hosp Infect, 2024;151:148-160. Doi: 10.1016/j.jhin.2024.05.003

11. Yu J, Liu Y, Qu R, Wang Z, Zhao Y, Zhao Y, et al. Evaluation of a clinical pharmacist-led antimicrobial stewardship program in a neurosurgical intensive care unit: a pre-and post-intervention cohort study. Front Pharmacol, 2023;14. Doi: 10.3389/fphar.2023.1263618

12. Ni XF, Yang CS, Bai YM, Hu ZX, Zhang LL. Drug-related problems of patients in primary health care institutions: a systematic review. Front Pharmacol, 2021;12. Doi: 10.3389/fphar.2021.698907

13. Lekpittaya N, Kocharoen S, Angkanavisul J, Siriudompas T, Montakantikul P, Paiboonvong T. Drug-related problems identified by clinical pharmacists in an academic medical centre in Thailand. J Pharm Policy Pract, 2024;17(1). Doi: 10.1080/20523211.2023.2288603

14. Pharmaceutical Care Network Europe Association. The PCNE Classification V9.1 [Internet]. 2003-2020 Pharmaceutical Care Network Europe Association; 2020 [Jun 1, 2024]. Available from: https://studylib.net/doc/25639956/417-pcneclassification-v9-1-final

15. Horodnycha O, Zimenkovsky A. Antibiotic therapy of adult inpatients with community-acquired pneumonia: a retrospective hospital-based study in Ukraine. Acta Pharm Sci, 2023;61(1). Doi: 10.23893/1307-2080.APS6102

16. Bekele F, Fekadu G, Bekele K, Dugassa D, Sori J. Drug-related problems among patients with infectious disease admitted to medical wards of Wollega University Referral Hospital: prospective observational study. SAGE Open Med, 2021;9. Doi: 10.1177/2050312121989625

17. Ünder D, Enver C, Demirci MY, Ayhan YE, Özgan B, İlerler EE, et al. Clinical pharmacist-led medication review in hospitalized confirmed or probable patients with COVID-19 during the first wave of COVID-19 pandemic. Turk J Pharm Sci, 2024;21(2):152-158. Doi: 10.4274/tjps.galenos.2023.47105

18. Barceló-Vidal J, Echeverría-Esnal D, Carballo N, De Antonio-Cuscó M, Fernández-Sala X, Navarrete-Rouco ME, et al. Drug-related problems in patients admitted for SARS-CoV-2 infection during the COVID-19 pandemic. Front Pharmacol, 2022;13:993158. Doi: 10.3389/fphar.2022.993158

19. Albayrak A, Başgut B, Bıkmaz GA, Karahalil B. Clinical pharmacist assessment of drug-related problems among intensive care unit patients in a Turkish university hospital. BMC Health Serv Res, 2022;22(1):79. Doi: 10.1186/s12913-022-07494-5

20. Çakır A, Memiş H, Gün ZÜ, Bıçakçıoğlu M. Evaluation of drug-related problems of intensive care unit patients by clinical pharmacists: a retrospective study. Turk J Pharm Sci, 2024;21(4):274-283. Doi: 10.4274/tjps.galenos.2023.44459

21. Durmuş M, Gün Z, Berktaş H. The evaluation and classification of drug-related problems by a clinical pharmacist in an internal diseases intensive care unit: a prospective cohort 7-month study. Istanbul J Pharm, 2024;54(1):1-11. Doi: 10.26650/IstanbulJPharm.2024.1327194

22. Ali Hussain Alsayed H, Saheb Sharif-Askari F, Saheb Sharif-Askari N, Halwani R. Clinical pharmacist interventions in an intensive care unit reduces ICU mortality at a tertiary hospital in Dubai, United Arab Emirates. Explor Res Clin Soc Pharm, 2024;14. Doi: 10.1016/j.rcsop.2024.100431

23. Kara E, Kelleci Çakır B, Sancar M, Demirkan K. Impact of clinical pharmacist-led interventions in Turkey. Turk J Pharm Sci, 2021;18(4):517-526. Doi: 10.4274/tjps.galenos.2020.66735

24. Pramotesiri P, Putthipokin K, Ruangritchankul S. Drug related problems among older inpatients at a tertiary care setting. J Clin Med, 2024;13(6). Doi: 10.3390/jcm13061638

25. Bektay MY, Sancar M, Karaköse OF, Durdu B, İzzettin FV. Identification of drug-related problems and investigation of related factors in patients with COVID-19: an observational study. Bezmialem Science, 2022 Dec;10(6):777-785. Doi: 10.14235/bas.galenos.2022.63935

26. Ohnuma T, Chihara S, Costin B, Treggiari MM, Bartz RR, Raghunathan K, et al. Association of appropriate empirical antimicrobial therapy with in-hospital mortality in patients with bloodstream infections in the US. JAMA Netw Open, 2023;6(1). Doi: 10.1001/jamanetworkopen.2022.49353

27. Hsueh SC, Chen PL, Ho CY, Hong MY, Lee CC, Ko WC. Comparing the prognostic impacts of delayed administration of appropriate antimicrobials in older patients with afebrile and febrile community-onset bacteremia. Antibiotics, 2024;13(5). Doi: 10.3390/antibiotics13050465

28. Jung-Poppe L, Nicolaus HF, Roggenhofer A, Altenbuchner A, Dormann H, Pfistermeister B, et al. Systematic review of risk factors assessed in predictive scoring tools for drug-related problems in inpatients. J Clin Med, 2022;11. Doi: 10.3390/jcm11175185

29. Tabah A, Buetti N, Staiquly Q, Ruckly S, Akova M, Aslan AT, et al. Epidemiology and outcomes of hospital-acquired bloodstream infections in intensive care unit patients: the EUROBACT-2 international cohort study. Intensive Care Med, 2023;49(2). Doi: 10.1007/s00134-022-06944-2

30. Fu M, Zhu Y, Wei G, Yu A, Chen F, Tang Y, et al. Evaluation of pharmacist-led medication reconciliation at county hospitals in China: a multicentre, open-label, assessor-blinded, non-randomised controlled study. J Glob Health, 2024;14. Doi: 10.7189/jogh.14.04058

31. Yakti O, Al-Badriyeh D, Rijims M, Abdelaal M, Alsoukhni O, Al Hail M, et al. Clinical pharmacists' interventions for preventing adverse events in critically ill neonates in Qatar: an economic impact analysis. J Pharm Policy Pract, 2024;17(1). Doi: 10.1080/20523211.2023.2291508

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6339

Development and validation of a versatile ultra performance liquid chromatography method for simultaneous estimation of selected antiviral drugs in bulk and dosage form

Divya NARLA[1*], Nagaraju PAPPULA[2], Prakash Nathaniel Kumar SARELLA[1]

1 Department of Pharmaceutical Analysis, Aditya College of Pharmacy, ADB road, Surampalem-533437, India
2 Department of Pharmaceutical Analysis, Hindu College of Pharmacy, Amaravathi Road, Guntur-522002, India

ABSTRACT

This research aimed to develop and validate an accurate Ultra performance Liquid Chromatography (UPLC) method with Photo Diode Array detection to simultaneously estimate Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate in their fixed dose combination. The developed method used Acetonitrile and pH 2.5 triethanolamine buffer in a 30:70 v/v ratio as the mobile phase at 1.0 mL/min flow rate and 0.50 µL injection volume. The analytes were separated on a BEH C18 column (1.8µ, 100×2.1mm) and detected at 265nm. Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate obeyed Beer's law in the ranges of 5–75 µg/mL, 20-300 µg/mL and 2.50–37.50 µg/mL respectively. The recovery for accuracy was 99-101%. Precision and robustness met acceptable limits. This stability indicating method could distinguish and quantify the compounds even with degradants. Thus, a specific, accurate and robust stability indicating method was developed to simultaneously quantify Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate in their combined dosage form.

Keywords: bictegravir, emtricitabine, tenofovir alafenamide fumarate, UPLC

*Corresponding author: Divya NARLA
E-mail: divyanarla@gmail.com
ORCIDs:
Divya NARLA: 0000-0002-3255-7108
Nagaraju PAPPULA: 0000-0001-8315-2859
Prakash Nathaniel Kumar SARELLA: 0000-0002-7386-6222
(Received 3 May 2023, Accepted 5 Aug 2024)

INTRODUCTION

Bictegravir (BIC) is an antiretroviral agent used to treat HIV infection in combination with other drugs[1]. It is an integrase strand transfer inhibitor (INSTI). The chemical name of Bictegravir sodium is 2,5-Methanopyrido[1',2':4,5] pyrazino[2,1-b][1,3]oxazepine-10-carboxamide, 2,3,4,5,7,9,13,13a-octahydro-8-hydroxy-7,9-dioxo-N-[(2,4,6-trifluorophenyl)methyl]-, sodium salt (1:1), (2R,5S,13aR). Bictegravir sodium has a molecular formula of $C_{21}H_{17}F_3N_3NaO_5$ and molecular weight 471.4. It appears as an off-white to yellow solid with 0.1 mg solubility in 1mL of water at 20°C. Bictegravir is an INSTI used only in combination with other antiretroviral drugs to treat HIV infection[2].

Emtricitabine (FTC) belongs to the nucleoside reverse transcriptase inhibitor (NRTI) class of antiretroviral drugs. It can be used with other antiretroviral agents to treat HIV infection and AIDS[3]. The chemical name of FTC is 4-amino-5-fluoro-1-(2R-hydroxymethyl-1,3-oxathiolan-5S-yl)-(1H)-pyrimidin-2-one. FTC is the (-) enantiomer of a thio analog of cytidine which differs from other cytidine analogs with a fluorine in the 5 positions. FTC has a molecular formula of $C_8H_{10}FN_3O_3S$ and molecular weight 247.2. It appears as a white to off-white powder with 112 mg solubility in 1mL of water at 25°C.

Tenofovir Alafenamide Fumarate (TAF) is a prodrug and HIV-1 reverse transcriptase inhibitor (NtRTI)[4].

The chemical name of Tenofovir alafenamide fumarate drug substance is L-alanine, N-[(S)-[[(1R)-2-(6-amino-9H-purin-9-yl)-1¬methylethoxy]methyl] phenoxyphosphinyl]-, 1-methylethyl ester, (2E)-2-butenedioate (2:1). It has an empirical formula of $C_{21}H_{29}O_5N_6P.½(C_4H_4O_4)$ and formula weight 534.5 g/mol. TAF appears as a white to off-white or tan powder with 4.7 mg solubility in 1 mL of water at 20°C. It is an NtRTI antiretroviral drug used with other drugs to treat HIV. The chemical structures of Bictegravir, Emtricitabine and Tenofovir Alafenamide Fumarate are shown in Figure 1.

The fixed dose combination of Bictegravir, Emtricitabine and Tenofovir alafenamide fumarate was approved by USFDA and is recommended for the treatment of patients suffering from chronic HIV infection with or without indication of compensated cirrhosis[5]. The objective of the present study is to develop and validate a simple, accurate and precise stability indicating UPLC method for the simultaneous estimation of BIC, FTC and TAF in pharmaceutical dosage forms, which would be applied for routine quality control of dosage form in the presence of degradants. UPLC was chosen over HPLC as it offers advantages of fast analysis, less solvent consumption, small sample size and increased sensitivity.

Various analytical methods have been reported for simultaneously estimating Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir Alafenamide Fumarate (TAF) in bulk and pharmaceutical formulations using LC-MS/MS[6-8] and high-performance liquid chromatography (HPLC)[9-16]. Ultra Performance Liquid Chromatography (UPLC) methods are scarcely available in literature[17]. An attempt was made to develop a stability indicating UPLC method to quantify BIC, FTC and TAF in pharmaceutical formulations. Though HPLC methods exist for simultaneously estimating BIC, FTC and TAF, no UPLC method has been reported. The current work aims to develop a stability indicating UPLC method to quantify these drugs in pharmaceutical formulations.

The developed UPLC method would provide improved sensitivity, speed and resolution over the existing HPLC techniques for analysis of BIC, FTC and TAF. The stability indicating nature of the method also allows determining the drugs in the presence of degradation products ensuring the quality and stability of pharmaceutical formulations.

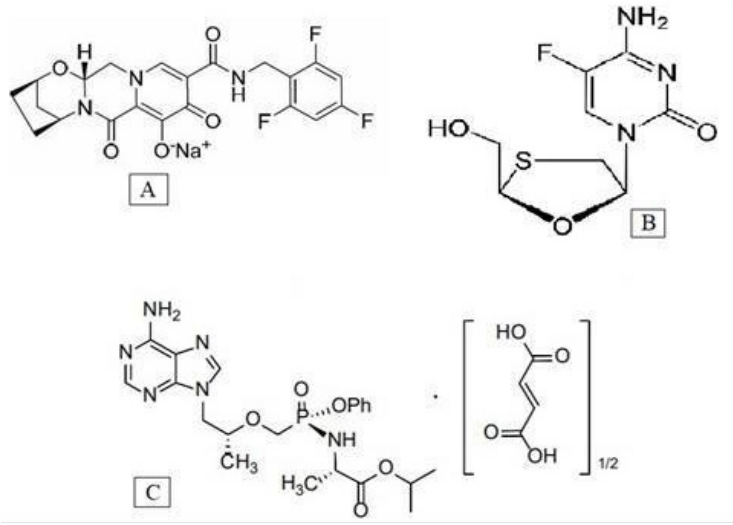


Figure 1. Chemical structures of Bictegravir (A), Emtricitabine (B), Tenofovir Alafenamide Fumarate (C)

METHODOLOGY

Reagents and chemicals

Reference standards of Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate were obtained as gift samples from Mylan, Hyderabad and Lupin Pharmaceuticals, Visakhapatnam. The fixed dose generic combination of Bictegravir/Emtricitabine/Tenofovir Alafenamide (50mg/200mg/25mg) was procured from commercial sources under the brand name Biktarvy®.

The following chemicals of chromatographic grade were used in the current study: Acetonitrile (UPLC Lichrosolv,Merck), Triethanolamine (Qualigens, India), Orthophosphoric acid (Merck, India) Hydrochloric acid (Finar, India), Sulphuric acid (Finar, India), Sodium Hydroxide (Finar, India), Hydrogen Peroxide (Finar, India).

The obtained reference standards and procured pharmaceutical products along with the chemicals were used in the present work to develop and validate the proposed UPLC method for simultaneous analysis of BIC, FTC and TAF.

Instrumentation and chromatographic conditions

Liquid chromatographic analysis to simultaneously estimate Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir Alafenamide fumarate (TAF) was performed using a Waters Acquity UPLC system equipped with quaternary pump, an inbuilt auto injector, a PDA 2996 detector and controlled by Empower 2 software.

Other equipments used included: An electronic balance (Shimadzu), pH meter (Adwa AD1020), Ultra sonicator (Labsoul, India), hot air oven (BiTechno, India), UV chamber (cole parmer, US).

Chromatographic separation of BIC, FTC and TAF was carried out on a BEH C18 column (100×2.1 mm,1.8 µm) at ambient temperature. A mobile phase containing Acetonitrile and TEA buffer (pH 2.5) in 30:70 v/v ratio was used at 1.0 mL/min flow rate with 0.5 µL injection volume. Detection of separate analytes was performed at 265 nm with 7 min runtime.

Preparation of standard solutions

Accurately weigh and transfer standardized amounts of Bictegravir (50 mg), Emtricitabine (200 mg) and Tenofovir alafenamide fumarate (25 mg) into 100 mL clean, dry volumetric flasks. Add a diluent solution of acetonitrile and buffer in 30:70 ratio and sonicate for 10 minutes. Bring the final volume to the appropriate level with the diluent solution to achieve concentrations of

500 micrograms per milliliter of Bictegravir, 2000 micrograms per milliliter of Emtricitabine and 250 micrograms per milliliter of Tenofovir alafenamide. Extract an additional 5 milliliters of this solution and dilute it further with the diluent to a final volume of 50 milliliters. This will produce a standard solution containing 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide.

Preparation of sample solutions

Five tablets of Biktarvy were weighed and finely ground into a powder. 350 milligrams of the powdered tablets were transferred into a 100-milliliter volumetric flask. 70 milliliters of diluent were added, and the solution was sonicated for approximately 30 minutes to dissolve the contents. The volume was then adjusted to the proper level with the diluent and filtered through a 0.45-micron filter. Five milliliters of the filtered sample stock solution were transferred to a 50-milliliter volumetric flask. The volume was adjusted to 50 milliliters with diluent to achieve concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine, and 25 micrograms per milliliter of Tenofovir alafenamide.

Method validation

The developed analytical method was validated in accordance with ICH guidelines for the following parameters[13]:

System suitability

The standard solutions were injected into the UPLC system, and the system suitability parameters were evaluated, including:

Theoretical plates: A measure of the effectiveness of the separation process. The number of theoretical plates reflected column efficiency.

Tailing factor: The asymmetry of the chromatographic peaks is indicated by tailing factor. Values close to 1 indicated symmetric peaks.

Resolution: The ability of the system to separate the compounds of interest into discrete peaks. Resolutions greater than 2 were considered acceptable.

These system suitability parameters were assessed to ensure the UPLC system and analytical column were performing adequately and suitable for the sample analysis. Acceptable values indicate the system could produce reproducible and precise results for the analysis.

Specificity

Method specificity is unequivocally assessed for the presence of interfering peaks by injecting blank (diluent) and placebo solutions. Absence of additional peaks at the same retention times of analytes indicates absence of interference and specificity of the method.

Linearity

Series of six standard solutions of known concentrations in triplicate were used to assess linearity range from peak area versus concentration. Calibration curves are plotted, and correlation coefficient, slope and intercept are calculated by using straight line equation.

Precision

The system precision or Intraday precision (Repeatability) was studied by repeated injection of replicates of standard solution containing 50 μg/mL of BIC, 25 μg/mL of TAF and 200 μg/mL of FTC for six times. The method precision was determined by injecting six solutions of sample into the UPLC system and calculating the percent relative standard deviation (%RSD) values. Six replicate injections of the standard solutions were run on the second day to assess how precisely the method could measure the concentrations of the compounds. The %RSD was calculated as the standard deviation of the six measurements divided by the mean, expressed as a percentage. Lower %RSD values indicated higher precision. Acceptable %RSD limits were established to ensure the method precision was sufficiently accurate and consistent. %RSD values within the acceptable range showed the method could produce reliable and reproducible results.

Accuracy

The accuracy of the UPLC method was assessed through recovery studies. The standard solution containing 500 μg/mL of BIC, 2000 μg/mL of FTC and 250 μg/mL of TAF was used to prepare solutions of 3 concentrations (n=3) at 50%, 100% and 150% levels of target assay. The accuracy was analyzed by the standard addition method. The % recovery and RSD were evaluated.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration data using the following equations:

$$LOD = \frac{3{\cdot}3\,\sigma}{s}$$

$$LOQ = \frac{10\,\sigma}{s}$$

Where:

Standard deviation of intercept = Variability in the intercept value from the calibration equation.

Slope = The slope of the calibration curve, which represents sensitivity.

LOD indicates the lowest concentration at which the presence of an analyte could be detected with reasonable certainty. LOQ refers to the lowest concentration that could be measured with acceptable accuracy, precision, and variability. Both LOD and LOQ provide measures of sensitivity and quantitative ability for the method.

These parameters were determined to establish the minimum amounts of the compounds that could be reliably detected (LOD) and precisely quantified (LOQ) using the analytical method. LOD and LOQ values had to meet method validation acceptance criteria.

Robustness

The robustness of the method was determined by deliberately changing the optimized analytical conditions in a controlled manner, including:

Mobile phase composition: The composition of acetonitrile and buffer in the mobile phase was varied by ± 5% to assess the method's tolerance.

Flow rate: The flow rate of the mobile phase was increased and decreased by ± 0.1 milliliters per minute to evaluate the robustness.

Column temperature: The temperature of the analytical column was raised and lowered by ± 5 degrees Celsius to determine if the method could withstand minor changes.

These variables were intentionally perturbed from the optimized conditions to evaluate the impact on method performance parameters like peak shape, resolution, theoretical plates, accuracy and precision. Limited deviations from the optimal values that still yielded acceptable results indicated a robust method.

A robust analytical method is less prone to variations from operational and environmental factors that could compromise the results. By subjecting the key method parameters to small, controlled changes, the robustness assessment evaluated the overall ruggedness and reliability of the procedure.

Solution stability

The stability of prepared standard solution was estimated by analyzing the solutions after 24hrs of storage at room temperature.

Forced degradation studies

The stability indicating nature of the method and identification of possible degradants were achieved by performing degradation studies under stressful conditions such as:

Acid hydrolysis: The standard solution was mixed with 0.1N hydrochloric acid and refluxed at 60°C for 30 minutes. The solution was then neutralized and diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system and chromatograms were assessed for sample stability.

Alkali degradation: 0.1N sodium hydroxide was added to the standard solution and refluxed at 60°C for 30 minutes. The solution was neutralized and diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system and chromatograms were assessed for sample stability.

Dry heat degradation: The standard solution was placed in an oven at 105°C for 6 hours. For UPLC analysis, the solution was diluted to concentrations of 50 micrograms per milliliter of Bictegravir, 200 micrograms per milliliter of Emtricitabine and 25 micrograms per milliliter of Tenofovir alafenamide. The solution was injected into the UPLC system to obtain chromatograms which were then assessed to indicate sample stability.

Oxidative degradation was studied by reflexing 1mL of standard solution and 1mL of 10% H_2O_2 at 60°C for 30mins. The resultant solution was neutralized and diluted to obtain concentrations of 50 µg/mL of Bictegravir, 200 µg/mL of Emtricitabine & 25 µg/mL of Tenofovir alafenamide and injected into UPLC system. The sample stability was assessed from the chromatograms obtained.

Photochemical stability of analyte was assessed by exposing the solution containing 500 µg/mL of Bictegravir, 2000 µg/mL of Emtricitabine & 250 µg/mL to UV light in UV chamber for 7days. The resultant solution was diluted and injected into UPLC system to record and assess the chromatograms.

Neutral Hydrolysis was performed by refluxing the drug in water for 6hrs at 60°C and diluted solution is injected into UPLC system to record chromatograms for stability assessment.

The degradation studies subjected the compounds to acid, alkali, heat and oxidation to evaluate the ability of the method to separate degradants from the analytes of interest. The method could be considered stability indicating if it could detect the formation of degradants under stressful conditions. Analysis of chromatograms allowed for the identification of possible degradation products.

Stability indicating methods are more robust and suitable for long term stability testing and estimation of shelf life. Degradation studies provide evidence of method specificity for the desired compounds in the presence of potential impurities or break down products.

RESULTS and DISCUSSION

Optimization of method was achieved by considering mobile phases with various solvents at different ratios with changing flow rates over columns with suitable stationary phase. The developed UPLC-PDA method was validated according to ICH guidelines for various chromatographic parameters to ensure suitability for the intended purpose.

Specificity

The specificity of the developed method was assessed by determining the ability to measure the analytes in the presence of likely components such as excipients, impurities, matrix, degradants, etc. Chromatograms of the following were evaluated for peaks that could indicate a lack of specificity:

Mobile phase alone: The chromatogram of just the mobile phase solvents was checked for any peaks that could interfere with analyte peaks. No peaks demonstrated the mobile phase would not compromise specifically.

Placebo solution: The chromatogram of a placebo solution containing excipients but not the active ingredients was analyzed for peaks at the retention times of the analytes. No peaks at the analyte retention times indicated no interference from excipients or matrix.

Blank: A blank sample with no active ingredients or excipients was injected to detect any impurities or system peaks at the analyte retention times. No peaks showed the blank would not impact specificity.

The absence of peaks in the chromatograms of the mobile phase alone, placebo solution, and blank demonstrated the specificity of the method. The method could accurately measure the analytes without interference from other components likely to be present.

Method specificity is the ability to assess unequivocally the analyte of interest in the presence of potential interferences. By evaluating chromatograms for interference at the retention times of interest, the specificity of the developed UPLC-PDA method was validated.

Figures 2, mentioned in the text, likely showed the chromatograms from the mobile phase alone, placebo solution, and blank that exhibited no peaks at the retention times of the analytes, thereby proving method specificity.

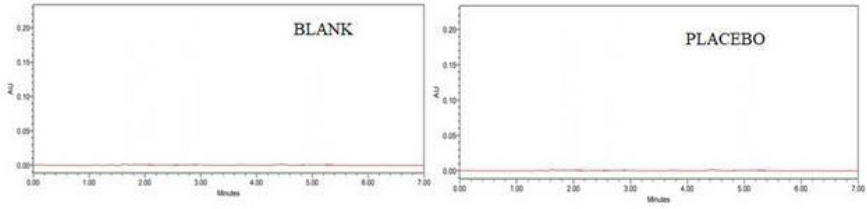


Figure 2. UPLC chromatogram of Blank and Placebo

System suitability

The performance of the UPLC system and suitability of the developed method for the intended purpose were verified by evaluating the system suitability parameters as mentioned in Table 1.

Acceptable limits for these parameters were established to ensure adequate system performance before proceeding to sample analysis. Results that fell within the acceptable range demonstrated the system could produce precise and accurate results. The results of system suitability and other validation parameters were reported in Table 1. As indicated, the results for resolution, plate count and tailing factor were found to lie within the acceptable limits. System suitability testing provides evidence that the system is capable of producing complete, separate, symmetrical and accurate measurements that meet specified requirements. By evaluating key performance indicators, system suitability verification confirms the system's quality, consistency and reliability for the intended analytical purpose. Only when system suitability was proven could the developed method be considered suitable and fit for the intended use of analyzing samples for the active ingredients Bictegravir, Emtricitabine and Tenofovir alafenamide.

Table 1. Results of system suitability and validation

Parameter	Bictegravir	Emtricitabine	Tenofovir alafenamide
USP Plate count	2874	8646	3383
USP tailing	1.11	1.04	1.06
Resolution	---	11.18	4.47
Retention time (Min)	1.910	5.020	2.739
Linearity range (µg/mL)	5-75	20-300	2.5-37.50
Correlation coefficient	0.9993	0.99906	0.99958
Slope	7303.16	8626.72	7639.59
Intercept	2892.52	14214.94	2997.09
LOD (µg/mL)	0.05	0.20	0.025
LOQ (µg/mL)	0.5	2.00	0.25
Flow rate Minus(%RSD)	0.15	0.53	0.66
Flow rate plus (%RSD)	0.06	0.35	0.15
Mobile Phase Minus (%RSD)	0.12	0.35	0.21
Mobile phase Plus (%RSD)	0.15	0.7	0.25
Assay	99.71%	99.04%	99.26%
Stability at room temperature (0-24 Hrs)	Stable	Stable	Stable
Stability at 2-8°C (0-24 Hrs)	Stable	Stable	Stable

%RSD-Percentage Relative Standard Deviation, LOD-Limit of Detection, LOQ-Limit of Quantification

Linearity

The linear relationship between analyte concentration and analytical response (peak areas) was determined for the method. Linearity was evaluated by obtaining the peak areas of replicate injections at different concentrations of the compounds within a specified range. The results were reported in Table 2 and calibration curves were shown in Figure 3. The correlation coefficients between concentration and response for the three analytes were above 0.999, indicating a high degree of linearity within the tested range. Linearity demonstrates the proportional and consistent response to increasing analyte amount across a defined concentration interval. It shows the method can accurately quantify the compounds over the expected or specified concentration range. Established linearity acceptance criteria, like minimum correlation coefficients, ensure the method has an adequate linear dynamic range for the intended application.

Results that meet and exceed the criteria substantiate the method's ability to quantify the compounds with acceptable accuracy at different concentrations.

Table 2. Linearity data

Bictegravir		Emtricitabine		Tenofovir alafenamide	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
0	0	0	0	0	0
5	46922	2.5	25879	20	233039
12.5	95366	6.25	50605	50	455421
25	179919	12.5	96490	100	867950
37.5	265022	18.75	150164	150	1218901
50	380055	25	192749	200	1771775
62.5	462116	31.25	237940	250	2186899
75	547335	37.5	291945	300	2610328

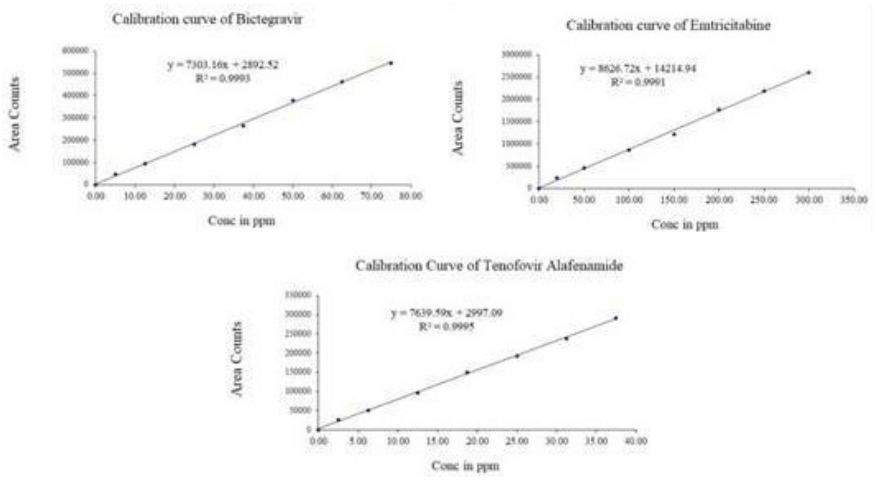


Figure 3. Calibration curves of BIC, FTC and TAF

Precision

The precision of an analytical method is typically expressed as either the standard deviation or percent relative standard deviation (%RSD). For this method, an acceptable %RSD value was established as not greater than 2.0. Repeatability (Intraday Precision) indicates the closeness of agreement between a series of measurements obtained under the same conditions. It

was determined by replicate injections of the same sample under a single set of conditions. The results of system precision and method precision were reported in Table 3.

Intermediate precision refers to the precision between laboratories or on different days. It was assessed by injecting the sample multiple times on different days. The results were provided in Table 4.

The percentage RSD values of 2% or less for precision and intermediate precision demonstrated good methods of precision and reproducibility. Higher %RSD values indicate greater variability and less reliable measurements. Methods with %RSD beyond the acceptable limit may not produce consistent or dependable results, especially for quantitative testing.

Table 3. Results of system and method precision

Sample No	Peak area response of drugs					
	Bictegravir		Tenofovir alafenamide		Emtricitabine	
	SP	MP	SP	MP	SP	MP
1.	384598	383598	192749	195483	1771809	1773276
2.	384223	381696	191930	194218	1773145	1761536
3.	377760	382323	193502	195368	1759786	1751822
4.	384743	382357	196369	194366	1780502	1744264
5.	381599	381758	194518	193369	1771283	1766622
6.	382243	382665	195558	193641	1764221	1783451
Average	382528	382399.5	194104	194407.5	1770124	1763495
STDEV	2673.66	695.84	1693.54	869.8158	7248.88	14238.09
%RSD	0.69	0.18	0.87	0.45	0.41	0.81

SP-System Precision, MP- Method Precision, STDEV – Standard deviation, %RSD – Percentage Relative Standard Deviation

Table 4. Results of intermediate precision

Inj No	Bictegravir				
	Day-1	Day-2	Average	STDEV	%RSD
1	384598	387759	386178.5	2235.16	0.58
2	384223	381695	382959	1787.57	0.47
3	377760	380724	379242	2095.86	0.55
4	384743	381596	383169.5	2225.27	0.58
5	381599	381691	381645	65.05	0.02
6	382243	383213	382728	685.89	0.18
	Emtricitabine				
1	192749	193985	193367	873.98	0.45
2	191930	192813	192371.5	624.38	0.32
3	193502	191740	192621	1245.92	0.65
4	196369	194196	195282.5	1536.54	0.79
5	194518	193532	194025	697.21	0.36
6	195558	194559	195058.5	706.40	0.36
	Tenofovir alafenamide fumarate				
1	1771809	1759787	1177199	8500.84	0.72
2	1773145	1763386	1178844	6900.66	0.59
3	1759786	1755593	1171794	2964.90	0.25
4	1780502	1768348	1182951	8594.18	0.73
5	1771283	1775920	1182403	3278.85	0.28
6	1764221	1756647	1173625	5355.63	0.46

Inj – Injection, STDEV – Standard Deviation, %RSD – Percentage Relative Standard Deviation

Accuracy

Accuracy of any developed analytical method indicates the degree of closeness between the measured value and the true value or reference value. Method accuracy was determined by performing recovery studies. The results of recovery studies in Table 5 confirm the adequate accuracy of developed method.

Table 5. Results of % recovery studies

% level	Bictegravir				Emtricitabine				Tenofovir alafenamide			
	A.A	A.R	%R	Mean %R ± RSD	A.A	A.R	%R	Mean %R ± RSD	A.A	A.R	%R	Mean %R ± RSD
50	25.1	24.75	98.6	98.43 ± 0.21	12.50	12.49	99.9	99.17 ± 0.6	100	98.94	98.9	98.81 ± 0.1
	25.2	24.83	98.5		12.50	12.35	98.9		100	98.7	98.7	
	25.1	24.64	98.2		12.50	12.34	98.7		100	98.8	98.8	
100	50.10	50.09	100	99.53 ± 0.43	25.0	24.89	99.6	98.77 ± 0.8	200	198.34	99.2	99.30 ± 0.3
	50.20	49.8	99.2		25.0	24.68	98.7		200	198.21	99.1	
	50.10	49.81	99.4		25.0	24.49	98.0		200	199.1	99.6	
150	75.20	73.78	98.1	98.16 ± 0.05	37.50	37.67	100.5	100.3 ± 0.3	300	295.14	98.4	99.30 ± 0.9
	75.40	74.02	98.2		37.50	37.48	99.9		300	197.77	99.3	
	75.30	73.97	98.2		37.50	37.67	100.5		300	300.53	100.2	

A.A – Amount Added, A.R – Amount Recovered, %R – Percentage Recovery, STDEV – Standard Deviation, %RSD – Percentage Relative Standard Deviation

Forced degradation studies

Degradation of analytes was induced by exposing the sample to various stress conditions and the chromatograms were studied for the presence of any degradant peaks without interfering with the analyte peaks. The degradation chromatograms were shown in Figure 4, and the results were given in Table 6.

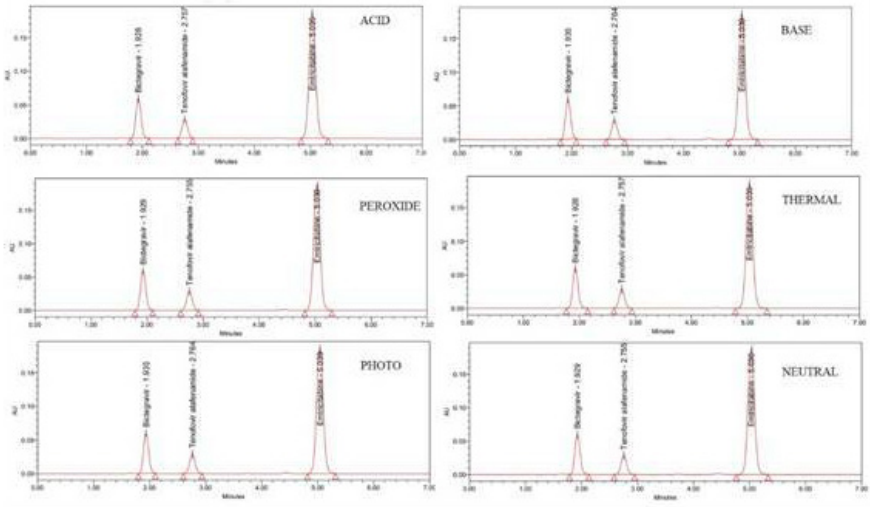


Figure 4. UPLC chromatograms of degradation studies

Table 6. Results of forced degradation studies

Stress Condition	Bictegravir			Emtricitabine			Tenofovir alafenamide		
	% D	P.A	P.T	% D	P.A	P.T	% D	P.A	P.T
Control	0.2	0.07	2.076	-0.7	0.07	2.022	0	0.483	2.199
Acid	1.8	0.071	2.107	2.7	0.087	2.033	3.2	0.233	2.209
Alkali	1.9	0.071	2.119	2.6	0.088	2.033	3.5	0.371	2.218
Oxidation	1.2	0.067	2.095	3.4	0.065	2.024	4.1	0.38	2.199
Thermal	0.8	0.083	2.115	3.4	0.088	2.032	4	0.325	2.226
Photo	1.6	0.076	2.122	3.4	0.088	2.035	3.4	0.454	2.219
Neutral	0.9	0.071	2.1	2.1	0.067	2.027	-3	0.438	2.213

%D – Percentage Degradation, P.A – Purity Angle, P.T – Purity Threshold

Assay

The developed method was applied for the assay of Biktarvy tablets. The assay values and the standard and sample chromatograms were shown in Table 7 & Figure 5.

Table 7. Assay results of marketed tablets

S.No	Parameter	Assay of Bictegravir %	Assay of Emtricitabine %	% Assay of TAF
1	Assay (Specification: NLT 98.00 % and NMT 102.00% w/w) (n=3)	99.82	99.63	99.57

NLT: Not less than, NMT: not more than, n=number of determination

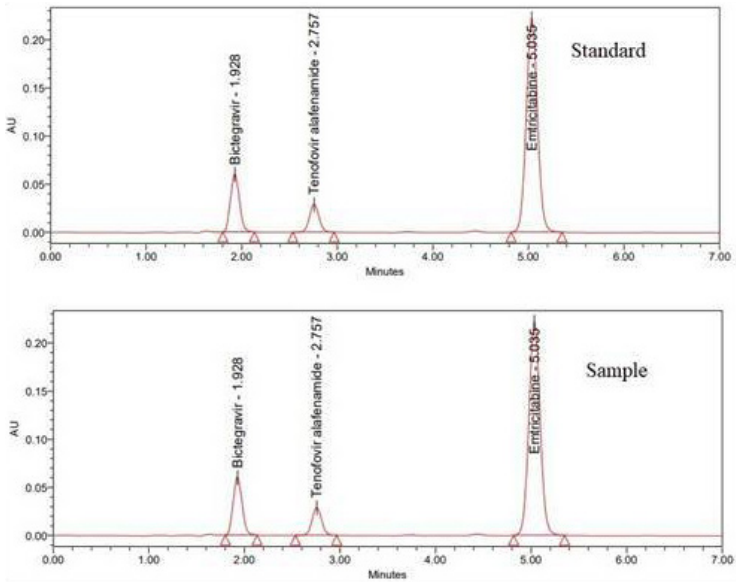


Figure 5. UPLC chromatograms of standard and sample

For the simultaneous estimation of BIC, FTC and TAF, a specific, accurate and suitable UPLC method was developed by applying different sets of conditions to achieve system suitability parameters within acceptable limits. A variety of mobile phase combinations in different proportions at different flow rates over different stationary phases were used to optimize the method.

The mobile phase consisting of acetonitrile and 0.1% TEA buffer pH 2.5 in a 30:70 volume ratio with a flow rate of 1.0 milliliters per minute was selected over a BEH C18 column as it provided better resolution and separation with an elution time of 3 minutes. Detection of the analytes was achieved at 265 nanometers using a PDA detector. The retention times were found to be 1.910, 5.020 and 2.739 minutes for Bictegravir (BIC), Emtricitabine (FTC) and Tenofovir alafenamide (TAF), respectively. The optimized chromatographic conditions were validated according to ICH guidelines to verify suitability for the intended use of the proposed method. Specificity of the developed method was indicated by the absence of interfering peaks. Key chromatographic parameters like plate count, tailing factor, resolution, peak area and retention times were evaluated for system suitability and found compliant with acceptable limits. Standard solutions in the concentration range of 18.75 to 112.5 µg/mL for BIC, 3.125 to 18.75 µg/mL for FTC, and 12.5 to 75 µg/mL for TAF showed a linear relationship with correlation coefficients above 0.999, demonstrating linearity. The proposed method was precise, accurate and robust for estimat-

ing BIC, FTC and TAF as %RSD values were less than 2.0%. When subjected to various stress conditions, the percentage degradation of BIC, FTC and TAF remained within limits. Degradation products were resolved from the analytes, indicating method specificity.

Table 8. Comparison between proposed method and reported method

Parameters	Proposed method			Reported method[17]		
	BIC	TAF	EMT	BIC	TA	EMT
Linearity (µg/mL)	5-75	2.5-37.5	20-300	12.5-75	6.25-37.5	50-300
LOD (µg/mL)	0.05	0.025	0.20	0.54	0.16	3.66
LOQ (µg/mL)	0.5	0.25	2.00	1.63	0.49	3.66

Upon comparison of the developed method with the reported method indicated that the developed method is more sensitive due to low values of LOD and LOQ, which can quantify the analytes in low concentrations (Table 8).

The proposed stability indicating UPLC method allows precise, linear, rapid and stable estimation of Bictegravir, Emtricitabine and Tenofovir Alafenamide fumarate in bulk and tablet dosage forms. The results of parameters validated were complying with the acceptance criteria given in ICH guidelines. Since the analytes were quantified with high sensitivity, better resolution and short retention times, the newly developed method can be choice for rapid determination of samples in routine quality control analysis of marketed formulation.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors significantly contributed to the present research work. The design, acquisition, analysis, drafting, critical revision, statistical analysis, and technical support of this work was contributed by Dr. Divya Narla. Supervision and critical revision of the manuscript was carried out by Dr. P. Nagaraju. Analysis and drafting of the manuscript were contributed by SPN Kumar.

FUNDING SOURCES

Not applicable.

ACKNOWLEDGMENTS

The authors take the privilege to express their gratitude to the Management, Principal of Aditya College of Pharmacy, for their continuous support.

REFERENCES

1. National Center for Biotechnology Information. PubChem compound summary for CID 90311989, Bictegravir [Internet]. 2023 [Mar 1, 2023]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Bictegravir

2. Gilead Sciences, Inc. Biktarvy prescribing information. Foster City (CA): Gilead Sciences, Inc.; 2018 Feb.

3. National Center for Biotechnology Information. PubChem compound summary for CID 60877, Emtricitabine [Internet]. 2021 [Mar 2, 2021]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Emtricitabine

4. National Center for Biotechnology Information. PubChem compound summary for CID 71492247, Tenofovir Alafenamide fumarate [Internet]. 2021 [Mar 2, 2021]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Tenofovir-Alafenamide-fumarate

5. U.S. Food and Drug Administration. Biktarvy (bictegravir, emtricitabine, and tenofovir alafenamide) tablets prescribing information [Internet]. Silver Spring (MD): FDA; 2019 [Mar 2, 2021]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/210251s006lbl.pdf

6. Raju VR, Ramana MV, Shyam T, Rajinikanth KN, Kumar VK, Raju NA. Bioanalytical method development and validation for simultaneous determination of bictegravir tenofovir and emtricitabine in human plasma by LC-MS/MS. World J Pharm Sci, 2018;7(12):441-463.

7. Prathipati PK, Mandal S, Destache CJ. Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC–MS/MS and its application to a pharmacokinetic study. J Pharm Biomed Anal, 2016;129:473-481. Doi: 10.1016/j.jpba.2016.07.040

8. Tanuja A, Ganapaty S. Bio-analytical method development and validation for simultaneous determination of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate in human plasma by LC-MS/MS, Indian J Pharm Educ, 2022;56(4):1190-1205. Doi: 10.5530/ijper.56.4.201

9. Kokkirala TK, Suryakala D. RP-HPLC method development and validation for the estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide in bulk and pharmaceutical dosage form. J Taibah Univ Sci, 2019;13(1):1137-1146. Doi: 10.1080/16583655.2019.1689601

10. Attaluri T, Ganapaty SE, Varanasi SNM. Development and validation of a stability-indicating RP-HPLC method for the simultaneous estimation of bictegravir, emtricitabine, and tenofovir alafenamide fumarate. Turk J Pharm Sci, 2021;18(4):410. Doi: 10.4274/tjps.galenos.2020.70962

11. Deepthi VSS, Narendra D. Method development and validation for simultaneous estimation of Emtricitabine, Bictegravir and Tenofovir alafenamide by RP-HPLC. Int J Pharm Sci Rev Res, 2019;58(2):54-59.

12. Sudha PDC, Sohail P, Avulapati U. Stability indicating RP-HPLC method for the simultaneous determination of emtricitabine, tenofovir, cobicistat and elvitegravir. Int J Pharm Sci & Res, 2020;11(3):1452-1466. Doi: 10.13040/IJPSR.0975-8232.11(3).1452-66

13. Yamana AV, Chandra Sekhar KB. Simultaneous HPLC method development and validation for quantification emtricitabine, bictegravir and tenofovir alafenamide in combined dosage form with stability studies. J Global Trends in Pharm Sci, 2020;11(3):8226-8234.

14. Meenakshi R, Shyam Sunder R. Method development and validation by RP-HPLC for simultaneous estimation of emtricitabine, bictegravir, tenofovir alafenamide in fixed dosage form. IAJPS, 2018;5(7):7123-7131.

15. Deepthi R, Gowri Sankar D. Novel stress indicating RP-HPLC method development and validation for the simultaneous estimation of Bictegravir, Emtricitabine and Tenofovir alafenamide. Indo Am J P Sci, 2019;6(6):13282-13291.

16. Abdul Sattar MD, Achanta S. Analytical method development and validation for the determination of Emtricitabine and Tenofovir Disoproxil Fumarate using reverse phase HPLC method in bulk and tablet dosage form. J Pharm Sci & Res, 2018;10(5):1207-1212.

17. Dadi V, Sowjanya G. Validated method for the simultaneous determination of emtricitabine, bictegravir and tenofovir alafenamide in pharmaceutical dosage form using UPLC. J Pharm Sci & Res, 2022;13(11):4536-4543. Doi: 10.13040/IJPSR.0975-8232.13(11).4536-43

18. International Conference on Harmonisation (ICH). ICH harmonised tripartite guideline Q2(R1): validation of analytical procedures – text and methodology. Geneva: ICH; 2005.

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6340

Cytotoxic activity of *Achillea arabica* Kotschy against renal cancer cell lines

Güneş Güniz GÜNGÖR[1], Fatma TOSUN[2*]

1 Istanbul Medipol University, Graduate School of Health Sciences, Department of Pharmacognosy, Istanbul, Türkiye
2 Istanbul Medipol University, School of Pharmacy, Department of Pharmacognosy, Istanbul, Türkiye

ABSTRACT

Achillea arabica Kotschy, belonging to the Asteraceae family, is a plant whose biological activities, such as wound healing, antimicrobial, anticancer, and antioxidant properties, have been evidenced by studies. It has been investigated for its cytotoxic activity in various cell lines, including AGS, MCF7, SW742, SKLC6, A375, PLC/PRF/5, HT29, and HepG2. However, no study has been conducted on any cell line related to kidney cancer. In this study, the cytotoxic activities of the extracts prepared from the root and aerial parts of the plant with dichloromethane, ethyl acetate, and methanol were investigated against A498 and UO31 kidney cancer cell lines. The highest activity was observed in the dichloromethane extract of the aerial parts of the plant on both cancer cell lines. The dichloromethane extract of the aerial parts showed 63% inhibition on the A498 cell line and 56% inhibition on the UO31 cell line at a concentration of 25 μg/mL. The results we have obtained have been of a quality that will lead to new research in this context.

Keywords: cytotoxic activity, *Achillea arabica*, renal cancer

INTRODUCTION

Cancer is one of the leading causes of death worldwide[1]. It is also a disease with typical properties such as abnormal cell growth, invasion, metastasis and mu-

* Corresponding author: Fatma TOSUN
E-mail: ftosun@medipol.edu.tr
ORCIDs:
Güneş Güniz GÜNGÖR: 0009-0002-0911-0946
Fatma TOSUN: 0000-0003-2533-5141
(Received 16 Jul 2024, Accepted 5 Sept 2024)

tations[2]. According to the studies, 2,001.140 new cancer cases and 611,720 cancer deaths are expected to occur in 2024. The fact that most of the drugs in cancer treatment often have side effects such as ischemic heart disease, vomiting, bone marrow suppression, myelosuppression, liver dysfunction, fatigue and hypertension, which further hinder the treatment process, has led researchers to search for different treatments[3]. Therefore, the discovery of new treatment strategies or chemotherapeutics with minimal or no side effects that are easily accessible, cost-effective, and more effective for treating this deadly disease has become one of the major goals in cancer therapy[4]. In addition, chemotherapy treatment significantly reduces the patient's quality of life as it damages normal cells. Therefore, it is very important to search for new anti-tumor agents that are specific to tumor cells[5].

Throughout the ages, it is known that people have utilized nature to meet their basic needs. This has also been the case for the use of natural products as medicines in the treatment of various diseases[6]. Plant extracts and plant-derived natural compounds such as glycosides, alkaloids, tannins, terpenes, coumarins and flavonoids are known to inhibit the growth of cancer cells such as kidney, lung, breast and colorectal cancer cells[7,8]. Kidney cancer (Renal Cell Carcinoma-RCC) is the second most common type of cancer of the urinary system[9]. Currently, RCC constitutes approximately 2-3% of all adult malignancies worldwide and ranks as the 12th most common malignancy and the third most common urogenital cancer[10]. RCC has a high metastatic potential to the lung, liver, bone, head and neck[11]. Approximately 30% of RCC cases are diagnosed at an advanced or metastatic stage, and according to the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) criteria, nearly 80% of these patients belong to the intermediate or low risk group[12]. Due to limited treatment options, less than 40% of patients survive for ≥5 years after diagnosis. Additionally, the lack of routine adjuvant therapy in the clinic is a key reason for the recurrence of kidney cancer, as kidney cancer is resistant to both chemotherapy and radiotherapy. Therefore, there is a need to search for new compounds and develop targeted therapies for kidney cancer[13].

The *Achillea* genus includes 138 accepted species distributed in Europe, Asia, North Africa, North and Central America, Alaska, and Greenland[14]. The genus *Achillea* is represented by 50 species in Turkey[15]. *Achillea* species were reported to contain polyphenols, flavonoids, phenolic and quinic acid derivatives, sesquiterpenes lactones and essential oils; and to have anticancer, antioxidant, antimicrobial, anti-inflammatory, analgesic, antipyretic, antidiabetic, antihelmintic and antihypertensive activities[16-24].

Previous studies have shown that the extracts of *Achillea arabica* have anticancer, antimicrobial, antioxidant, anti-inflammatory, anticholinesterase, analgesic, anxiolytic and wound-healing effects[17,23-30] and contain phenolic compounds, flavonoids, sesquiterpenes lactones and essential oils[17,29-38].

When cytotoxic activity studies conducted on different cell lines were compiled to determine the anticancer efficacy of *A. arabica*, no study was found to be conducted on the A498 and UO31 kidney cancer cell lines.

METHODOLOGY

Plant material

Achillea arabica Kotschy used in this study was collected from Adana-Saimbeyli region on June 12, 2016. Herbarium specimens of the plant identified by Prof. Mecit Vural are registered in Istanbul University Faculty of Pharmacy Herbarium (ISTE 115056).

Preparation of extracts

The extraction process was conducted using solvents of increasing polarity, as they selectively consume compounds with different chemical structures based on their polarities. Additionally, considering the possibility of degradation with heat, the extraction process was carried out at room temperature. The pulverized plant material was successively macerated with dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) at room temperature. After each extraction with a solvent, the plant material was dried to remove the solvent completely and then subjected to extraction with the next solvent. After consumption, the extracts filtered through filter paper were concentrated using a rotary evaporator at 40°C.

Cytotoxicity assay on cancer cells

In-vitro 2Day XTT cytotoxic activity test[39] was applied to extracts of the roots and aerial parts. The 2Day XTT bioactivity assay is an *in-vitro* colorimetric cytotoxic activity test. XTT bioactivity assays were performed at the NCI MTP Experiment Development and Imaging Department. Kidney cancer cell lines (A498, UO31) were used in XTT cytotoxic activity tests. Sanguinarine chloride hydrate was used as a control in the experiment. The assay was performed as described previously[8].

RESULTS and DISCUSSION

In this study, the *Achillea arabica* plant was investigated for the first time in terms of cytotoxic activity on A498 and UO31 cell lines in kidney cancer. The

extract yields obtained by DCM, EtOAc and MeOH extraction of the aerial parts and roots of the plant are given in Table 1. The yield of the extract is also important for the calculation of the plant material to be used for the subsequent isolation of the active compounds.

As a result of the cytotoxic activity studies of the extracts obtained from the aerial parts and roots of the plant, the % inhibition values they showed on A498 and UO31 kidney cancer cell lines are given in Table 2.

Table 1. % yield obtained by extraction of the aerial parts and roots of *A. arabica*

	yield (a/a) %	
Extracts	**Aerial parts**	**Roots**
1	1.32%	1.18%
2	0.69%	0.68%
3	3.64%	3.36%

1: DCM extract; 2: EtOAc extract; 3: MeOH extract

Table 2. Cytotoxic activities of the extracts (25 ug/mL concentration)

	inhibition %					
	A498			UO31		
Plant parts	1	2	3	1	2	3
Roots	40	45	54	43	53	53
Aerial parts	63	41	46	56	43	54

1: DCM extract; 2: EtOAc extract; 3: MeOH extract

Cytotoxic activity was determined on the kidney cancer cell lines (A498 and UO31) by *in-vitro* 2Day XTT cytotoxic activity assay. On the A498 cancer cell line, the root MeOH extract, and the aerial parts DCM extract exhibited over 50% inhibition at a concentration of 25 µg/mL, while on the UO31 cell line, the root EtOAc and MeOH extracts, as well as the aerial parts DCM and MeOH extracts, showed over 50% inhibition at a concentration of 25 µg/mL. The highest activity on both cancer cell lines was observed with the DCM extract of the aerial parts. The DCM extract of the aerial parts exhibited 63% inhibition on the A498 cell line and 56% inhibition on the UO31 cell line at a concentration of 25 µg/mL.

In recent years, the therapeutic potential of medicinal plant extracts in preventing and treating cancer has attracted scientists' attention. Approximately 75% of approved cancer drugs have been developed based on agents of natural source. Plant-derived active compounds support treatment at all stages of cancer and are multi-targeted and non-toxic[40].

Previously, *A. arabica* has been tested against HeLa (cervical cancer), AGS (gastric adenocarcinoma), MCF7 (breast cancer), SW742 (colorectal adenocarcinoma) SKLC6 (lung cancer), A375 (skin cancer), PLC/PRF/5 (liver cancer)[24,30]. In another study, *A. arabica* (*A. biebersteinii*) extracts prepared with hexane, chloroform, and methanol were found to be effective in HT-29 (colorectal carcinoma) cell line, increased the activity of the 5-FU compound used in treatment, induced apoptosis by regulating PTEN/Akt/mTOR signaling pathway, and inhibited angiogenesis[41]. Ag-NPs were synthesized using *A. arabica* (*A. biebersteinii*) flower extract has been reported to induce apoptosis on MCF-7 cell line and can be considered a potential chemotherapeutic agent in treating breast cancer[42]. This study represents the first investigation on the cytotoxic activity of *A. arabica* on A498 and UO31 cell lines.

Previous studies reported that the extracts of the *Achillea* species exhibited cytotoxic activities against different cancer cell lines[17-22,43,44], but there are no reports in the literature dealing with the cytotoxic activities of the *Achillea* species on the renal cancer cell lines. Therefore, our study is important in terms of being the first cytotoxic activity study on the kidney cancer cell lines not only on the *A. arabica* species but also on the *Achillea* genus and contributed to the literature on this regard.

In conclusion, bioactivity-guided fractionation of the DCM extract of the aerial parts, exhibited the highest activity on both cancer cell lines, is planned to isolate and identify the compounds responsible for the activity.

STATEMENT OF ETHICS

There is no ethical statement provided.

CONFLICT OF INTEREST STATEMENT

The authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

These authors contributed to the work equally.

FUNDING SOURCES

There are no sources of funding indicated.

ACKNOWLEDGMENTS

We thank Dr. John A. Beutler, Molecular Targets Laboratory, CCR, NCI, Frederick, MD, U.S.A., for the cytotoxic activity testing.

We thank Prof. M. Vural for identifying of plant material.

REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin, 2022;72(1):7-33. Doi: 10.3322/caac.21708

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin, 2018;68(6):394-424. Doi: 10.3322/caac.21492

3. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. CA Cancer J Clin, 2024;74(1):12-49. Doi: 10.3322/caac.21820

4. Banik K, Khatoon E, Harsha C, Rana V, Parama D, Thakur KK, et al. Wogonin and its analogs for the prevention and treatment of cancer: a systematic review. Phytother Res, 2022;36(5):1854-1883. Doi: 10.1002/ptr.7386

5. Deb DD, Parimala G, Devi SS, Chakraborty T. Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. Chem Biol Interact, 2011;193(1):97-106. Doi: 10.1016/j.cbi.2011.05.009

6. Majolo F, Delwing LKDOB, Marmitt DJ, Bustamante-Filho IC, Goettert MI. Medicinal plants and bioactive natural compounds for cancer treatment: important advances for drug discovery. Phytochem Lett, 2019;31:196-207. Doi: 10.1016/j.phytol.2019.04.003

7. Isyaka SM, Mas-Claret E, Langat MK, Hodges T, Selway B, Mbala BM. Cytotoxic diterpenoids from the leaves and stem bark of *Croton haumanianus* (Euphorbiaceae). Phytochem, 2020;178:112455. Doi: 10.1016/j.phytochem.2020.112455

8. Tosun F, Aytar EC, Beutler JA, Wilson JA, Miski M. Cytotoxic sesquiterpene coumarins from the roots of *Heptaptera cilicica*. Rec Nat Prod, 2021;15(6):529-536. Doi: 10.25135/rnp.242.21.02.1990

9. Mihoğlugil F, Akalgan D, Tosun F. Cytotoxicity screening of some Turkish plants against renal cancer cells. J Res Pharm, 2023;27(2):636-641. Doi: 10.29228/jrp.346

10. Yu X, Du Z, Zhu P, Liao B. Diagnostic, prognostic, and therapeutic potential of exosomal microRNAs in renal cancer. Pharmaco Rep, 2024;76:273-286. Doi: 10.1007/s43440-024-00568-7

11. Kweon HT, Yoo JS, Hong, YT. Tongue metastasis from renal cell carcinoma: a rare case presentation. Ear Nose Throat J, 2024;1-3. Doi: 10.1177/01455613231226038

12. Lalani AKA, Heng D, Basappa NS, Wood L, Iqbal N, McLeod D. Evolving landscape of first-line combination therapy in advanced renal cancer: a systematic review. Ther Adv Med Oncol, 2022;14:1-17. Doi: 10.1177/17588359221108685

13. Zhu C, Na N, Sheng H, Feng B, Wang H, Zhu P. Ginkgolic acid inhibits the growth of renal cell carcinoma cells via inactivation of the EGFR signaling pathway. Exp Ther Med, 2020;19(4):2949-2956. Doi: 10.3892/etm.2020.8570

14. Plants of the World Online. *Achillea arabica* Kotschy [Internet]. Kew Royal Botanic Gardens [Jul 16, 2024]. Available from: https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:173817-1/

15. Güner A, Aslan S, Ekim T, Vural M, Babaç MT, editors. Türkiye bitkileri listesi (damarlı bitkiler). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını; 2012.

16. Saeidnia S, Gohari AR, Mokhber-Dezfuli N, Kiuchi FA. Review on phytochemistry and medicinal properties of the genus *Achillea*. DARU: J Pharm Sci, 2011;19(3):173-186.

17. Barda C, Grafakou ME, Tomou EM, Skaltsa H. Phytochemistry and evidence-based traditional uses of the genus *Achillea* L.: an update (2011–2021). Sci Pharm, 2021;89(4):50-86. Doi: 10.3390/scipharm89040050

18. Awad BM, Habib ES, Ibrahim AK, Wanas AS, Radwan MM, Helal MA, et al. Cytotoxic activity evaluation and molecular docking study of phenolic derivatives from *Achillea fragrantissima* (Forssk.) growing in Egypt. Med Chem Res, 2017;26:2065-2073. Doi: 10.1007/s00044-017-1918-6

19. Tsiftsoglou OS, Krigas N, Gounaris C, Papitsa C, Nanouli M, Vartholomatos E, et al. Isolation of secondary metabolites from *Achillea grandifolia* Friv. (Asteraceae) and main compounds' effects on a glioblastoma cellular model. Pharmaceutics, 2023;15(5):1383-1397. Doi: 10.3390/pharmaceutics15051383

20. Mohammed HA, Abd-Elraouf M, Sulaiman GM, Almahmoud SA, Hamada FA, Khan RA, et al. Variability in the volatile constituents and biological activities of *Achillea millefolium* L. essential oils obtained from different plant parts and by different solvents. Arab J Chem, 2023;16(9):1-15. Doi: 10.1016/j.arabjc.2023.105103

21. Taşkın D, Doğan M, Ermanoğlu M, Ermanoglu M, Arabaci T. Achillea goniocephala extract loaded into nanochitosan: in vitro cytotoxic and antioxidant activity. Clin Exp Health Sci, 2021;11(4):659-666. Doi: 10.33808/clinexphealthsci.972180

22. Eruygur N, Ataş M, Tekin M, Cevik, O. Evaluation of in vitro antioxidant, antimicrobial and cytotoxic activities of crude ethanol extract and fractions of Achillea sintenisii Hub. Mor. Clin Exp Health Sci, 2023; 13(3):517-524. Doi: 10.33808/clinexphealthsci.1058614

23. Raudone L, Radušiene J, Seyis F, Yayla F, Vilkickyte G, Marksa M, et al. Distribution of phenolic compounds and antioxidant activity in plant parts and populations of seven underutilized wild *Achillea* species. Plants, 2022;11(3):447-466. Doi: 10.3390/plants11030447

24. Yilmaz MA, Ertaş A, Yener İ, Türkmenoğlu FP, Ölmez ÖT, Öztürk M, et al. Chemical fingerprints and bioactivities of 12 Anatolian Achillea species by LC-MS/MS with chemometric approach: novel phytonutrients, natural food preservatives and chlorogenic acid sources. Turk J Bot, 2022;46(5):473-489. Doi: 10.55730/1300-008X.2723

25. Toplan GG, Taşkın T, İşcan G, Göger F, Kürkçüoğlu M, Civaş A, et al. Comparative studies on essential oil and phenolic content with in vitro antioxidant, anticholinesterase, antimicrobial activities of *Achillea biebersteinii* Afan. and *A. millefolium* subsp. *millefolium* Afan. L. growing in Eastern Turkey. Molecules, 2022;27(6):1956. Doi: 10.3390/molecules27061956

26. Bashi DS, Fazly Bazzaz BS, Sahebkar A, Karimkhani MM, Ahmadi A. Investigation of optimal extraction, antioxidant, and antimicrobial activities of *Achillea biebersteinii* and *A. wilhelmsii*. Pharm Biol, 2012;50(9):1168-1176. Doi: 10.3109/13880209.2012.662235

27. Akkol EK, Koca U, Pesin I, Yilmazer D. Evaluation of the wound healing potential of *Achillea biebersteinii* Afan. (Asteraceae) by *in vivo* excision and incision models. eCAM, 2011;1-7. Doi: 10.1093/ecam/nep039

28. Varasteh-Kojourian M, Abrishamchi P, Matin MM, Asili J, Ejtehadi H, Khosravitabar F. Antioxidant, cytotoxic and DNA protective properties of *Achillea eriophora* DC. and *Achillea biebersteinii* Afan. extracts: a comparative study. Avicenna J Phytomed, 2017;7(2):157-168.

29. Bariş Ö, Güllüce M, Şahin F, Özer H, Kiliç H, Özkan H, et al. Biological activities of the essential oil and methanol extract of Achillea biebersteinii Afan. (Asteraceae). Turk J Biol, 2006;30(2):65-73. Doi: 10.5897/JMPR10.560

30. Ghavami G, Sardari S, Shokrgozar MA. Anticancerous potentials of *Achillea* species against selected cell lines. J Med Plants Res, 2010;4(22):2411-2417. Doi: 10.5897/JMPR10.560

31. Al-Shuneigat JM, Al-Sarayreh SA, Al-Qudah MA, Al-Saraireh YM. Antibacterial and antibiofilm activity of essential oil of *Achillea biebersteinii* and its mode of action. J Pharm Pharmacogn Res, 2020;8(2):155-166.

32. Cirak C, Radusiene J, Raudone L, Vilkickyte G, Seyis F, Marksa M, Yayla F. Phenolic compounds and antioxidant activity of *Achillea arabica* populations. S Afr J Bot, 2022;147:425-433. Doi: 10.1016/j.sajb.2022.02.006

33. Oskay E, Yesilada A. Four flavonoids and three other constituents from *Achillea biebersteinii*. J Nat Prod, 1984;47(4):742-742. Doi: 10.1021/np50034a041

34. Abd-Alla H, Shalaby N, Hamed M, El-Rigal NS, Al-Ghamdi S, Bouajila J. Phytochemical composition, protective and therapeutic effect on gastric ulcer and a-amylase inhibitory activity of *Achillea biebersteinii* Afan. Arch Pharm Res, 2015;39:10-20. Doi: 10.1007/s12272-014-0544-9

35. Şabanoğlu S, Gökbulut A, Altun ML. Characterization of phenolic compounds, total phenolic content and antioxidant activity of three *Achillea* species. J Res Pharm, 2019;23(3):567-576. Doi: 10.12991/jrp.2019.164

36. Gawel-Beben K, Strzepek-Gomółka M, Czop M, Sakipova Z, Głowniak K, Kukula-Koch W. *Achillea millefolium* L. and *Achillea biebersteinii* Afan. hydroglycolic extracts-bioactive ingredients for cosmetic use. Molecules, 2020;24:3368. Doi: 10.3390/molecules25153368

37. Chialva F, Monguzzi F, Manitto P, Akgül, A. Essential oil constituents of *Achillea biebersteinii* Afan. J Essent Oil Res, 1993;5(1):87-88. Doi: 10.1080/10412905.1993.9698176

38. Tosun F, Kürkçüoğlu M. The essential oils of two Achillea L. species from Turkey. Acta Pharm Sci, 2018;56(2):59-66. Doi: 10.23893/1307-2080.aps.05611

39. Devkota KP, Covell D, Ransom T, McMahon JB, Beutler JA. Growth inhibition of human colon carcinoma cells by sesquiterpenoids and tetralones of *Zygogynum calothyrsum*. J Nat Prod, 2013;76:710-714. Doi: 10.1021/np400042q

40. Wani ZA, Guru SK, Rao AS, Sharma S, Mahajan G, Behl A. A novel quinazolinone chalcone derivative induces mitochondrial dependent apoptosis and inhibits PI3K/Akt/mTOR signaling pathway in human colon cancer HCT-116 cells. Food Chem Toxiol, 2016;87:1-11. Doi: 10.1016/j.fct.2015.11.016

41. Erdoğan MK, Ağca CA, Aşkın H. *Achillea biebersteinii* extracts suppress angiogenesis and enhance sensitivity to 5-fluorouracil of human colon cancer cells via the PTEN/AKT/mTOR pathway *in vitro*. Asian Pac J Trop Biomed, 2020;10(11):505-515. Doi: 10.4103/2221-1691.294091

42. Baharara J, Namvar F, Ramezani T, Mousavi M, Mohamad R. Silver nanoparticles biosynthesized using *Achillea biebersteinii* flower extract: apoptosis induction in MCF-7 cells via caspase activation and regulation of Bax and Bcl-2 gene expression. Molecules, 2015;20(2):2693-2706. Doi: 10.3390/molecules20022693

43. Boutennoun H, Boussouf L, Rawashdeh A, Al-Qaoud K, Abdelhafez S, Kebieche M, et al. *In vitro* cytotoxic and antioxidant activities of phenolic components of Algerian *Achillea odorata* leaves. Arab J Chem, 2017;10(3):403-409. Doi: 10.1016/j.arabjc.2014.05.013

44. Csupor-Loffler B, Hajdu Z, Zupko I, Rethy B, Falkay G, Forgo P, et al. Antiproliferative effect of flavonoids and sesquiterpenoids from *Achillea millefolium* on cultured human tumour cell lines. Phytother Res, 2009;23(5):672-676. Doi: 10.1002/ptr.2697

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6341

The physicochemical and sensory properties, and the impact on probiotic viability during storage of probiotic ice cream containing cocoa, walnut, persimmon, and cinnamon

Burcu ÇAKMAK SANCAR[1*], Canan HECER[2], Meryem AKHAN[1], Burak ERİM[1], Ayşe Gül ÇEÇEN[3]

1 Istanbul Esenyurt University, Faculty of Health Sciences, Nutrition and Dietetics Department, 34510 Istanbul, Türkiye
2 Kıbrıs Batı University, Faculty of Health Sciences, Nutrition and Dietetics Department, 99450 Famagusta, Cyprus
3 Istanbul Sabahattin Zaim University, Faculty of Health Sciences, Nutrition and Dietetics Department, 34303 Istanbul, Türkiye

ABSTRACT

In this study, the effect of probiotics in flavored ice creams was investigated and ice creams containing different concentrations of cocoa, walnut, date and cinnamon were prepared. Two different probiotics (*Lactobacillus acidophilus* and *Bifidobacterium animalis* subps. *lactis* BB-12) were added to each sample and stored at -18°C for 60 days. The effects of storage on pH, titratable acidity, dry matter, fat and ash, sensory properties and probiotic viability were investigated. Increasing ingredient concentrations and storage time did not affect the dry matter, fat and ash values of the ice creams ($p>0.05$). Especially in ice creams containing 20% walnut and 0.4% cinnamon, pH increased significantly ($p<0.05$), and titratable acidity decreased ($p<0.05$). Probiotic counts were performed on days 1, 15, 30, 45 and 60. Although probiotic bacteria decreased during storage, by day 45 of storage all ice cream samples were above the minimum probiotic concentration required to produce a probiotic effect.

Keywords: probiotic, *Lactobacillus*, *Bifidobacterium*, ice cream, prebiotic

*Corresponding author: Burcu ÇAKMAK SANCAR
E-mail: burcucakmak@esenyurt.edu.tr
ORCIDs:
Burcu ÇAKMAK SANCAR: 0000-0002-0737-7009
Canan HECER: 0000-0003-1156-9510
Meryem AKHAN: 0000-0001-8065-8635
Burak ERİM: 0000-0003-1927-4549
Ayşe Gül ÇEÇEN: 0009-0006-3056-604X
(Received 12 Jul 2024, Accepted 12 Sept 2024)

INTRODUCTION

Ice cream is a dairy product widely consumed in all age groups all over the world due to its taste, cooling effect and high nutritional value[1,2]. The addition of various ingredients to ice cream, which has a higher carbohydrate, fat and protein content compared to milk, further increases its nutritional value[3]. In recent years, with the increasing awareness of consumers about health and nutrition, interest in the consumption of foods containing more bioactive substances, probiotics and prebiotics has increased[4]. Defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as "living microorganisms that provide health benefits to the host when administered in adequate amounts", probiotics have been reported to have health benefits such as protection of intestinal flora, anticarcinogenic effect, lowering serum cholesterol levels and blood pressure[5,6]. Probiotic foods are expected to contain more than 106 colony forming units (CFU) of live probiotic microorganisms per ml or gram. The most important points to be considered in the selection of probiotic microorganisms are that they remain viable during the processing and storage of foods, during intestinal transit, and that they confer potential health benefits on consumers[7]. *Lactobacillus* and *Bifidobacterium* species are the most widely used microorganisms in the probiotic food industry[2,8]. However, it has been reported that *Lactobacillus* are more resistant than *Bifidobacterium* because they are more resistant to low pH values and can adapt more easily to environments such as milk[9].

The addition of probiotic cultures to ice cream gives the product functional properties. In addition, the use of prebiotic sources creates a symbiotic effect by increasing the viability of probiotic microorganisms[10]. Ice cream is a good food for probiotic microorganisms because its nutrients such as milk protein, fat and lactose provide a suitable environment for probiotics[2,11]. Plants containing bioactive compounds such as phenolic compounds, chlorophyll and carotenoids increase the survival of probiotic microorganisms by reducing their oxidative stress[12]. Cinnamon is one of the most widely used spices with prebiotic properties and therapeutic applications and has strong antioxidant and anti-inflammatory activities[10]. Cocoa contains flavonoids and phenolic acids, which have high antioxidant activity. Furthermore, cocoa has prebiotic activity and can support the growth of beneficial bacteria[13]. Walnuts contain many nutrients such as unsaturated fatty acids, fiber, minerals, vitamins, phytosterols and polyphenols[14]. Persimmon is a rich source of vitamins, minerals and dietary fiber and may enhance the health benefits of probiotic ice cream[15,16].

The aim of the study was to produce ice creams containing *Lactobacillus acidophilus* and *Bifidobacterium animalis* subps. *lactis* BB12 (*Bifidobacterium* BB-12) with different concentrations of ingredients such as cocoa, walnuts, persimmon and cinnamon and to investigate their organoleptic properties and the effect of added ingredients on the viability of probiotics and physicochemical properties of ice cream.

METHODOLOGY

Materials

Ice cream was produced with UHT milk obtained from grocery stores (Pınar, Izmir, Turkey). *Lactobacillus acidophilus* (ATCC 4356, Kwik-Stik) and *Bifidobacterium* Bb-12 (Chr. Hansen, Hoersholm Denmark) were used as probiotics. White sugar (Bor Şeker, Niğde, Turkey), salep (Salep Evi, Samsun, Turkey), cream (İçim, Sakarya, Turkey), skim milk powder (Bağdat, Ankara, Turkey), emulsifier (Danisco, Turkey), cocoa (Dr. Oetker, Izmir, Turkey), walnut (Tadım, Kocaeli, Turkey), cinnamon (Baghdad, Ankara, Turkey) and persimmon from the market were used in the production of ice cream.

Preparation of probiotic ice cream

A total of 17 different ice creams were prepared, including one control (no ingredient). These ice creams were prepared in 4 different flavors; cocoa, walnut, cinnamon and persimmon (2 different proportions of each flavor). In addition, 2 different probiotic microorganisms, *L. acidophilus* and *Bifidobacterium* BB-12, were used.

Ice creams were produced in Istanbul Esenyurt University laboratory using WMF brand D-89343 model ice cream machine (China). The ingredients used in ice cream production and their usage percentages are given in Table 1.

Table 1. Ingredients used in functional ice cream production and their usage percentages

Components	K*	A1*	A5*	C10*	C20*	H10*	H30*	T4*	T8*
Milk	74.8%	74.1%	71.1%	67.3%	59.8%	67.3%	52.4%	74.5%	74.2%
Skim milk powder	2.2%	2.2%	2.1%	2%	1.8%	2%	1.6%	2.2%	2.2%
Emulsifier	0.5%	0.5%	0.5%	%0.4	0.4%	0.4%	0.3%	0.5%	0.5%
Cream	5.2%	5.2%	4.9%	4.7%	4.1%	4.7%	3.7%	5.2%	5.2%
Salep	0.8%	0.7%	0.7%	0.7%	0.6%	0.7%	0.5%	0.8%	0.7%
Sugar	16.5%	16.3%	15.6%	14.8%	13.2%	14.8%	11.5%	16.4%	16.3%
Cocoa		1%	5%						
Walnut				10%	20%				
Cinnamon								0.4%	0.8%
Persimmon						10%	30%		

* K: Plain ice cream, control; A1: ice cream with 1% cocoa; A5: ice cream with 5% cocoa; C10: ice cream with 10% walnuts; C20: ice cream with 20% walnuts; H10: ice cream with 10% persimmon; H30: ice cream with 30% persimmon; T4: ice cream with 0.4% cinnamon; T8: ice cream with 0.8% cinnamon

Probiotic strains were incubated in MRS Broth at 37°C for 24 hours to obtain the desired probiotic density[17]. The density was determined using McFarland standards.

Probiotic cultures (*L. acidophilus* and *Bifidobacterium* BB-12) were inoculated at 107 level into the ice cream mixture kept in the refrigerator overnight. Then, probiotic ice cream samples were produced by mixing in an ice cream machine for 20 minutes.

Microbiological analysis

Ice creams were inoculated with *L. acidophilus* and *Bifidobacterium* BB-12 and stored at -18°C. Live probiotic bacteria counts were determined on days 0, 15, 30, 45 and 60 during storage. Maximum Recovery Diluent (Neogen VCM0085A) was used for serial dilutions of ice creams. The viability of *L. acidophilus* and *Bifidobacterium* BB-12 during storage was determined by incubation for 72 hours at 37°C under anaerobic conditions on MRS Agar supplemented with 0.05% Clindamycin (Merck 1.10660) and MRS Agar supplemented with 0.05% L-cysteine, respectively. Colony numbers were calculated by converting to log CFU g^{-1} [18,19].

Physicochemical analysis

Ice cream samples were thawed at room temperature and pH values were determined by pH meter (HANNA instruments, HI 221). Titratable acidity, total solid, fat (Gerber method) and ash values were determined according to AOAC 947.05, TS ISO 3728, AOAC 952.06, AOAC 930.30, respectively. All measurements were performed on day 0 and 60 days of storage with 3 repetitions[20-23].

Sensory analysis

All ice cream samples were sensory evaluated for taste, texture, color and overall acceptability by 20 panelists (17 female, 3 male) trained in sensory evaluation. A hedonic scale ranging from 1 to 5 was used in the evaluation process (1 being very bad and 5 being very good). All samples were coded with 3-digit numbers. In addition, during the evaluation, panelists were asked to clean their mouths with water after each tasting.

Statistical analysis

IBM SPSS 25.0 program was used for all statistical analyses. Significant differences between samples were determined using One-Way ANOVA test and Independent t-test. A $p<0.05$ level was used to define significant differences. Graphic plots were made in GrapPad Prism 9.1.1 program (mean ± standard deviation).

RESULTS and DISCUSSION

Survivability of probiotics during storage time

To study the effect of different concentrations of certain ingredients on the viability of two different probiotics in ice cream, the samples were stored at -18°C for 60 days. Table 2 shows the changes in the number of probiotic bacteria during storage of probiotic ice creams containing different concentrations of ingredients. During 60 days of storage, the number of probiotic bacteria decreased in ice creams except C10L and C20L ($p< 0.01$), while the change in the number of probiotics in C10L and C20L samples was insignificant ($p>0.01$). On the 45th day of storage, the decrease in probiotic microorganisms in A1L, C10L, C20L, H10L, A1B, A5B, C10B, C20B samples was statistically insignificant. The decrease in probiotic bacteria populations during storage at -18°C may be thought to be due to the limited ability of microorganisms to adapt to low temperatures[24].

Table 2. Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* subps. lactis BB-12 (log CFU g-1) in ice cream samples over a 60-day storage period

Days	KL	A1L	A5L	C10L	C20L	H10L	H30L	T4L	T8L
0	7.38 ± 0.13^{a}	6.84 ± 0.20^{a}	6.98 ± 0.08^{a}	6.91 ± 0.49^{a}	6.08 ± 0.34^{a}	6.92 ± 0.28^{a}	6.78 ± 0.51^{a}	6.95 ± 0.62^{a}	7.10 ± 0.61^{a}
15	6.41 ± 0.09^{b}	6.71 ± 0.24^{a}	5.97 ±0 .89^{b}	7.02 ± 0.23^{a}	6.32 ± 0.43^{a}	6.58 ± 0.23^{a}	6.51 ± 0.37^{ab}	6.35 ± 0.30^{ab}	6.79 ± 0.26^{ab}
30	6.26 ± 0.10^{b}	6.46 ± 0.39^{a}	6.35 ± 0.18^{ab}	6.52 ± 0.47^{a}	6.17 ± 0.72^{a}	6.61 ± 0.41^{a}	6.63 ± 0.44^{a}	5.8 4± 0.84^{b}	6.72 ± 0.35^{ab}
45	6.13 ± 0.14^{b}	6.40 ± 0.16^{a}	6.34 ± 0.37^{ab}	6.58 ± 0.48^{a}	6.20 ± 0.22^{a}	6.72 ± 0.09^{a}	6.53 ± 0.47^{ab}	6.37 ± 0.34^{ab}	6.09 ± 0.27^{bc}
60	5.76 ± 0.39^{c}	5.30 ± 0.27^{b}	5.57 ± 0.36^{b}	6.50 ± 0.25^{a}	6.28 ± 0.19^{a}	5.50 ± 0.54^{b}	5.61 ± 0.61^{b}	6.27 ± 0.13^{ab}	5.86 ± 0.55^{c}
Days	**KB**	**A1B**	**A5B**	**C10B**	**C20B**	**H10B**	**H30B**	**T4B**	**T8B**
0	7.65 ± 0.22^{a}	7.17 ± 0.21^{a}	6.93 ± 0.20^{a}	7.13 ± 0.18^{a}	6.94 ± 0.12^{c}	7.43 ± 0.08^{ab}	7.67 ± 0.16^{a}	7.05 ± 0.08^{a}	7.13 ± 0.05^{a}
15	6.9 5± 0.11^{b}	6.95 ± 0.19^{a}	6.89 ± 0.08^{a}	7.17 ± 0.11^{a}	7.28 ± 0.06^{a}	7.27 ± 0.17^{ab}	6.99 ± 0.08^{c}	6.60 ± 0.49^{ab}	6.91 ± 0.07^{bc}
30	6.97 ± 0.29^{b}	6.96 ± 0.11^{a}	6.65 ± 0.31^{a}	6.98 ± 0.09^{a}	7.11 ± 0.03^{b}	7.17 ± 0.29^{b}	7.42 ± 0.25^{ab}	6.10 ± 0.07^{c}	6.94 ± 0.06^{b}
45	6.94 ± 0.23^{b}	6.90 ± 0.14^{a}	6.77 ± 0.10^{a}	7.04 ± 0.08^{a}	6.95 ± 0.04^{c}	7.49 ± 0.05^{a}	7.13 ± 0.14^{bc}	6.33 ± 0.25^{bc}	6.81 ± 0.06^{c}
60	5.22 ± 0.15^{c}	5.80 ± 0.07^{b}	5.96 ± 0.39^{b}	5.81 ± 0.20^{b}	5.52 ± 0.13^{d}	6.13 ± 0.08^{c}	5.77 ± 0.16^{d}	5.26 ± 0.10^{d}	5.77 ± 0.06^{d}

* Mean ± standard deviation. $^{a-d}$ -- Mean values shown with different letters in the columns show statistically significant difference ($p<0.01$). KL: control sample containing *L. acidophilus*, A1L: sample containing 1% cocoa and *L. acidophilus*, A5L: sample containing 5% cocoa and *L. acidophilus*, C10L: sample containing 10% walnuts and *L. acidophilus*, C20L: sample containing 20% walnuts and *L. acidophilus*, H10L: sample containing 10% persimmon and *L. acidophilus*, H30L: sample containing 30% persimmon and *L. acidophilus*, T4L: sample containing 0.4% cinnamon and *L. acidophilus*, T8L: sample containing 0.8% cinnamon and *L. acidophilus*. KB: Control sample containing *Bifidobacterium* BB-12, A1B: sample containing 1% cocoa and *Bifidobacterium* BB-12, A5B: sample containing 5% cocoa and *Bifidobacterium* BB-12, C10B: sample containing 10% walnuts and *Bifidobacterium* BB-12, C20B: 20% walnuts and *Bifidobacterium* BB-12, H10B: 10% persimmon and *Bifidobacterium* BB-12, H30B: 30% persimmon and *Bifidobacterium* BB-12, T4B: 0.4% cinnamon and *Bifidobacterium* BB-12, T8B: 0.8% cinnamon and *Bifidobacterium* BB-12.

Probiotic foods should contain probiotic microorganisms in numbers ranging from 106 to 109 CFU g^{-1} (6 to 9 log CFU g^{-1}) during shelf life[25]. At the end of 60th day, it was observed that C10L, C20L, T4L and H10B samples had probiotic food characteristics. In other words, 10% and 20% walnuts and 0.4% cinnamon in ice creams containing *L. acidophilus* and 10% persimmon in ice creams containing *Bifidobacterium* BB-12 are thought to be effective on probiotic viability. However, it was observed that the number of probiotic bacteria was above 6 log CFU g^{-1} in all samples during 30 and 45 days of storage.

L. acidophilus was inoculated at an average level of 6.88 and *Bifidobacterium* BB-12 at an average level of 7.23 in the ice cream samples produced. When the logarithmic decreases in the number of probiotic microorganisms were compared; the number of probiotic bacteria survived at a higher level compared to the control samples except for the samples containing 0.4% cinnamon (T4L and T4B). This finding suggests that the applied concentrations of all ingredients except 0.4% cinnamon may be due to the prebiotic effect up to 30 and 45 days.

When the logarithmic decrease of the control samples without any ingredient (CL and CB) at the end of 60 days was analyzed, it was observed that the logarithmic decrease of the ice cream containing *L. acidophilus* was lower. This finding supports the hypothesis that *Lactobacillus* species are more resistant than *Bifidobacterium* species[9]. Although there was a significant decrease in the number of probiotics on the 15th and 45th days of the control samples ($p<0.01$), the number of viable probiotic bacteria was above 6 log CFU g^{-1} at the end of the 45th day. Differently, in a study by Turgut and Çakmakçı (2009) investigating the possibility of using some probiotic bacteria species in ice cream production, no significant difference was observed in the numbers of *L. acidophilus* and *B. bifidum* in ice cream samples after 15 and 45 days of storage at -20°C[26].

All ingredients were applied at two different concentrations. When the effect of increasing concentrations on probiotic viability was evaluated, it was observed that only increasing the walnut concentration increased the number of probiotics ($p<0.01$).

When probiotic viability was evaluated in ice cream samples with 1% and 5% cocoa at the end of 60 days of storage, a significant decrease in the number of probiotics was observed ($p<0.01$). In a study by Laličić-Petronijević et al. on probiotic viability in milk and dark chocolate, it was reported that the survival rate of *L. acidophilus* in dark chocolate containing 75% cocoa part was very good at 4 and 25°C for 180 days; *B. lactis* decreased faster[27]. The difference in the viability of *L. acidophilus* with increasing cocoa portion is thought to be due to different storage temperatures.

The change in *L. acidophilus* count with increasing walnut concentration was insignificant ($p>0.01$), while *Bifidobacterium* BB-12 count increased until day 30 ($p<0.01$). At the end of 60 days, the number of *L. acidophilus* was at the desired level for probiotic food (10^6 to 10^8 CFU g^{-1}), while *Bifidobacterium* BB-12 remained below this level. In the study of Salik and Aslaner on probiotic ice cream containing saruc, the decrease in the number of probiotic bacteria on days 15 and 30 as the saruc concentration increased was statistically insignificant. However, similarly, the probiotic microorganism maintained its viability throughout 60 days of storage and remained at the level of 106 to 108 CFU g^{-1} [28].

Although the antibacterial effect of cinnamon against food pathogens has been reported in various studies[29,30], when the effect of increasing cinnamon concentration on the viability of two different probiotic bacteria was investigated in this study, it was found that increasing cinnamon concentration provided a higher survival rate of both probiotic bacteria on day 30. Similarly, Gunes-Bayir et al. found that cinnamon increased the number of probiotic bacteria in yogurts produced by adding the same amount of propolis and increasing concentrations (0.3%, 1%, 2.5%) of cinnamon[31].

During the 45-day storage period, the logarithmic decrease in the ice cream samples with persimmon was very low compared to the control samples, so it is seen that persimmon also shows prebiotic effect. There is no study on the effect of persimmon on probiotic viability.

Since most probiotic bacteria thrive in anaerobic environment, the fact that ice cream is not stored in anaerobic environment may explain the decrease in the number of probiotic microorganisms. Furthermore, the decrease in the probiotic population during the freezing process may be due to damage to bacterial cells due to thermal shock caused by the freezing process[32,33].

Physicochemical properties of probiotic ice cream products

The evaluation of the physicochemical properties of probiotic ice cream samples is given in Table 3. On the 60th day of storage, the ash values of probiotic ice creams ranged between 1.08% and 1.28%, fat values ranged between 5.91% and 6.31%, and dry matter values ranged between 32.70% and 44.76%. The changes in dry matter, ash, acidity and fat values of all ice cream samples during the 60-day storage period were insignificant ($p>0.05$). Also, increasing ingredient concentrations did not affect ash, fat and dry matter values.

Table 3. Physico-chemical properties of ice cream products produced with *L. acidophilus* and *Bifidobacterium* BB-12 at 0 and 60 days of storage

L. acidophilus (log CFU g^{-1})										
	Days	**KL**	**A1L**	**A5L**	**C10L**	**C20L**	**H10L**	**H30L**	**T4L**	**T8L**
pH	0	6.1 9± 0.06^{b}	6.44 ± 0.13^{b}	6.38 ± 0.07^{b}	6.33 ± 0.13^{b}	6.79 ± 0.03^{a}	6.40 ± 0.13^{b}	6.41 ± 0.17^{b}	6.25 ± 0.14^{a}	6.25 ± 0.18^{b}
	60	6.89 ± 0.10^{a}	6.93 ± 0.03^{a}	6.97 ± 0.05^{a}	6.54 ± 0.04^{a}	6.73 ± 0.02^{b}	6.92 ± 0.03^{a}	6.91 ± 0.02^{a}	6.24 ± 0.02^{b}	6.87 ± 0.03^{a}
Titration	0	0.38 ± 0.02^{a}	0.29 ± 0.02^{a}	0.22 ± 0.02^{a}	0.33 ± 0.03^{a}	0.18 ± 0.02^{a}	0.25 ± 0.03^{a}	0.26 ± 0.03^{a}	0.26 ± 0.02^{b}	0.27 ± 0.01^{a}
	60	0.14 ± 0.02^{b}	0.12 ± 0.01^{b}	0.10 ± 0.05^{b}	0.22 ± 0.01^{b}	0.18 ± 0.01^{a}	0.11 ± 0.01^{b}	0.12 ± 0.01^{b}	0.33 ± 0.02^{a}	0.13 ± 0.01^{b}
Dry matter	0	36.92 ± 0.07^{a}	37.04 ± 0.08^{a}	38.10 ± 0.94^{a}	37.90 ± 0.13^{a}	39.12 ± 0.53^{a}	38.73 ± 0.18^{a}	40.50 ± 0.86^{a}	36.96 ± 0.25^{a}	37.01 ± 0.56^{a}
	60	37.63 ± 0.98^{a}	37.15 ± 0.18^{a}	39.37 ± 0.46^{a}	37.87 ± 0.54^{a}	39.29 ± 1.06^{a}	37.99 ± 1.99^{a}	41.25 ± 0.37^{a}	36.99 ± 0.62^{a}	38.01 ± 0.53^{a}
Ash	0	1.19 ± 0.04^{a}	1.24 ± 0.06^{a}	1.22 ± 0.04^{a}	1.18 ± 0.04^{a}	1.25 ± 0.04^{a}	1.13 ± 0.13^{a}	1.26 ± 0.04^{a}	1.22 ± 0.07^{a}	1.27 ± 0.03^{a}
	60	1.16 ± 0.06^{a}	1.21 ± 0.08^{a}	1.25 ± 0.03^{a}	1.23 ± 0.06^{a}	1.24 ± 0.04^{a}	1.13 ± 0.08^{a}	1.21 ± 0.04^{a}	1.19 ± 0.06^{a}	1.28 ± 0.03^{a}
Fat	0	6.10 ± 0.16^{a}	6.25 ± 0.17^{a}	6.16 ± 0.22^{a}	6.33 ± 0.07^{a}	6.32 ± 0.19^{a}	6.17 ± 0.34^{a}	6.19 ± 0.45^{a}	6.21 ± 0.35^{a}	5.99 ± 0.58^{a}
	60	6.11 ± 0.43^{a}	6.14 ± 0.15^{a}	6.19 ± 0.23^{a}	6.23 ± 0.26^{a}	6.31 ± 0.29^{a}	6.26 ± 0.19^{a}	6.24 ± 0.23^{a}	6.17 ± 0.17^{a}	6.11 ± 0.16^{a}
Bifidobacterium animalis subps. lactis BB-12 (log CFU g^{-1})										
	Days	**KB**	**A1B**	**A5B**	**C10B**	**C20B**	**H10B**	**H30B**	**T4B**	**T8B**
pH	0	6.12 ± 0.06^{b}	6.27 ± 0.07^{b}	6.40 ± 0.13^{b}	6.29 ± 0.08^{b}	6.41 ± 0.11^{b}	6.19 ± 0.01^{b}	5.97 ± 0.05^{b}	6.32 ± 0.06^{b}	6.27 ± 0.02^{b}
	60	6.95 ± 0.03^{a}	6.87 ± 0.02^{a}	6.81 ± 0.02^{a}	6.85 ± 0.05^{a}	6.92 ± 0.02^{a}	6.79 ± 0.01^{a}	6.87 ± 0.03^{a}	7.97 ± 1.74^{a}	6.89 ± 0.02^{a}
Titration	0	0.38 ± 0.02^{a}	0.28 ± 0.01^{a}	0.25 ± 0.01^{a}	0.28 ± 0.01^{a}	0.16 ± 0.02^{a}	0.35 ± 0.03^{a}	0.40 ± 0.01^{a}	0.32 ± 0.04^{a}	0.35 ± 0.02^{a}
	60	0.13 ± 0.02^{b}	0.13 ± 0.02^{b}	0.18 ± 0.01^{b}	0.15 ± 0.01^{b}	0.10 ± 0.01^{b}	0.17 ± 0.02^{b}	0.13 ± 0.01^{b}	0.06 ± 0.02^{b}	0.14 ± 0.02^{b}
Dry matter	0	32.05 ± 0.10^{a}	32.31 ± 0.37^{a}	34.22 ± 0.76^{a}	38.51 ± 0.59^{a}	42.98 ± 0.83^{a}	34.93 ± 0.18^{a}	39.66 ± 0.57^{a}	34.28 ± 1.11^{a}	34.53 ± 0.93^{a}
	60	32.70 ± 0.25^{a}	33.24 ± 0.43^{a}	34.73 ± 0.57^{a}	39.13 ± 1.41^{a}	44.76 ± 2.57^{a}	34.47 ± 1.62^{a}	38.65 ± 0.85^{a}	36.66 ± 3.55^{a}	36.71 ± 5.09^{a}
Ash	0	1.24 ± 0.09^{a}	1.22 ± 0.05^{a}	1.25 ± 0.08^{a}	1.24 ± 0.12^{a}	1.26 ± 0.09^{a}	1.08 ± 0.08^{a}	1.19 ± 0.06^{a}	1.21 ± 0.11^{a}	1.22 ± 0.07^{a}
	60	1.21 ± 0.08^{a}	1.19 ± 0.16^{a}	1.22 ± 0.03^{a}	1.19 ± 0.09^{a}	1.23 ± 0.13^{a}	1.13 ± 0.09^{a}	1.21 ± 0.04^{a}	1.19 ± 0.06^{a}	1.22 ± 0.06^{a}
Fat	0	5.85 ± 0.43^{a}	6.11 ± 0.76^{a}	5.96 ± 0.55^{a}	6.28 ± 0.27^{a}	6.23 ± 0.47^{a}	6.20 ± 0.20^{a}	6.24 ± 0.08^{a}	6.22 ± 0.19^{a}	6.00 ± 0.19^{a}
	60	6.07 ± 0.22^{a}	5.91 ± 0.35^{a}	6.11 ± 0.27^{a}	6.30 ± 0.17^{a}	6.20 ± 0.18^{a}	6.10 ± 0.36^{a}	6.27 ± 0.11^{a}	6.21 ± 0.26^{a}	6.25 ± 0.11^{a}

* Mean ± standard deviation. $^{a-b}$ -- Mean values shown with different letters in the columns show statistically significant difference ($p<0.05$). KL: control sample containing *L. acidophilus*, A1L: sample containing 1% cocoa and *L. acidophilus*, A5L: sample containing 5% cocoa and *L. acidophilus*, C10L: sample containing 10% walnuts and *L. acidophilus*, C20L: sample containing 20% walnuts and *L. acidophilus*, H10L: sample containing 10% persimmon and *L. acidophilus*, H30L: sample containing 30% persimmon and *L. acidophilus*, T4L: sample containing 0.4% cinnamon and *L. acidophilus*, T8L: sample containing 0.8% cinnamon and *L. acidophilus*. KB: Control sample containing *Bifidobacterium* BB-12, A1B: sample containing 1% cocoa and *Bifidobacterium* BB-12, A5B: sample containing 5% cocoa and *Bifidobacterium* BB-12, C10B: sample containing 10% walnuts and *Bifidobacterium* BB-12, C20B: 20% walnuts and *Bifidobacterium* BB-12, H10B: 10% persimmon and *Bifidobacterium* BB-12, H30B: 30% persimmon and *Bifidobacterium* BB-12, T4B: 0.4% cinnamon and *Bifidobacterium* BB-12, T8B: 0.8% cinnamon and *Bifidobacterium* BB-12.

The reason for the lack of a significant difference in ash values may be the low ingredient concentrations. The studies of Şentürk et al. (2023) and Haghani et al. (2021) support these results[2,34]. Differently, Kotan (2018) stated that the ash content in ice cream decreased with increasing blueberry concentration[35].

The highest fat content was found in walnut ice cream (6.20% to 6.33%). Regarding the change in fat values, different results were obtained in some studies. Karaman et al. (2014) and Haghani et al. (2021) reported that fat content decreased as the concentration increased in ice creams with CCP[2,36].

Dry matter results were similar to the study of Haghani et al., and it was reported that dry matter content increased with increasing CCP levels[2]. Akalın et al. (2017) reported that the addition of 5 different dietary fibers (apple, orange, oat, bamboo and wheat) to ice cream increased the total solids content[37]. The increase in the dry matter content of ice cream samples can be attributed to moisture loss during storage[38]. However, it has been reported that ice creams with lower dry matter have more water content and therefore form more ice crystals during freezing, which affects the ice cream texture[39]. In the study by Şentürk et al. differently, the dry matter content decreased with the increase in the amount of blueberry and jujube fruit. This decrease was explained by the high moisture content of the added fruits[34].

The addition of cocoa, walnuts, persimmon and cinnamon to ice cream generally increased pH, but this increase was statistically significant ($p<0.05$) in ice cream samples containing 20% walnuts and 0.4% cinnamon. Titratable acidity decreased, this decrease was statistically significant in ice cream samples containing 0.4% cinnamon and *L. acidophilus* ($p<0.05$). In the study by Şentürk et al., there were no regular changes in pH values with the addition of blueberry and jujube fruit purees to ice cream; titratable acidity decreased insignificantly compared to the control sample[34]. In the study by Öztürk et al., the pH value decreased insignificantly, while the titratable acidity increased insignificantly in probiotic ice creams enriched with white and blue myrtle fruits[40].

Changes in pH and acidity were irregular with increasing ingredient concentration. The pH increased and titratable acidity decreased with increasing walnut and cinnamon concentrations. This change was statistically significant in ice creams containing *L. acidophilus* ($p<0.05$), but insignificant in ice creams containing *Bifidobacterium* BB-12 ($p>0.05$). The change in pH and acidity due to the increase in concentration of other ingredients was not significant ($p>0.05$). It is thought that fatty acid and acidic phenolic compounds contained in walnut and cinnamon with high antioxidant content affect pH and acidity[41,42].

Sensory analyses of probiotic ice cream products

The results of the sensory evaluation of the ice cream samples in terms of taste, structure/texture, color and general palatability are given in Figure 1 and Figure 2. As a result of the metabolic activities of probiotic microorganisms, components that may adversely affect the taste and aroma of the product may be formed. For example, acetic acid is produced during fermentation and storage of *Bifidobacterium* spp.[32].

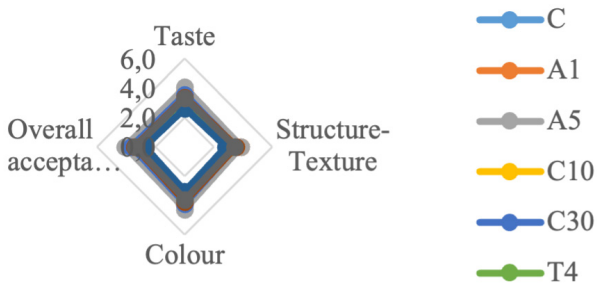


Figure 1. *L. acidophilus* sensory evaluation results

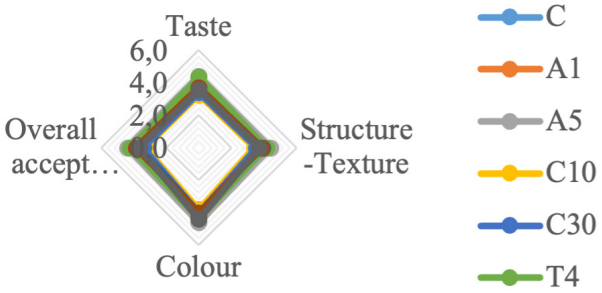


Figure 2. *Bifidobacterium* BB-12 sensory evaluation results

The lowest taste, structure/texture, color and overall liking scores belonged to sample T8L with scores of 2.6, 2.8, 3.0, 2.8, respectively. The highest taste, structure/texture, color and overall liking scores belonged to sample A5B with scores of 4.4, 4.4, 4.4, 4.6, 4.4, respectively.

Adding various concentrations of ingredients to ice cream affected the overall acceptability either positively or negatively. As the concentration of dates increased, the overall acceptability score decreased. The most preferred ice cream was the one containing 5% cocoa.

In conclusion; it is very important to use the appropriate probiotic microorganism at the appropriate dose in probiotic food production[43,44]. *Bifidobacterium* and *Lactobacillus* are commonly used probiotic microorganisms in foods and

Lactobacilli have been reported to be more resistant[2,9]. In this study, *Lactobacillus acidophilus* and *Bifidobacterium* BB-12 were used separately and the effect of the added ingredients was examined. At the end of 60 days, it was observed that there was a lower logarithmic decrease in CL compared to the control samples and the decrease in the walnut ice cream containing *L. acidophilus* was insignificant.

The viability of probiotic microorganisms in food during production and storage is very important in terms of the number of microorganisms that can show the probiotic effect during consumption[7]. The data obtained from the study showed that different concentrations of cocoa, walnuts, persimmon and cinnamon added to ice cream had a positive effect on probiotic viability until the end of the 45th day of storage. In other words, according to this study, the required level of probiotic viability in ice cream continued for 45 days.

Walnut showed the strongest prebiotic effect on day 45. Because both the number of probiotic microorganisms increased with the increase in walnut concentration and the highest number of probiotics was seen in ice cream with 20% walnut on the 45th day.

Sensory wise, the most liked ice cream was the ice cream with 5% cocoa and the least liked ice cream was the ice cream with 0.8% cinnamon.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Planing and Design: C.H., B.Ç.S., M.A.; Materials and Methods: B.Ç.S., M.A.; Laboratory Studies and Literature Review: B.Ç.S., M.A., B.E., A.G.Ç.; Article Writing, Review and Editing: B.Ç.S., M.A., B.E., A.G.Ç. and C.H.

FUNDING SOURCES

This study was supported by Istanbul Esenyurt University as Infrastructure Project No. ALT-2021/01-A.

ACKNOWLEDGMENTS

The authors thank to Istanbul Esenyurt University for the research support.

REFERENCES

1. Göktaş H, Dikmen H, Bekiroğlu H, Cebi N, Dertli E, Sağdıç O. Characteristics of functional ice cream produced with probiotic *Saccharomyces boulardii* in combination with *Lactobacillus rhamnosus* GG. LWT, 2022;153:1-8. Doi: 10.1016/j.lwt.2021.112489

2. Haghani S, Hadidi M, Pouramin S, Adinepour F, Hasiri Z, Moreno A, et al. Application of cornelian cherry (*Cornus mas* L.) peel in probiotic ice cream: functionality and viability during storage. Antioxidants, 2021;10(11):1777. Doi: 10.3390/antiox10111777

3. Pehlivcanoğlu H, Sunal Z, Yaman M, Aksoy A. Investigation of the bioaccessibility of functional ice cream with blueberry enriched with whey protein gel. JOTAF, 2024;21(3):795-806. Doi: 10.33462/jotaf.1399931

4. Soukoulis C, Fisk ID, Bohn T. Ice cream as a vehicle for incorporating health-promoting ingredients: conceptualization and overview of quality and storage stability. CRFSFS, 2014;13(4):627-655. Doi: 10.1111/1541-4337.12083

5. Food and Agriculture Organization of the United Nations, World Health Organization. Guidelines for the evaluation of probiotics in food: report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food [Internet]. London, Ontario, Canada: 2002 [Jul 12, 2024]. Available from: https://www.mhlw.go.jp/file/05-Shingikai-11121000-Iyakushokuhinkyoku-Soumuka/0000197343.pdf

6. Latif A, Shehzad A, Niazi S, Zahid A, Ashraf W, Iqbal MW, et al. Probiotics: mechanism of action, health benefits and their application in food industries. Front microbiol, 2023;14:1216674. Doi: 10.3389/fmicb.2023.1216674

7. El-Sohaimy SA, Hussain MA. Functional probiotic foods development: trends, concepts, and products. Fermentation, 2023;9(3):249. Doi: 10.3390/fermentation9030249

8. Kahraman B, Şenol BM, Dertli E, Arıcı M. A comparative study of Bacillus spp. isolated from various sources and commercial food supplements and evaluation of some probiotic properties. JOTAF, 2024;21(1):265-276. Doi: 10.33462/jotaf.1329533

9. Ross RP, Desmond C, Fitzgerald GF, Stanton C. Overcoming the technological hurdles in the development of probiotic foods. J Appl Microbiol, 2005;98(6):1410-1417. Doi: 10.1111/j.1365-2672.2005.02654.x

10. Sarmadi B, Musazadeh V, Dehghan P, Karimi E. The effect of cinnamon consumption on lipid profile, oxidative stress, and inflammation biomarkers in adults: an umbrella meta-analysis of randomized controlled trials. NMCD, 2023;33(10):1821-1835. Doi: 10.1016/j.numecd.2023.03.010

11. Hwang JY, Shyu YS, Hsu CK. Grape wine lees improves the rheological and adds antioxidant properties to ice cream. LWT, 2009;42(1):312-318. Doi: 10.1016/j.lwt.2008.03.008

12. Holkem AT, Silva MPD, Favaro-Trindade CS. Probiotics and plant extracts: a promising synergy and delivery systems. Crit Rev Food Sci Nutr, 2023;63(28):9561-9579. Doi: 10.1080/10408398.2022.2066623

13. Martin MÁ, Ramos S. Impact of cocoa flavanols on human health. FCT, 2021;151:112121. Doi: 10.1016/j.fct.2021.112121

14. Binici Hİ, Şat İG, Aoudeh E. Nutritional composition and health benefits of walnut and its products. Atatürk Univ J Agric Fac, 2021;52(2):224-230. Doi: 10.17097/ataunizfd.843028

15. Kaur N, Kumari A, Agarwal A, Sabharwal M, Dipti S. Utilisation of *Diospyros kaki* L. (persimmon) as a functional ingredient to produce functional foods: a review. Nutr Food Sci, 2022;52(7):1083-1099.

16. Zahe AA, Mohammed ZS, Awaad EA, Salem AA, Atwa ESH. Production of a healthy yoghurt drink fortified with persimmon fruits. Afr J Biol Sci, 2023;19(1):135-146. Doi: 10.21608/ajbs.2023.304837

17. Alamprese C, Foschino R, Rossi M, Pompei C, Corti S. Effects of *Lactobacillus rhamnosus* GG addition in ice cream. Int J Dairy Technol, 2005;58(4):200-206. Doi: 10.1111/j.1471-0307.2005.00214.x

18. Smanalieva J, Iskakova J, Fischer P. Investigation of the prebiotic potential of rice varieties for *Lactobacillus acidophilus* bacteria. Eur Food Res Technol, 2021;247:1815-1824. Doi: 10.1007/s00217-021-03754-6

19. Zhang L, Huang S, Ananingsih VK, Zhou W, Chen XD. A study on *Bifidobacterium lactis* Bb12 viability in bread during baking. J Food Eng, 2014;122:33-37. Doi: 10.1016/j.jfoodeng.2013.08.029

20. Turkish Standards Institution (TSE). TS ISO 3728: Ice cream — Determination of total solids amount — Reference method. Ankara: TSE; 2010.

21. Association of Official Analytical Chemists (AOAC). Official Method 947.05: Acidity of milk — Titrimetric method. Rockville (MD): AOAC International; 2015.

22. Association of Official Analytical Chemists (AOAC). Official Method 952.06: Fat in ice cream and frozen desserts. Rockville (MD): AOAC International; 2015.

23. Association of Official Analytical Chemists (AOAC). Official Method 930.30: Ash of dried milk. Rockville (MD): AOAC International; 2015.

24. Senanayake SA, Fernando S, Bamunuarachchi A, Arsekularatne M. Application of *Lactobacillus acidophilus* (LA 5) strain in fruit-based ice cream. Food Sci Nutr, 2013;1(6):428-431. Doi: 10.1002/fsn3.66

25. Casarotti SN, Penna ALB. Acidification profile, probiotic in vitro gastrointestinal tolerance and viability in fermented milk with fruit flours. Int Dairy J, 2015;41:1-6. Doi: 10.1016/j.idairyj.2014.08.021

26. Turgut T, Cakmakci S. Investigation of the possible use of probiotics in ice cream manufacture. Int. J Dairy Technol, 2009;62(3):444-451. Doi: 10.1111/j.1471-0307.2009.00494.x

27. Laličić-Petronijević J, Popov-Raljić J, Obradović D, Radulović Z, Paunović D, Petrušić, M, et al. Viability of probiotic strains *Lactobacillus acidophilus* NCFM® and *Bifidobacterium lactis* HN019 and their impact on sensory and rheological properties of milk and dark chocolates during storage for 180 days. J Funct Foods, 2015;15:541-550. Doi: 10.1016/j.jff.2015.03.046

28. Salik MA, Arslaner A. The quality characteristics and shelf life of probiotic ice cream produced with Saruç and *Saccharomyces boulardii*. Int Food Res J, 2020;27(2):234-244.

29. Choi YJ, Jin HY, Yang HS, Lee SC, Huh CK. Quality and storage characteristics of yogurt containing *Lacobacillus sakei* ALI033 and cinnamon ethanol extract. JAST, 2016;58:16. Doi: 10.1186/s40781-016-0098-0

30. Nematollahi Z, Ebrahimi M, Raeisi M, Shahamat YD, Moghadam MG, Hashemi M, et al. The antibacterial activity of cinnamon essential oil against foodborne bacteria: a mini-review. JHEHP, 2020;6(3):101-105. Doi: 10.29252/jhehp.6.3.1

31. Güneş-Bayır A, Bilgin MG, Güçlü D, Pogda S, Dadak A. Preparation and evaluation of novel functional fermented dairy products containing propolis and cinnamon. JFST, 2022;59:2392-2401. Doi: 10.1007/s13197-021-05255-6

32. Mohammadi R, Mortazavian AM, Khosrokhavar R, da Cruz AG. Probiotic ice cream: viability of probiotic bacteria and sensory properties. Ann Microbiol, 2011;61:411-424. Doi: 10.1007/s13213-010-0188-z

33. de Souza EL, de Albuquerque TMR, Dos Santos AS, Massa NML, de Brito Alves JL. Potential interactions among phenolic compounds and probiotics for mutual boosting of their health-promoting properties and food functionalities–a review. Crit Rev Food Sci Nutr, 2019;59(10):1645-1659. Doi: 10.1080/10408398.2018.1425285

34. Şentürk G, Akın N, Konak Göktepe Ç, Denktaş B. The effects of blueberry (*Vaccinium corymbosum* L.) and jujube fruit (*Ziziphus jujube*) on physicochemical, functional, and sensorial properties, and probiotic (*Lactobacillus acidophilus* DSM 20079) viability of probiotic ice cream. Food Sci Nutr, 2024;12(4):2747-2759. Doi: 10.1002/fsn3.3955

35. Kotan TE. Mineral composition and some quality characteristics of ice creams manufactured with the addition of blueberry. GIDA, 2018;43(4):635-643. Doi: 10.15237/gida.GD18042

36. Karaman S, Toker ÖS, Yüksel F, Çam M, Kayacier A, Doğan M. Physicochemical, bioactive, and sensory properties of persimmon-based ice cream: technique for order preference by similarity to ideal solution to determine optimum concentration. JDS, 2014;97(1):97-110. Doi: 10.3168/jds.2013-7111

37. Akalın AS, Kesenkas H, Dinkci N, Unal G, Özer E, Kınık O. Enrichment of probiotic ice cream with different dietary fibers: structural characteristics and culture viability. JDS, 2018;101(1):37-46. Doi: 10.3168/jds.2017-13468

38. Singh A, Bajwa U, Goraya RK. Effect of storage period on the physicochemical, sensory and microbiological quality of bakery flavoured ice cream. IJERA, 2014;4(8):80-90.

39. Pintor A, Escalona-Buendía HB, Totosaus A. Effect of inulin on melting and textural properties of low-fat and sugar-reduced ice cream: optimization via a response surface methodology. Int Food Res J, 2017;24(4):1728-1734.

40. Öztürk Hİ, Demirci T, Akın N. Production of functional probiotic ice creams with white and dark blue fruits of *Myrtus communis*: the comparison of the prebiotic potentials on *Lactobacillus casei* 431 and functional characteristics. LWT, 2018;90:339-345. Doi: 10.1016/j.lwt.2017.12.049

41. Gülsoy E, Kaya E, Türkhan A. Bazı ceviz (*Juglans regia* L.) çeşitlerinin antioksidant aktiviteleri ve fenolik madde içeriklerinin belirlenmesi. Anadolu Tarım Bilimleri Dergisi, 2021;36(1):55-62. Doi: 10.7161/omuanajas.751354

42. Akbari V, Jamei R, Heidari R, Esfahlan AJ. Antiradical activity of different parts of Walnut (*Juglans regia* L.) fruit as a function of genotype. Food Chem, 2012;135(4):2404-2410. Doi: 10.1016/j.foodchem.2012.07.030

43. Talwalkar A, Kailasapathy K. The role of oxygen in the viability of probiotic bacteria with reference to *L. acidophilus* and *Bifidobacterium* spp. Curr Issues Intest Microbiol, 2004;5(1):1-8.

44. Ventura M, Perozzi G. Introduction to the special issue "probiotic bacteria and human gut microbiota". Genes Nutr, 2011;6(3):203-204. Doi: 10.1007/s12263-011-0241-y

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6342

Formulation of granules from *Pleurotus ostreatus* mushroom with potentialities for developing solid dosage forms

Daily ARIAS-RAMOS[1], Idelsy CHIL-NÚÑEZ[1], Humberto J. MORRIS-QUEVEDO[2*], Julio A. ROJAS-VARGAS[3], Yaixa BELTRÁN-DELGADO[2], Yamila LEBEQUE-PÉREZ[2], Paul COS[4]

1 Universidad de Oriente, Faculty of Natural and Exact Sciences, Pharmacy Department, Santiago de Cuba, Cuba
2 Universidad de Oriente, Center for Studies on Industrial Biotechnology (CEBI), Santiago de Cuba, Cuba
3 Universidad de Oriente, Faculty of Natural and Exact Sciences, Chemistry Department, Santiago de Cuba, Cuba
4 University of Antwerp, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Department of Pharmaceutical Sciences, Antwerp, Belgium

ABSTRACT

The study was aimed to evaluate the potential of *Pleurotus ostreatus* granules as a raw material for developing solid dosage forms. Drug-excipient compatibility was assayed using 1:1 binary mixtures and the ratio of formulation components was optimised with a D-optimal design considering flow properties as response variables. Three batches of granules were produced with the optimised mixture by the wet granulation method. The quality of the granules was evaluated based on physical, rheological, chemical, and microbiological parameters. The concentration of phenolic compounds in the binary mixtures remained unchanged at 30°C, but decreased at 45°C and 60°C. Moreover, a quadratic model was used to fit the response variables. Mixing design allowed selecting the best excipient ratio. The granules showed a residual moisture content and a particle size lesser than 5% and 350 μm, respectively, as well as, excellent flow and compressibility properties, and optimal microbiological quality.

* Corresponding author: Humberto J. MORRIS-QUEVEDO
E-mail: jquevedo@uo.edu.cu
ORCIDs:
Daily ARIAS-RAMOS: 0000-0003-0128-9558
Idelsy CHIL-NÚÑEZ: 0000-0003-4661-0472
Humberto J MORRIS-QUEVEDO: 0000-0002-3916-8594
Julio A ROJAS-VARGAS: 0000-0002-8877-1592
Yaixa BELTRÁN-DELGADO: 0000-0002-7149-5069
Yamila LEBEQUE-PÉREZ: 0000-0002-6680-6301
Paul COS: 0000-0003-4361-8911
(Received 28 Jul 2024, Accepted 10 Feb 2025)

Keywords: *Pleurotus ostreatus*, granules, phenolic compounds, flow properties, solid dosage forms

INTRODUCTION

Currently, there are numerous pharmaceutical and commercial products that utilise granules as a solid dosage form. Granules can serve as a precursor for other solid dosage forms, such as capsules and tablets [1]. They are composed of agglomerations of smaller particles with sufficient strength to allow their handling. Granules are primarily used when the Active Pharmaceutical Ingredient (API) is sensitive to moisture, has stability problems, or poor flow properties[2].

The powder derived from the fruiting body of the edible and medicinal mushroom *Pleurotus ostreatus* (oyster mushroom) is considered a nutraceutical preparation due to its abundance of bioactive compounds, including polysaccharides, proteins, amino acids, polyphenols, vitamins and fatty acids[3]. Pharmacologically, *P. ostreatus* has a wide range of activities, such as immunomodulatory[4], antioxidant[4], hypoglycaemic[5], anti-tumour[6] and antibacterial[7] properties.

In a previous publication, an evaluation of the technological, biochemical, and microbiological properties of *P. ostreatus* powder was performed to determine the quality parameters for its potential use as an API. The powder possessed good organoleptic properties, a rich nutraceutical composition, and adequate microbiological quality. However, rheological parameters indicated poor flowability, which negatively impacts in the development of solid dosage forms[8].

Preformulation studies are a critical stage in the drug development process. They involve the characterisation of physical, chemical, and mechanical properties to enable the design of dosage forms with greater stability, safety, and efficacy. Experimental designs and drug-excipient compatibility studies are useful tools to achieve a more stable formulation while reducing the investment of time, resources, and effort[9].

In view of the increasing prevalence of non-communicable diseases (NCDs), such as type II Diabetes Mellitus, the World Health Organization recommends exploring alternative therapies[10]. In this context, *P. ostreatus* extracts reduced the high blood glucose levels in hyperglycaemic rats[11], and in hyperglycaemic mice[5]. Moreover, ethanolic extracts of this mushroom exhibited an antihyperglycemic effect in high sucrose high fat diet streptozotocin induced diabetes in rats[12]. In this way, dietary phenolic compounds can be considered a potential

strategy in the development of pharmaceutical approaches that aim to reduce complications resulting from the progression of this metabolic pathology[13]. *Pleurotus florida* (=*P. ostreatus*) with a high concentration of total phenolics showed effective antioxidant and antidiabetic effects under *in vitro* conditions[14].

Moreover, most of the pharmaceutical formulations employed for animals closely resemble those utilised in human medication (including capsules, tablets, powders, and so on)[15]. Therefore, mushrooms granules can be considered in manoeuvring the innovative drug delivery systems for veterinary therapeutics.

This research was aimed to investigate the potential of *P. ostreatus* granules as a precursor for developing solid dosage forms with nutraceutical potential, including anti-diabetic effects in humans as well as veterinary applications. The study highlights research-development activities in the field of mushrooms natural products as an environmentally friendly, safe and viable alternative for third world countries. At least until we know, this is the first report of a comprehensive design and characterisation of mushroom granules with applications in food and pharmaceutical industries.

METHODOLOGY

Mushroom material

Pleurotus ostreatus CCEBI-3024 (Pleurotaceae) is a cultivated strain deposited in the Culture Collection of the Center for Studies on Industrial Biotechnology (CEBI, Universidad de Oriente, Cuba). Experts from the *Centro Oriental de Ecosistemas y Biodiversidad* (BIOECO, Santiago de Cuba, Cuba) confirmed the taxonomic identification. Slants with potato dextrose agar (PDA) solid medium, incubated at 37°C for 7 days, were used for strain conservation.

Excipients

Colloidal silicon dioxide (Aerosil 200, Evonik Resource Efficiency GmbH, Germany), microcrystalline cellulose (Avicel PH 101, JRS Pharma, Germany), magnesium stearate (Sudeep Pharma Pvt. Ltd, India), polyvinylpyrrilidone (K-25, O-BASF, Germany) and lactose monohydrate (Molkerei MEGGLE Wasserburg GmbH & Co. KG, Germany) were used as excipients. These Generally Accepted as Safe (GRAS) excipients were selected on the basis of their multifunctionality in the formulation of pharmaceuticals from natural sources[16]. All other chemicals and solutions used were of pharmaceutical grade.

Obtaining *Pleurotus ostreatus* powder

Powder preparation from the fruiting bodies of *P. ostreatus* mushroom was carried out as described by Arias-Ramos et al.[8]. Briefly, the fruiting bodies were harvested and cut into small pieces of approximately 1 cm^2. They were dried in an oven at 45°C for 24 h (VENTICELL, Spain). The dried material was ground in a blade mill (Retsch GM 200, Germany) to obtain a powder with a grain size <250 μm, and stored in plastic bags, protected from light and moisture, for further use.

Drug-excipients compatibility study

Binary mixtures of *P. ostreatus* powder with each of the excipients were prepared in a 1:1 ratio to assess compatibility. The substances were mixed in a mortar and pestle, and the resulting mixture was passed through a 350 μm mesh sieve (TSS-200, Utrecht, Germany) to homogenise the particle size. Then, 10 g of each mixture was placed in amber bottles with ground-glass stoppers. The mixtures were stored for 30 days at 30, 45 and 60°C in an oven (VENTICELL, Spain). Total phenolic compounds were quantified in the powder and in each mixture by the Folin-Ciocalteu method[17], after 0, 7, 15, 21 and 30 days of treatment.

D-optimal design

A D-optimal mixture design was used to determine the proportions of mixture components corresponding to the optimal rheological parameters of the granules (Design Expert 13.0 software, Stat-Ease, Inc., Minneapolis, MN, USA). The responses selected as indicative of the presence of drug-excipient interactions were Carr's index, Hausner's ratio, Flow rate and Angle of repose. The empirical models estimated to find the optimal formulation were plotted as contour plots.

A range of 0 to 46% of the independent variables (coded as 0 and 1) was used to optimise the composition of the excipient mixture. Five excipients, commonly used for preparing solid dosage forms from natural products were selected[18]. As previously mentioned, *P. ostreatus* powder was chosen based on its nutraceutical composition -both nutrients and mycochemicals, like phenolic compounds[3].

The granules were prepared for a total quantity of 100 g with a constant content of the active ingredient (50%), colloidal silicon dioxide (2%) and magnesium stearate (2%). Restrictions were applied to the remaining components in order to respect the actual amounts used in the pharmaceutical formulations

(Table 1). The evaluated responses were fitted to a quadratic model linking product properties with product composition (Equation 1):

$$Y = \beta 1xMCC + \beta 2xLM + \beta 3xPVP + \beta 12xMCCxLM + \beta 13xMCCxPVP + \beta 23xLMxPVP \quad (1)$$

where: Y represents the response; β1, β2 and β3 represent the effect relative to the concentrations of Microcrystalline Cellulose (MCC), Lactose Monohydrate (LM) and Polyvinylpyrrolidone (PVP) (coded values); β12, β13 and β23 represent the interaction effect between the three factors.

The best granules were selected taking into account the increase in flowability and compressibility, aspects related to the response variables evaluated.

Table 1. Experimental matrix of the D-Optimal Mixing Design used in the preparation of *P. ostreatus* granules (components levels are expressed in %)

Components	**Low levels (%)**	**High levels (%)**
A: Microcrystalline Cellulose (MCC)	0	1
B: Lactose Monohydrate (LM)	0	1
C: Polyvinylpyrrolidone (PVP)	0	1
Partial mixture	**46**	
Colloidal Silicon Dioxide (CSD)	2	
Magnesium Stearate (MS)	2	
Pleurotus ostreatus powder	50	
Partial mixture	**54**	
Total mixture	**100**	

Preparation of granules

The wet granulation method was used to produce the granules. All powders were first sieved through a 177 μm mesh (TSS-200, Utrecht, Germany). Then, *P. ostreatus* powder was mixed with CSD, followed by the addition of the remaining excipients in a horizontal laboratory mixer (Eureka AR-400, USA). PVP was used as a binder. The wet mass was dried in a vacuum oven (Sartorius, Germany) at 40°C for 12 h, and then, the dry mass was ground in a blade mill (Retsch GM 200, Germany) and sieved through a 350 μm mesh (TSS-200, Utrecht, Germany).

The best granules were selected considering fluidity and compressibility. Three batches of 200 g each were evaluated. The physical, technological, chemical and microbiological quality was assessed.

Quality evaluation of *Pleurotus ostreatus* granules

Moisture content

The moisture content was determined by the infrared gravimetric method using a thermogravimetric balance (model MB-110, MRC, Germany).

Scanning electron microscope - energy dispersive X-ray Spectrometer analysis

Surface morphology of the *P. ostreatus* granules and the chemical composition of the excipients were studied using a field emission Scanning Electron Microscope (SEM, FE, TESCAN Mira3, Brno, Czech Republic) coupled with Energy Dispersive X-ray Spectrometer (EDS, Oxford Instrument, INCAx-act, Abingdon, Oxfordshire, UK). The sample was coated with a 10 nm gold layer to ensure electronic conductivity. SEM images were taken with a view field of 1000, 200, 100 and 50 μm.

Particle size

The mean particle size of granules was studied using the vibration sieving method in a mechanical sieve (TSS-200, Utrecht, Germany)[8].

Rheological analysis

The following parameters were determined: Carr's index, Hausner's ratio, flow rate, and angle of repose[8,18].

Determination of total phenolic content and HPLC analysis of phenolic compounds

The determination of total phenolic content was carried out according to Beltrán et al.[19]. with slight modifications. Briefly, 1.5 mL of 10% Folin-Ciocalteu reagent was added to 1 mL of aqueous extracts of *P. ostreatus* granules and allowed to stand for 5 min at room temperature. Then, 2 mL of a saturated Na_2CO_3 solution was added. After one hour in the absence of light, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Genesis 10S, Thermo Fisher Scientific, Waltham, MA, USA). Tannic acid at concentrations of 6.25, 12.5, 25.0 and 50.0 μg/mL was used as standard (calibration curve $y=0.0076x - 0.4224$, $r^2=0.9917$). *The results were expressed as microgram tannic acid equivalents per mL of granules extract.*

Moreover, the HPLC analysis of phenolic compounds contained in mushroom granules was performed as a fingerprint and quality criteria. An aqueous extract of *P. ostreatus* granules was adjusted to a concentration of 5 mg/mL in 50% methanol and filtered through a 0.22 μm membrane filter before chromatographic

analysis (E-Merck, Darmstadt, Germany). The individual phenolic compounds were identified by using an Agilent 1200 series HPLC system with degasser, quaternary pump, automatic injection, thermostatic column compartment and a diode array detector (UV-DAD) (Agilent Technologies, Eindhoven, Netherlands). Detection was carried out at 280 nm as a preferred wavelength. Reverse-phase chromatographic analysis was carried out using a Phenomenex Luna C-18 column (250 × 4.6 mm i.d., particle size 5 μm, Phenomenex B.V., Utrecht, The Netherlands) at 26°C. Running conditions consisted of a gradient mixture of a solvent A (0.1% aqueous formic acid solution) and solvent B (acetonitrile), with a flow rate of 1 mL/min, and a run time of 65 min. The phenolic compounds were identified according to their UV spectra and by retention times (Rt), in comparison with authentic standards: gallic acid, pyrogallol, homogentisic acid, protocatechuic acid, chlorogenic acid, caffeic acid, vanillin, feluric acid, naringin, naringenin, hesperetin. Acetonitrile and formic acid were of HPLC grade and obtained from Fisher Chemical TM (Loughborough, UK), and the phenolic standard compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Microbiological analysis

The microbiological stability of the *P. ostreatus* granules was evaluated to assess their susceptibility to the presence of filamentous fungi, yeasts, *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacteriaceae and Candida albicans* in accordance to the microbiological quality acceptance criteria[20].

Statistical analysis

The software Design-Expert version 13.0 (Stat-Ease, Inc., Minneapolis, MN, USA), the Microsoft Excel included in the Microsoft Office package, and the software Statgraphics Centurion XV version 15.2.14 (Statgraphics Technologies, Inc., The Plains, VA, USA) were used for the mathematical processing and statistical analysis of data.

The statistical parameters used to evaluate and select the best fitting model were the coefficient of determination(R^2), adjusted coefficient of determination (adjusted R^2), coefficient of variation (CV), standard deviation, predicted residual sum of squares (PRESS), and the lack of fit and regression data (p value and F value). The positivity of the coefficient in the equation of the best-fitting model represents the positive contribution to the response, and vice versa. For a better explanation, a contour plot and a three-dimensional response surface were additionally generated for each response.

The physicochemical, technological and chemical parameters of granules were expressed as mean ± standard deviation of each batch, and the means were compared with ANOVA coupled with the Tukey's Least and Maximum Significant Difference test in order to identify significant differences. In the case of the mean particle size distribution, the normality of the results was assessed by the Kolmogorov-Smirnov test. A significance level of 95% was considered in the analysis.

RESULTS and DISCUSSION

Preformulation of *Pleurotus ostreatus* granules

The selection of granulation technique depends on the characteristics of the product and its manufacture requirements. In the context of our investigation, the direct method is not considered a viable option, because of the *P. ostreatus* powder exhibits inadequate flow properties. Although dry granulation has a reduced production cost compared to wet granulation, it is an unfavourable alternative as judged by the high friability of the product and the presence of fines. On the contrary, wet granulation facilitates closed processing, enhances drying efficiency, and improves rheological properties, rendering a cost-effective process for applications in the pharmaceutical industry[1].

An optimal pharmaceutical formulation is achieved through the appropriate selection of excipients. Drug-excipient compatibility studies help to choose excipients that will not interfere with the drug. Incompatibilities can arise from a covalent chemical reaction between the API and excipients, resulting in an intrinsic degradation of the API. Among the extrinsic factors, temperature is one of the most affecting compatibility[21].

Phenolic compounds, which have been extensively studied for their pharmacological properties, are found in the fruiting bodies of the mushroom *P. ostreatus*[22,23]. Therefore, total phenolic content was selected as a chemical marker of API in the formulations.

The compatibility results for the five drug-excipient mixtures regarding phenolic concentration at different temperatures are shown in Figure 1. Phenolic content at 30 ºC remained stable between 97.36% and 98.43% of the initial values during the 30-day test period. However, the concentration of phenolic compounds varied between 93 to 86% and 77 to 70% of the initial concentration at temperatures of 45ºC and 60ºC, respectively.

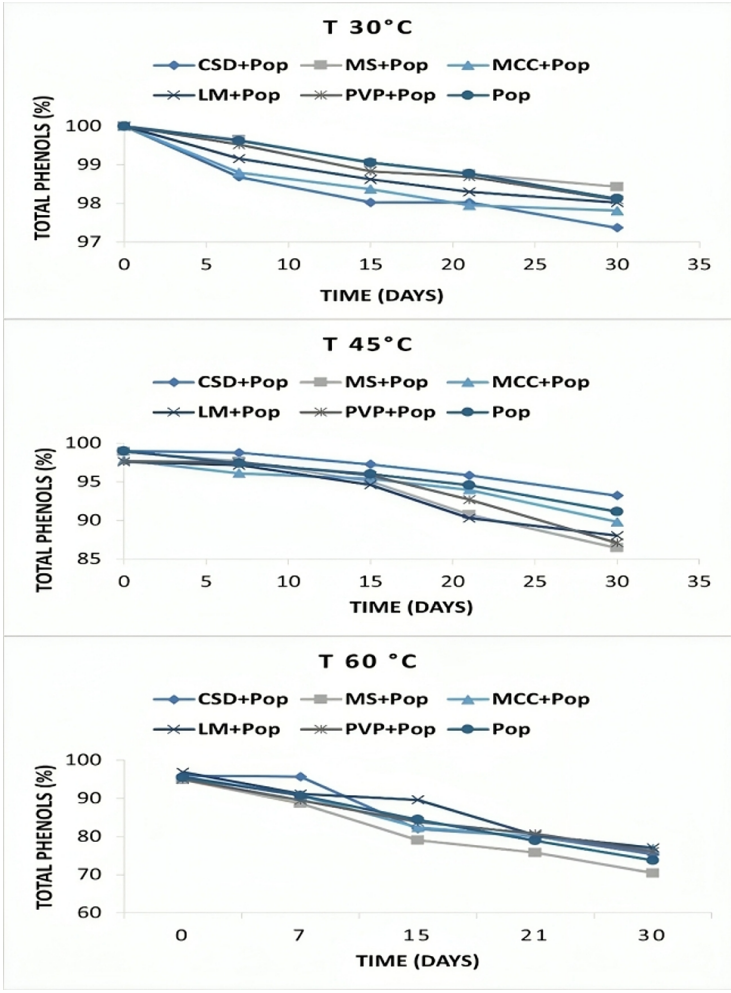


Figure 1. Concentration of total phenols at 30, 45 and 60 ºC for 30 days

Legend: *P. ostreatus* powder (Pop), colloidal silicon dioxide (CSD), magnesium stearate (MS), Microcrystalline Cellulose (MCC), Lactose Monohydrate (LM), Polyvinylpyrrolidone (PVP)

The decrease in the concentration of the bioactive compounds selected as chemical markers could be related to oxidation processes of phenolics. Though, it is important to note that the decrease in the concentrations was not significant when the mixtures were subjected to changing temperatures. Presumably, the decrease observed in phenol concentration may be due to oxidation reactions of phenolics rather than the presence of any other excipient, which should be confirmed using advanced analytical techniques such as HPLC or FTIR. However, the impact of excipients on the long-term stability of phenolic

compounds has been demonstrated. The carboxyl groups present in MCC and PVP allow the formation of esters, influenced by temperature and moisture. These esters can undergo hydrolysis, leading to a decrease in the concentration of phenolic compounds. This is supported by the fact that the process also occurred with powders treated individually at the same temperatures.

Rodriguez et al.[24], when evaluating the possible interactions between excipients and *Tamarindus indica* Soft Extract as the active ingredient, reported that the low differences in polyphenol content could be influenced by the sensitivity of the analytical method used in the determination.

Table 2 shows the results of the 15 runs according to the model describing the combination between different levels of the independent factors (MCC, LM and PVP). The experimental matrix showed the different combinations resulting from the D-optimal mixing design. The quality of each excipient combination was evaluated by considering the effect on rheological properties: Carr's index (CI), Hausner's ratio (HR), Flow rate (FR) and Angle of repose (AR).

Table 2. Experimental matrix of the mix design and results of the evaluation of the quality of the granules

Runs	Independent factors (%)			Response variables			
	MCC	LM	PVP	CI (%)	HR	FR (g cm^{-2} s^{-1})	AR (°)
1	1	0	1	20.26	1.25	6.16	34.68
2	0	1	1	10.25	1.1	8.77	24.98
3	1	0	1	17.96	1.2	6.25	34.94
4	1	1	0	17.86	1.22	6.99	32.98
5	0.83	0.58	0.58	17.35	1.21	6.46	34.03
6	0.5	0.5	1	10.12	1.1	8.77	25.3
7	0.5	1	0.5	20.89	1.26	6.93	31.46
8	0.33	0.83	0.83	12.82	1.14	8.36	25.47
9	1	1	0	17.8	1.22	7.03	33.02
10	0.58	0.83	0.58	20.9	1.26	6.65	31.12
11	0.83	0.83	0.33	20.08	1.25	6.3	33.86
12	1	0.5	0.5	20.76	1.25	6.86	33.94
13	0	1	1	10.27	1.11	8.76	25.16
14	0.83	0.33	0.83	20.02	1.25	6.84	33.11
15	0.5	0.5	1	15.42	1.19	7.96	28.42

In the statistical analysis, a quadratic model was suggested as the best model to analyse the flowability of the evaluated mixtures (Table 3) with p-values<0.05 in all the cases. Similarly, the lack of fit was negligible in all cases, thus indicating a low probability of error. The sum of squares and mean square ratio for the CI (10.71), HR (7.87), FR (19.81) and AR (26.12) showed values greater than 4.77 extracted from the Fisher-Snedecor law table at α=5% for (5.9) as degrees of freedom[25]. The coefficient of determination was 0.85, 0.81, 0.91 and 0.93 for CI, HR, FR and AR, respectively. The adjusted R-squared (R^2 -adj) for the evaluated responses were 0.97, 0.71, 0.87 and 0.89, respectively. Thus, there is evidence that all significant terms with values close to the R^2 values are part of the empirical models.

Table 3. Summary of Analysis of Variance (ANOVA) results for each response variable in the evaluation of granules flow properties in the optimisation process

	Carr's index (%)	**Hausner's ratio**	**Flow rate (g cm^{-2} s^{-1})**	**Angle of repose (°)**
Model	significant	significant	significant	significant
Coefficient of determination (R^2)	0.8561	0.8139	0.9167	0.9355
Adjusted coefficient of determination (adjusted R^2)	0.7762	0.7105	0.8704	0.8997
Coefficient of variation	11.48	2.68	4.81	3.95
Standard Deviation	1.93	0.03	0.35	1.22
Predicted residual sum of squares (PRESS)	83.73	0.02	3.44	35.53
Lack of fit	Not significant	Not significant	Not significant	Not significant
p-value	0.0014	0.0042	0.0001	<0.0001
F value	10.71	7.87	19.81	26.12

Figure 2a shows the influence of the evaluated excipients on the CI. The CI was favoured by the influence of PVP first, followed by LM. It was noticed that the higher the amount of these excipients, the lower the CI. This effect is largely due to the role of PVP as a binder in wet granulation process. In the case of LM, the fine particle size allows better mixing with other ingredients and helps to utilise the binder more efficiently[16]. The influence of these components and the interactions between them are also shown in the coded equation 2 for the CI.

The HR presented a similar pattern as the CI; this confirmed its relationship in predicting the flow properties of a powdery solid. The equation coded for the HR (3) showed a lower value for the influence of PVP and the interaction between LM and PVP in the evaluated granules as well as a negative coefficient for the interaction between MCC and LM. This result corroborated that LM and PVP were the excipients that most favoured the decrease in the HR, aided by MCC. The lowest values of the HR were obtained as the amount of PVP increased (Figure 2b)[18].

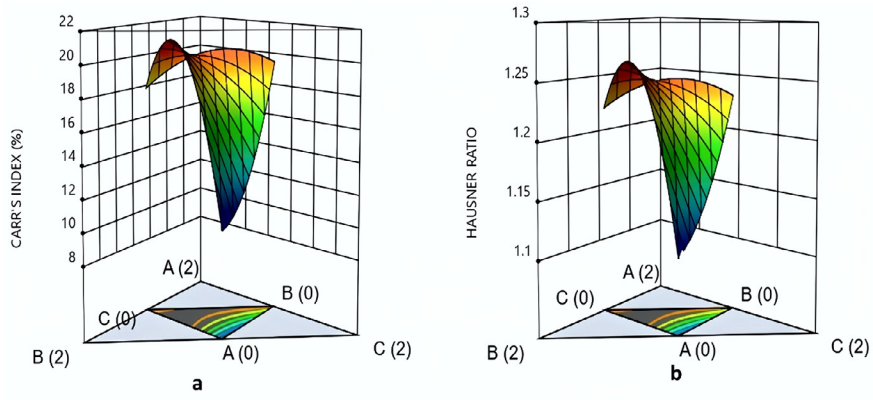


Figure 2. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for the Carr's index (a) and Hausner's ratio (b) of granules.

For the FR (Figure 3a), the independent variable PVP showed the greatest influence, followed by MCC and LM (coded equation 4). In this case, the better the rheological properties of the granules and the rounder the shape of the particles composing them, the higher the values of FR.

$$HR = 1.14CM + 1.47LM + 0.56PVP - 0.35CMLM + 1.51CMPVP + 0.33LMPVP \quad (3)$$

The AR values obtained in the test runs allowed to classify the flow properties between excellent and passable (Figure 3b), probably due to that PVP, as a binder in the mixture, could be responsible for making the granules more spherical and homogenising the powder mass[18]. This implies that the lower values were favoured by increasing the amount of PVP (equation 5).

$$AR = 50.41MCC + 40.26LM + 0.57PVP - 48.91MCCLM + 37.79MCCPVP + 17.10LMPVP \quad (5)$$

The Design Expert software allowed the exploration of the experimental regions and the delimitation of the points where the response variables evaluated had the most fitted values. The evaluation of the best possible formulation

among those obtained in the factorial design yielded the following results: minimum MCC and maximum LM and PVP, with a desirability of 0.719 (Figure 4), which is the formulation selected for the preparation of *P. ostreatus* granules.

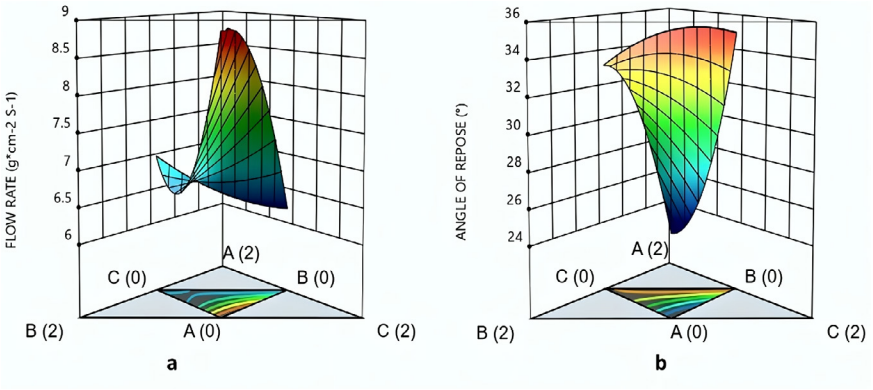


Figure 3. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for the flow rate (a) and angle of repose (b) of granules

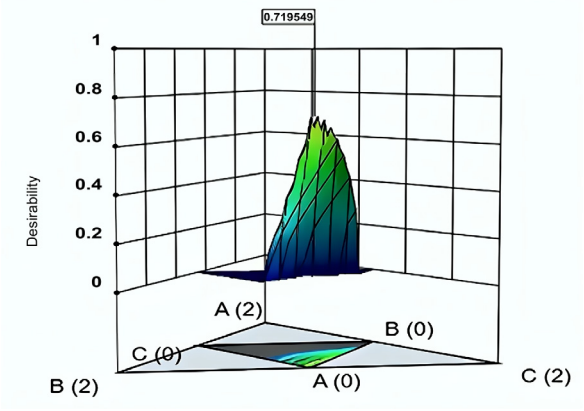


Figure 4. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for desirability of granules

Quality of *Pleurotus ostreatus* granules

Moisture is a critical variable in the characterisation of materials such as powders and granules. There is a correlation between a higher amount of moisture absorbed or contained in the material and the increased difficulty in compression, mixing and other handling operations in the manufacture of pharmaceutical products, whether mechanical or manual, due to the increased cohesion forces between the particles of the material. According to the literature, the optimum residual moisture in pharmaceutical granules should be below 5%[26].

The results of the residual moisture in the three batches of *P. ostreatus* granules are shown in Table 4. A minimum value of 2.76% and a maximum value of 4.68% were obtained, and the differences between batches were no significant. The results obtained are within the range established for products of natural origin (maximum 10%)[24]. Taking into account that this product will act as an intermediate, the residual moisture values required are low. The moisture of a granule for use as an intermediate could not only affect the size of the granule, but could also exceed the desired size and lead to complications, such as, microbial contamination. Thus, the residual moisture in obtained granules was optimal and sufficient.

In this case, as the product is derived from the fruiting bodies of *P. ostreatus*, the drying process also increases the probability of degradation of the secondary metabolites responsible for the pharmacological action[27]. Moisture content is a critical property to consider when ensuring the shelf life and stability of nutraceutical/pharmaceutical products. An excess of water may result in physical or chemical instability resulting from microbial growth[28].

Table 4. Residual moisture of three batches of *P. ostreatus* granules

Batches	Residual moisture (%)
1	3.79 ± 0.76
2	3.39 ± 0.45
3	4.08 ± 0.44
F - Ratio	0.74
p-value	0.5158

Similarly, the efficiency of the drying process was confirmed by the moisture content of the granules, which ensures their microbiological safety. The link between the moisture content of natural products and the effectiveness of the drying process has been demonstrated in several studies[26,29].

The morphological characterisation of granules, as solid raw materials or intermediates in pharmaceutical formulations is critical, influencing many of their properties[30]. Figure 5 shows the results of scanning electron microscopy of *P. ostreatus* granules. Visible granules can be observed without dust particles, but with some degree of breakage (Figure 5A).

Figures 5B, 5C and 5D reveal the low surface porosity of the granules; this is an important parameter, as it affects the disintegration time, both of the granules as a solid dosage form and of tablets made from the intermediate product. For example, it can influence factors, such as, the flowability and dissolution rate of the product[31].

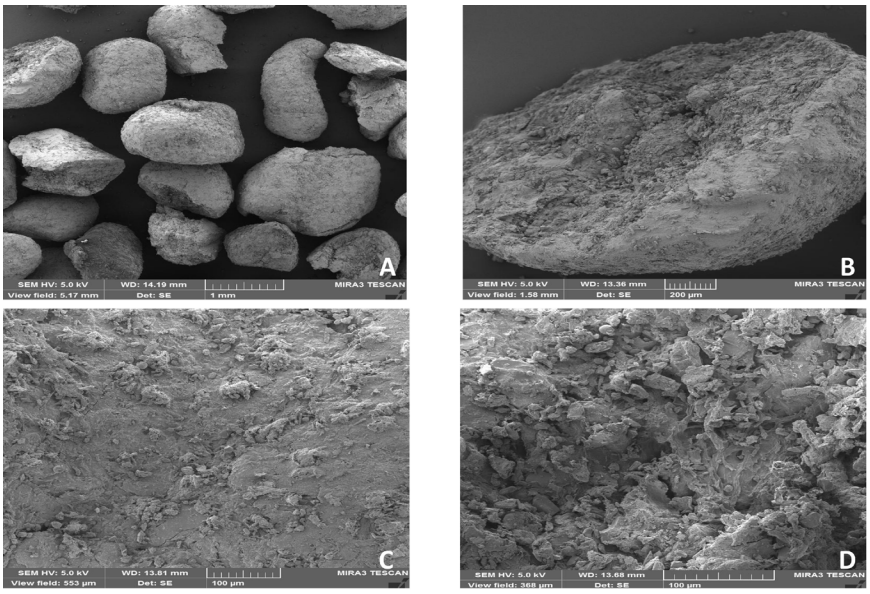
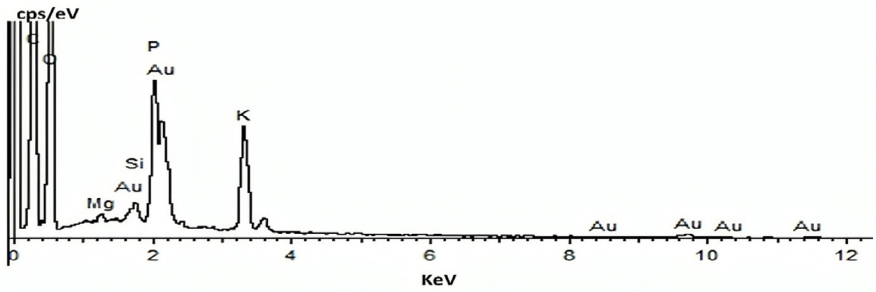


Figure 5. Scanning Electron Microscope Electronic images of *P. ostreatus* granules (A: 5.17 mm; B: 1.58 mm; C: 553 µm and D: 368 µm)

The composition of the granules was analysed by X-ray energy dispersive spectrometry (Figure 6). A certain amount of gold was found in the samples, which is the effect of sputtering with gold on the surface of the samples. With regard to the other constituents, no significant differences were observed in carbon and oxygen contents due to the different amounts of excipients (lactose monohydrate, magnesium stearate, polyvinylpyrrolidone and microcrystalline cellulose) used in granules preparation. The presence of silicon and magnesium is related to the concentrations of colloidal silicon dioxide and magnesium stearate. These agents are employed as moisture absorbers and flow enhancers, respectively, as well as lubricants. Potassium and phosphorus are derived from *P. ostreatus* mushroom biomass. Potassium was the main potential health element detected, followed by phosphorus[32]; mushroom powder could also contribute to magnesium content in granules.

The particle size of any material or substance has a direct effect on its rheological behaviour, modifies its properties, and increases or decreases its quality as a raw material or intermediate product for compression. In general, small particle sizes (<0.05 mm) have greater cohesiveness, which reduces their fluidity. However, with a smaller particle size, the surface area of the substance is increased and therefore, it would have greater solubility[33].



Element	Atomic number	series	App Conc	Intensity Corrn.	Weight %	Weight % Sigma	Atomic %
C	6	K-series	6.65	0.6288	47.71	0.56	56.75
O	8	K-series	5.09	0.5110	45.09	0.53	40.26
Mg	12	K-series	0.03	0.7968	0.16	0.04	0.09
Si	14	K-series	0.05	0.9257	0.25	0.04	0.13
P	15	K-series	0.88	1.3075	3.02	0.10	1.39
K	19	K-series	0.80	0.9627	3.76	0.09	1.38
Totals					100.00		100.00

Figure 6. X-ray diffraction analysis of the macro- and microelemental composition of *P. ostreatus* granules

An average particle size>0.1 μm was observed in the three batches of evaluated granules (Table 5). A particle size between 0.2 and 4 mm is commonly found in pharmaceutical granules. Small particle size can adversely affect flow characteristics[34]. In this case, the positive effect of the excipients used in the formulation of the granules, as a solid and/or intermediate dosage form for the production of capsules or tablets, can also be mentioned. When used as a filler, lactose monohydrate tends to increase particle size, but when combined with binders (e.g. polyvinylpyrrolidone), desiccants, and flow enhancers (e.g. colloidal silicon dioxide), and lubricants (e.g. magnesium stearate), it ensures further consolidation of granules[35]. These results are supported by the statistical analysis, which showed no significant differences among the three formulations.

Table 5. Mean particle size of *P. ostreatus* granules

Batches	Mean particle size (μm)
1	317.50 ± 14.03
2	326.18 ± 10.78
3	317.83 ± 5.09
F-Ratio	0.43
p-value	0.6702

The particle size was highest between the 350 and 177 μm sieves, with a distribution towards normality, proven by the Kolmogorov-Smirnov test (p-value of 0.53). These results corroborate those related to the particle shape, which showed that as the sphericity increased, the irregular particle shape and the roughness surface decreased[36].

Particle size distribution has a direct impact on the development and manufacture of dosage forms. It directly influences the efficacy, stability and safety of preparations[37].The drying method and type of raw material can both affect the particle size, and it is important to consider these factors to ensure optimal product quality during the processing, handling and storage of solid raw materials[29].

The results of the rheological evaluation of the granules of the three formulations studied are presented in Table 6. No statistical differences were observed in the formulations, which showed favourable flow and compressible behaviour, making them suitable for use in the production of solid dosage forms, as reported in the literature[21]. When a material is porous, it contacts more of the medium and is easier to disperse; therefore, less porous raw materials can cause problems when dispersing the product[38].

Table 6. Results of rheological properties of *P. ostreatus* granules

Batches	CI (%)	RH	FR (g cm^{-2} s^{-1})	AR (°)
1	11.20 ± 0.56	1.13 ± 0.01	8.72 ± 0.46	25.85 ± 1.08
2	11.23 ± 0.05	1.13 ± 0.00	8.21 ± 0.51	23.29 ± 2.75
3	10.45 ± 0.86	1.12 ± 0.01	8.76 ± 0.73	24.03 ± 0.76
p-value	0.3884	0.3906	0.6058	0.3888
F-Ratio	1.11	1.10	0.55	1.11

In this work, the composition of the particles influenced the results obtained for rheological properties. The cohesion between the particles lead to an adequate flow of the material, since a product composed of particles that tend to be spherical and without a high electrostatic charge between them and their walls, results in a material with adequate flow properties[30].

Particle size affects the flowability of powders derived from natural products, leading to problems with compaction, segregation and handling[39]. Juarez-Enriquez et al.[28] studied the structural changes in the matrix of pharmaceutical and food powders, resulting from water adsorption, particle agglomeration and powder caking, which are critical control points for improved flowability.

Varun and Ghoroi[40] reported that powdered solids are non-porous, and they tend to form spherical particles. On the other hand, Zolotov et al.[41] informed that solids derived from natural products have small and irregular particles, which can be reconstituted by the addition of excipients to fill the porous surface and ensure adequate fluidity. This is related with the fact that larger particle sizes result in lower cohesive forces between them.

Although the experimental conditions may affect the results obtained here, these tests allow the assessment of the appropriate use of selected excipients to improve the granulometric and rheological properties of the powdered solid from *P. ostreatus* through granule design. Successful use of lubricants can be indicated by a solid product with appropriate flow characteristics. These agents help to reduce cohesion and friction between particles[42].

Table 7 shows the total phenolic content in the three evaluated batches; no significant differences were found at 95% confidence level. Our previous studies reported that the total phenolic content of mushroom products is influenced by cultivation conditions and extraction solvents. These findings highlight the importance of the good cultivation practices and standardized extraction methods in the production of high-quality mushroom products. It is recommended to use solid-state fermentation for obtaining fruiting bodies under controlled cultivation conditions to ensure consistent and safe products for use as functional foods, nutraceuticals and bioactive compounds[4].

Table 7. Concentration of phenolic compounds in *P. ostreatus* granules

Batches	Total phenols (mg/100 g, d.w.)
1	34.52 ± 1.03
2	33.08 ± 1.01
3	34.23 ± 1.00
F-Ratio	1.69
p-value	0.2610

Aqueous extracts were prepared using water as the extraction solvent to quantify total phenolics in *P. ostreatus* granules. Compared to organic solvents, water was chosen because of its safety, economy and flexibility. Due to its polar nature, it is suitable for extracting compounds, such as polyphenols, which have a chemical structure based on aromatic rings substituted by hydroxyl groups. This structure facilitates their solubility in polar solvents, whether in free or conjugated form. This is particularly important for aqueous extraction of these compounds with applications in the development of functional foods, nutraceuticals and pharmaceutical formulations[43].

Beltrán et al.[44] reported the total phenolic content of *P. ostreatus* extracts obtained with solvents of different polarity, and the highest values were achieved with the most polar solvents (water and 50% ethanol) with values of 138.4 and 86.37 mg/100 g (d.w.), respectively. Peraza et al.[45] informed a total phenolic content of 73 mg/100 g in *P. ostreatus* extracts. The study also concluded that genetic differences between species and cultivation conditions, such as the use of commercial or wild substrates, could be responsible for the differences in phenolics concentration.

A number of phenolic compounds with anti-diabetic properties have been identified, including anthocyanins, ellagitannin, luteolin, rosmarinic acid, catechin, resveratrol, rutin, quercetin, diosmetin and myricetin. Therefore, phenolic compounds may be a promising therapeutic option for the treatment of type II Diabetes Mellitus[46].

The HPLC profile of the phenolic compounds identified in the aqueous extracts of mushroom granules as a fingerprint and quality criteria is shown in Figure 7. The five main peaks identified correspond to gallic acid, homogentisic acid, chlorogenic acid, caffeic acid and naringin. The differences in the structure of the phenolic compounds might have influenced the biological activities. For example, the antioxidant activity of the compound structure was reported to be dependent on the number and distribution (*ortho* and *para* positions) of the active group (OH)[47]. Therefore, correlation analysis between phenolic compounds and the potential anti-diabetic activities are worthwhile to be studied further.

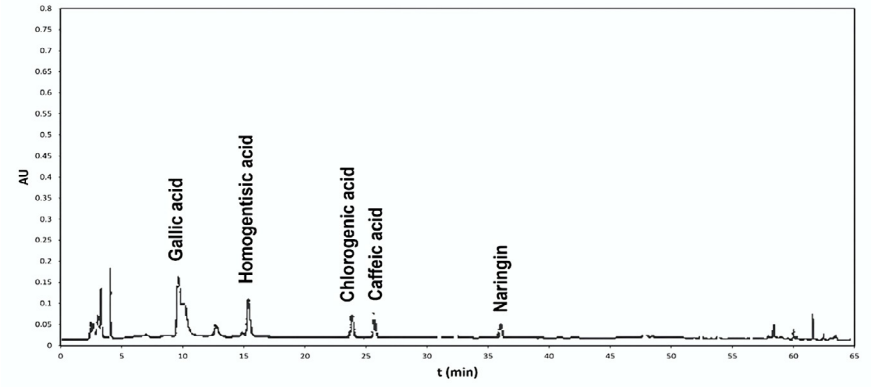


Figure 7. HPLC profile of the aqueous extract derived from P. *ostreatus* mushroom granules used as a fingerprint and quality criteria.

It is important to note that in a shelf stability study of granules from *P. ostreatus* mushroom, conducted over a 12-month period[48], no significant differences in the total phenolic content were shown up to the first six months, and a decreasing of only 5% was observed at the end of the experiment. On the other hand, the HPLC profile showed a similar pattern during the overall experimental time.

Contamination by microorganisms can alter the physical, chemical and therapeutic properties of a medicinal product and transform active ingredients into toxic substances[49]. Microbiological evaluation of *P. ostreatus* granules indicates the absence of pathogenic microbes which may hinder the use of granules in the development of nutraceuticals (Table 8).

Proper dehydration is crucial to prevent the growth of filamentous fungi and yeasts, which can grow in products with moisture levels below 50%. To ensure that the product is safe for human consumption, it is important to maintain low levels of bacteria, fungi and yeasts. It is also important to prevent the presence of harmful microorganisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacteriaceae* and *Candida albicans*[50].

The *in vitro* activation of the microbial autolytic system of the microorganisms tested was reported in a hot water extract of *Pleurotus sp*. mycelia[4]. In addition to mycelia, the antibacterial potential of *P. ostreatus* fruiting bodies was informed by Gutef et al.[51].

Table 8. Microbiological counts in *Pleurotus ostreatus* granules

TESTS	RESULT	LIMITS[20]
Total bacterial count	≤10 cfu/mL	$\leq 10^3$ cfu/mL
Total count of fungi and yeasts	≤10 cfu/mL	$\leq 10^2$ cfu/mL
Pseudomonas aeruginosa	Absence	Absence
Sthaphylococcus aureus	Absence	Absence
Enterobacteriae	Absence	Absence
Candida albicans	Absence	Absence

Recent studies have evaluated the phytochemical composition and *in vitro* antimicrobial activity of wild *P. ostreatus*. Results showed its antimicrobial effects against all tested microorganisms, and the aqueous extracts were more effective than methanol extracts, with different degrees of inhibition against both bacteria and fungi, including *Staphylococcus sp*., *Escherichia coli*, yeasts and moulds. The reported antimicrobial activity could be related to the presence of phenolic compounds, among other metabolites[7].

In the shelf stability study of granules from *P. ostreatus* mushroom, conducted over a 12-month period[48], no significant changes were found throughout the research period in the moisture content (2.8 and 4.7%) ($p<0.05$). Moreover, the microbiological analysis did not indicate the presence of pathogenic microorganisms.

Within the scope of this study, the potential of *P. ostreatus* for developing solid dosage forms with applications in food and pharmaceutical industries for human and veterinary applications, was demonstrated by obtaining mushroom granules with good flow and compressibility properties as well as appropriate chemical and microbiological quality.

Further *in vitro* and/or *in vivo* studies are needed to validate the anti-diabetic potential of the granules. Preliminary studies with the water-extract obtained from mushroom granules showed good activity in the α-glucosidase and α-amylase inhibition assays, as well as, in molecular docking studies (data not shown, VLIR – UOS Project CU 2019-2024 IUC 030A105). This approach would contribute to the diversification of mushroom commercial products with increased efficacy, stability and quality.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided herein.

AUTHOR CONTRIBUTIONS

Conceptualization/ Design: HJ Morris, I Chil, P Cos; Acquisition of data: D Arias-Ramos, JA Rojas, Y Beltrán, Y Lebeque; Analysis of data/ Software: HJ Morris, I Chil, D Arias-Ramos, JA Rojas, Y Beltrán; Writing –Original Draft Preparation: D Arias-Ramos, I Chil, JA Rojas; Supervision: HJ Morris, P Cos; Financial support/ Project: HJ Morris, Y Lebeque, P Cos; Review & Editing: HJ Moris, I Chil.

FUNDING SOURCES

Cuban Agriculture Ministry (Minag) through the Sectoral Program of Animal and Plant Health contributes to the financial support of the project PS-223MY003131 “Bioprospecting of basidiomycete fungal preparations as a source of bioactive agents for animal and plant health” (2023-2025).

Belgian Development Cooperation - Flemish Interuniversity Council - University Cooperation for Development (VLIR - UOS, Belgium) for financial support through a P3 project "Biopharmaceutical Products from Natural Sources in the Development of Biotechnology" (grant number CU 2019 IUC 030A105).

Cuban Ministry of Science, Technology and Environment (CITMA) through the Project PT241SC003-003 belonging to the Territorial Program of "Development of Health Products and Services".

ACKNOWLEDGMENTS

The authors would like to thank to the funding sources mentioned above for the financial support for acquiring laboratory equipment, reagents and consumables used in this research.

REFERENCES

1. Awad A, Trenfield SJ, Basit AW. Solid oral dosage forms. In: Adejare A, editor. The science and practice of pharmacy. Academic Press; 2021. p. 333-358. Doi: 10.1016/B978-0-12-820007-0.00019-2

2. Wang B, Sun X, Xiang J, Guo X, Cheng Z, Liu W, et al. A critical review on granulation of pharmaceuticals and excipients: principle, analysis and typical applications. Powder Technol, 2022;401:117329. Doi: 10.1016/j.powtec.2022.117329

3. Lebeque Y, Morris HJ, Beltrán Y, Llauradó G, Gaime-Perraud I, Meneses M, et al. Proximal composition, nutraceutical properties, and acute toxicity study of culinary-medicinal oyster mushroom powder, *Pleurotus ostreatus* (Agaricomycetes). Int J Med Mushrooms, 2018;20(12):1185-1195. Doi: 10.1615/IntJMedMushrooms.v20.i12.60

4. Morris HJ, Llauradó G, Beltrán Y, Lebeque Y, Batista PL, Moukha S, et al. Immunomodulating and antitumor properties of *Pleurotus* sp. in Cuba. In: Sánchez JE, Mata G, Royse DJ, editors. Updates on tropical mushrooms. Basic and Applied Research. San Cristóbal de Las Casas, Chiapas: El Colegio de la Frontera Sur; 2018. p. 159-180.

5. Xiong M, Huang Y, Liu Y, Huang M, Song G, Ming Q, et al. Antidiabetic activity of ergosterol from *Pleurotus ostreatus* in KK-Ay mice with spontaneous type 2 diabetes mellitus. Mol Nutr Food Res, 2018;62(1):e1700444. Doi: 10.1002/mnfr.201700444

6. Waktola G, Temesgen T. Pharmacological activities of oyster mushroom (*Pleurotus ostreatus*). Nov Res Microbiol J, 2020;4(2):688-695. Doi: 10.21608/nrmj.2020.84017

7. Ogidi OI, Oguoma LMO, Adigwe PC, Anthony BB. Phytochemical properties and *in-vitro* antimicrobial potency of wild edible mushrooms (*Pleurotus ostreatus*) obtained from Yenagoa, Nigeri. J Phytopharmacol, 2021;10(3):180-184. Doi: 10.31254/phyto.2021.10306

8. Arias-Ramos D, Chil-Núñez I, Morris HJ, Camacho-Pozo M, Lebeque-Pérez Y, Llauradó-Maury G, et al. Technological, biochemical and microbiological evaluation of dehydrated *Pleurotus ostreatus* powder for nutraceutical applications. Curr Nutr Food Sci, 2022;18(5):507-514. Doi: 10.2174/1573401318666220211101143

9. Ahirwar K, Shukla R. Preformulation studies: a versatile tool in formulation design. In: Shukla R, Kuznetsov A, Ali A. Drug formulation design. London: IntechOpen; 2023. Doi: 10.5772/intechopen.110346

10. Verma S, Gupta M, Popli H, Aggarwal G. Diabetes mellitus treatment using herbal drugs. Int J Phytomedicine, 2018;10(1):1-10.

11. Oloke J. Oyster mushroom (*Pleurotus species*); a natural functional food. J Microbiol Biotechnol Food Sci, 2017;7(3):254. Doi: 10.15414/jmbfs.2017/18.7.3.254-264

12. Samuel C, Prince NO, Augustine A, Comfort C, Young CU, Chidinma OC. Antihyperglycemic effect of ethanol extract of fruiting bodies of organically cultivated *Pleurotus ostreatus* in high sucrose high fat diet streptozotocin induced diabetes in rats. IJPSAT, 2021;28(2):94-104.

13. Farias DP, Fernandes de Araújo F, Neri-Numa IA, Pastore G. Antidiabetic potential of dietary polyphenols: a mechanistic review. Food Res Int, 2021;145:110383. Doi: 10.1016/j.foodres.2021.110383

14. Krishna K, Murugan J, Khan H, Kumar M, Veeramanikandan V, Hatamleh A, et al. Exploring the therapeutic potential of edible *Pleurotus* mushroom species for oxidative stress and diabetes management. J King Saud Univ Sci, 2023;35(9):102926. Doi: 10.1016/j.jksus.2023.102926

15. Unde JS, Ahirwar K, Kumar A, Alshehri SA, Wahab S, Kesharwani P, et al. Manoeuvring the innovative drug delivery systems for veterinary therapeutics: present day demand. Eur Polym J, 2024;215:11324. Doi: 10.1016/j.eurpolymj.2024.113244

16. Rowe RC, Sheskey P, Quinn M. Handbook of pharmaceutical excipients. 6th ed. London: Pharmaceutical Press and the American Pharmacists Association; 2009.

17. González A, Nobre C, Simoes LS, Cruz M, Loredo A, Rodríguez-Jasso RM, et al. Evaluation of functional and nutritional potential of a protein concentrate from *Pleurotus ostreatus* mushroom. Food Chem, 2021;346:128884. Doi: 10.1016/j.foodchem.2020.128884

18. Rodríguez-Amado JR, Lafourcade-Prada A, Arranz JCE, Morris HJ, Colarte AI, Carvalho JCT. Optimization of a novel tablets formulation using D-optimal mixture design. Afr J Pharm Pharmacol, 2015;9(14):474-483. Doi: 10.5897/AJPP2014.4296

19. Beltrán-Delgado Y, Morris HJ, Domínguez OD, Batista-Corbal P, Llauradó-Maury G. Composición micoquímica y actividad antioxidante de la seta Pleurotus ostreatus en diferentes estados de crecimiento. Acta Biol Colomb, 2021;26(1):89-98. Doi: 10.15446/abc.v26n1.84519

20. Cengiz G, Yapar EA. Method decision for determining specific microorganisms in pharmaceutical products: an overview. Univers J Pharm Res, 2020;5(5). Doi: 10.22270/ujpr.v5i5.492

21. Gibson M. Pharmaceutical preformulation and formulation: a practical guide from candidate drug selection to commercial dosage form. 2nd ed. New York: CRC Press; 2016.

22. Venturella G, Ferraro V, Cirlincione F, Gargano ML. Medicinal mushrooms: bioactive compounds, use, and clinical trials. Int J Mol Sci, 2021;22(2):634. Doi: 10.3390/ijms22020634

23. Seethapathy P, Thangaraj P, Pandita A, Sankaralingam S, Pandita D. Oyster Mushroom (Pleurotus ostreatus). In: Pandita D, Pandita A, editors. Mushrooms: nutraceuticals and functional foods. 1st ed. Boca Raton (FL): CRC Press; 2023. p. 302-321. Doi: 10.1201/9781003322238-18

24. Rodríguez-Amado JR, Lafourcade A, Escalona JC, Pérez R, Keita H, Morris HJ, et al. Antioxidant and hepatoprotective activity of a new tablets formulation from *Tamarindus indica* L. Evid Based Complement Alternat Med, 2016;3918219. Doi: 10.1155/2016/3918219

25. Karoui IJ, Hammami M, Abderrabba M. The use of D-Optimal Mixture Design in the development of date stone and spirulina tablet formulation as a phycocyanin dietary supplement. C R Chim, 2023;26(S1):1-14. Doi: 10.5802/crchim.222

26. Salústio PJ, Inácio C, Nunes T, Sousa e Silva JP, Costa PC. Flow characterization of a pharmaceutical excipient using the shear cell method. Pharm Dev Technol, 2020;25(2):237-244. Doi: 10.1080/10837450.2019.1688348

27. Velu G, Palanichamy V, Rajan AP. Phytochemical and pharmacological importance of plant secondary metabolites in modern medicine. In: Roopan SM, Madhumitha G, editors. Bioorganic phase in natural food: an overview. Cham: Springer International Publishing; 2018. p. 135-156. Doi: 10.1007/978-3-319-74210-6_8

28. Juarez-Enriquez E, Olivas GI, Zamudio-Flores PB, Perez-Vega S, Salmeron I, Ortega-Rivas E, et al. A review on the influence of water on food powder flowability. J Food Process Eng, 2022;45(5):e14031. Doi: 10.1111/jfpe.14031

29. Aziz M, Yusof Y, Blanchard C, Saifullah M, Farahnaky A, Scheiling G. Material properties and tableting of fruit powders. Food Eng Rev, 2018;10(2):66-80. Doi: 10.1007/s12393-018-9175-0

30. Bansal AK, Balwani G, Sheokand S. Critical material attributes in wet granulation. In: Narang AS, Badawy S, editors. Handbook of pharmaceutical wet granulation theory and practice in a quality by design paradigm. Amsterdam: Academic Press; 2019. p. 421-453. Doi: 10.1016/B978-0-12-810460-6.00009-9

31. Bhujbal SV, Mitra B, Jain U, Gong Y, Agrawal A, Karki S, et al. Pharmaceutical amorphous solid dispersion: A review of manufacturing strategies. Acta Pharm Sin B, 2021;11(8):2505-2536. Doi: 10.1016/j.apsb.2021.05.014

32. Doroški A, Klaus A, Režek Jambrak A, Djekic I. Food waste originated material as an alternative substrate used for the cultivation of oyster mushroom (*Pleurotus ostreatus*): a review. Sustainability, 2022;14(19):12509. Doi: 10.3390/su141912509

33. Ulusoy U. A review of particle shape effects on material properties for various engineering applications: from macro to nanoscale. Minerals, 2023;13(1):91. Doi: 10.3390/min13010091

34. Drewnick F, Pikmann J, Fachinger F, Moormann L, Sprang F, Borrmann S. Aerosol filtration efficiency of household materials for homemade face masks: Influence of material properties, particle size, particle electrical charge, face velocity, and leaks. Aerosol Sci Technol, 2021;55(1):63-79. Doi: 10.1080/02786826.2020.1817846

35. Chaerunisaa AY, Sriwidodo S, Abdassah M. Microcrystalline cellulose as pharmaceutical excipient. In: Ahmad U, Akhtar J, editors. Pharmaceutical formulation design-recent practices. London: IntechOpen; 2019. p. 41-61. Doi: 10.5772/intechopen.88092

36. Kudo Y, Yasuda M, Matsusaka S. Effect of particle size distribution on flowability of granulated lactose. Adv Powder Technol, 2020;31(1):121-127. Doi: 10.1016/j.apt.2019.10.004

37. Arshad MS, Zafar S, Yousef B, Alyassin Y, Ali R, AlAsiri A, et al. A review of emerging technologies enabling improved solid oral dosage form manufacturing and processing. Adv Drug Deliv Rev, 2021;178:113840. Doi: 10.1016/j.addr.2021.113840

38. Hernández O, Baltazar E, González E, Contreras LM. Production of directly compressible excipients with mannitol by wet granulation: rheological, compressibility and compactibility characterization. Farmacia, 2019;67(6):973-985. Doi: 10.31925/farmacia.2019.6.7

39. Stavrou AG, Hare C, Hassanpour A, Wu C-Y. Investigation of powder flowability at low stresses: Influence of particle size and size distribution. Powder Technol, 2020;364:98-114. Doi: 10.1016/j.powtec.2020.01.068

40. Varun N, Ghoroi C. Engineered inhalable micro-balloon shaped drug particles for carrier-free dry powder inhalation (DPI) application. Powder Technol, 2022;408:117705. Doi: 10.1016/j.powtec.2022.117705

41. Zolotov SA, Demina NB, Zolotova AS, Shevlyagina NV, Buzanov GA, Retivov VM, et al. Development of novel darunavir amorphous solid dispersions with mesoporous carriers. Eur J Pharm Sci, 2021;159:105700. Doi: 10.1016/j.ejps.2021.105700.

42. Chendo C, Pinto JF, Paisana MC. Comprehensive powder flow characterization with reduced testing. Int J Pharm, 2023;642:123107. Doi: 10.1016/j.ijpharm.2023.123107

43. Lefebvre T, Destandau E, Lesellier E. Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. J Chromatogr A, 2021;1635:461770. Doi: 10.1016/j.chroma.2020.461770

44. Beltrán Y, Morris HJ, Aguirre RI, Quevedo Y, Armando T, Vázquez R. et al. Phenolic content and *in-vitro* antioxidant activities of fruiting bodies extracts from the oyster mushroom *Pleurotus ostreatus*. Presented at: 15th International Conference on Oxidative Stress Reduction, Redox Homeostasis & Antioxidants; 2015 Jun 22–24; Institut Pasteur, Paris, France. J Int Soc Antioxid Nutr Health. 2015;1(1):[Special Issue for 15th International Antioxidants Congress].

45. Yildiz O, Can Z, Laghari AQ, Şahin H, Malkoç M. Wild edible mushrooms as a natural source of phenolics and antioxidants. J Food Biochem, 2015;39(2):148-154. Doi: 10.1111/jfbc.12107

46. Li W, Yuan G, Pan Y, Wang C, Chen H. Network pharmacology studies on the bioactive compounds and action mechanisms of natural products for the treatment of diabetes mellitus: a review. Front Pharmacol, 2017;8:248511. Doi: 10.3389/fphar.2017.00074

47. Munteanu I.G, Apetrei C. Analytical methods used in determining antioxidant activity: a review. Int J Mol Sci, 2021,22(7):3380. Doi: 10.3390/ijms22073380

48. Arias-Ramos D, Chil-Núñez I, Morris-Quevedo HJ. Shelf stability study of granules from *Pleurotus ostreatus* mushroom. Tecnol Quím, 2025;45(2). In press.

49. Ratajczak M, Kubicka M, Kamińska D, Sawicka P, Długaszewska J. Microbiological quality of non-sterile pharmaceutical products. Saudi Pharm J, 2015;23(3):303-307. Doi: 10.1016/j.jsps.2014.11.015

50. Riesute R, Salomskiene J, Moreno DS, Gustiene S. Effect of yeasts on food quality and safety and possibilities of their inhibition. Trends Food Sci Technol, 2021;108:1-10. Doi: 10.1016/j.tifs.2020.11.022

51. Gutef AH, Al-Attraqchi AA, Tawfeeq AT, Saheb HB. Evaluation of antibacterial potential of fruiting body extracts of *Pleurotus ostreatus in vitro* and *in vivo* study. Drug Inven Today, 2020;14(6).

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6343

Development of multi-unit pellet system tablets containing acyclovir: Effect of fillers on drug release and tablet quality

Miroslava ŠPAGLOVÁ[1*], Patrícia JACKULIAKOVÁ[1], Dominika ŽIGRAYOVÁ[1], Martina PAPADAKOS[2], Juraj PIEŠŤANSKÝ[1,3]

1 Comenius University Bratislava, School of Pharmacy, Department of Galenic Pharmacy, Bratislava, Slovakia
2 IQVIA, Research and Development Solutions, Bratislava, Slovakia
3 Slovak Academy of Sciences, Institute of Neuroimmunology, Bratislava, Slovakia

ABSTRACT

The study aimed to formulate multi-unit pellet system (MUPS) tablets containing the antiviral drug acyclovir (ACV) and investigate the impact of different tablet fillers on the quality parameters and drug release profiles. Acyclovir-loaded pellets were prepared using the extrusion-spheronization method and then compressed into MUPS tablets with one of the following fillers: starch, Avicel® or lactose. The results showed that the choice of filler significantly affected the mechanical properties of the MUPS tablets. Tablets containing Avicel® exhibited the highest hardness and longest disintegration time, while those with lactose had the lowest strength. Only the Avicel®-containing tablets met all the pharmacopeial quality requirements. However, the type of filler did not have a significant effect on the *in vitro* dissolution profiles of acyclovir from the MUPS tablets. Regardless of the filler, the drug release was faster in the simulated gastric fluid (pH 1.2) compared to the simulated intestinal fluid (pH 6.8). Kinetic modeling revealed that the Weibull model best described the drug release mechanism for all three formulations. The findings underscore the importance of selecting appropriate excipients when formu-

* Corresponding author: Miroslava ŠPAGLOVÁ
E-mail: spaglova@fpharm.uniba.sk
ORCIDs:
Miroslava ŠPAGLOVÁ: 0000-0002-8462-2565
Patrícia JACKULIAKOVÁ: 0009-0006-4125-6194
Dominika ŽIGRAYOVA: 0009-0005-3406-2546
Martina PAPADAKOS: 0000-0002-8414-3054
Juraj PIEŠŤANSKY: 0000-0003-4736-4592
(Received 16 Aug 2024, Accepted 3 Dec 2024)

lating MUPS tablets to achieve the desired mechanical properties and drug release characteristics.

Keywords: multi-unit pellet system, acyclovir, *in vitro* dissolution, chitosan, Avicel®

INTRODUCTION

In the pharmaceutical industry, the continuous pursuit of advanced oral drug delivery systems is paramount for enhancing patient compliance, improving therapeutic efficacy, and achieving optimal drug bioavailability. Among these systems, multiple unit pellet systems (MUPS) have garnered significant attention due to their ability to merge the advantages of both pellets and tablets. MUPS tablets, composed of drug-loaded pellets compressed into a single dosage form, offer numerous benefits such as uniform distribution of the active pharmaceutical ingredient (API), minimized risk of dose dumping, and potential for controlled and sustained release profiles. The mentioned attributes are essential for maintaining consistent therapeutic drug levels and improving patient adherence to prescribed medication regimens[1,2].

Acyclovir (ACV), an antiviral drug extensively utilized in the treatment of herpes simplex virus and varicella-zoster virus infections[3], serves as an exemplary model drug for MUPS formulation. Categorized under the biopharmaceutical classification system (BCS) as a class III compound[4], ACV is characterized by adequate aqueous solubility but low intestinal permeability. However, in some countries, 800 mg tablets are also available, placing them within BCS as a class IV[5]. This presents a notable challenge for oral delivery, as the drug's bioavailability is limited by its inability to be efficiently delivered through the gastrointestinal membrane. Therefore, innovative formulation strategies are crucial to improve the bioavailability and effectiveness of acyclovir, thus meeting the clinical need for effective antiviral treatment.

The process of pelletization through extrusion and spheronization is a well-established technique in the pharmaceutical industry for producing spherical pellets with high mechanical strength and uniform size distribution[6]. The pellets can be either filled into the capsules or compressed into the MUPS tablets[7], offering the advantage of multiparticulate dosage forms that combine the benefits of immediate and controlled release mechanisms. The formulation of MUPS tablets is preferred over capsules for several reasons. Tablets have a higher production rate and rapidly disintegrate into primary microparticles. Additionally, MUPS tablets are less likely to stick to the esophageal

lining when swallowed and are more difficult to tamper with, addressing major concerns associated with hard capsules[8,9]. Taking tablets with food, especially high-calorie food, can cause them to remain in the stomach for too long. This can be disadvantageous, particularly for enterosolvent tablets, because their onset of action may be significantly delayed. MUPS tablets rapidly disintegrate in the stomach into individual pellets, which are less affected by the emptying of gastric contents. As a result, they pass freely through the pylorus into the small intestine[10]. The speed at which pellets move from the stomach to the small intestine is determined by their density. Pellets with a density of around 1.5 g/cm^3 exit the stomach more quickly than those with a density greater than 2 g/cm^3. Pellets that are smaller than 2 mm and have a density of less than 2 g/cm^3 pass through the pyloric sphincter as rapidly as liquids, whether the stomach is empty or after a meal[11].

The selection of excipients, particularly binders and fillers, plays a critical role in the formulation process, influencing the physical and mechanical properties of the pellets and the resulting MUPS tablets. Chitosan, a naturally derived biopolymer with favorable biocompatibility and mucoadhesive properties, is employed in this study as a binder. Chitosan's unique characteristics, including its ability to form a gel-like matrix and facilitate drug release through matrix erosion and diffusion[12], make it an ideal candidate for modulating the release profile of ACV from the pellets. The incorporation of chitosan in pellets can modify the drug release profile. It can slow down the drug release by forming a gel layer around the pellets, hindering drug diffusion[13]. A decisive influence also has molecular weight of chitosan. Higher molecular weight chitosan tends to retard drug release more effectively compared to lower molecular weight chitosan[14]. Using chitosan in combination with other oppositely charged polymers can further modulate the drug release from pellets. The interaction between the oppositely charged chitosan and alginate can create a more controlled release profile[15]chitosan and sodium alginate, alone and in combination, on the ability of formulations containing a model drug (paracetamol. Increasing the chitosan content in pellets can lead to lower porosity and a rougher surface, which can also influence the drug release kinetics[16].

Additionally, the choice of filler - potato starch, lactose, or Avicel® - is crucial, as it can significantly affect the mechanical integrity, disintegration time, and drug release kinetics of the MUPS tablets[8]. Each filler exhibits distinct properties that can influence the overall performance of the tablet, necessitating a comprehensive evaluation to determine the optimal formulation[13]. In addition to ensuring the quality of MUPS tablets, it is important to choose correct shape

and size of tablets because it can affect the tolerability and swallowability of the dosage form by the patient[8].

The study aims to formulate acyclovir-loaded MUPS tablets and investigate the impact of different fillers on the tablets' quality parameters and drug release profiles. The study encompasses a detailed assessment of the physical characteristics of the tablets, including weight variation, hardness, friability, and disintegration time, adhering to pharmacopeial standards. *In vitro* dissolution testing evaluating the release profiles of ACV in simulated gastric or simulated intestinal fluid, provides insights into the drug's release behavior under physiological conditions. Understanding the mechanisms of drug release from MUPS tablets is essential for optimizing their formulation. Drug release from MUPS tablets can be influenced by several factors, including the physical properties of the pellets, the type of binder and filler used, and the compression force applied during tablet formation. The findings from this study have significant implications for the pharmaceutical industry, particularly in the formulation of oral drug delivery systems. By optimizing the formulation of acyclovir-loaded MUPS tablets, this research addresses key challenges associated with the oral delivery of BCS class III drugs. The insights gained can guide the development of robust, patient-friendly dosage forms that enhance therapeutic efficacy and improve patient compliance.

METHODOLOGY

Material

ACV was obtained from Union Quimico Farmaceutica S.A. (Barcelona, Spain). Chitosan (medium molecular weight 90–310 kDa, degree of deacetylation 82%) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Methocel K100M; co-processed microcrystalline cellulose with lactose monohydrate and natrium carboxymethyl cellulose (Specicel®140) were supplied by Dow Chemical Company (Midland, Michigan, USA). Microcrystalline cellulose (Avicel® PH 102), acetic acid, sodium chloride, hydrochloric acid and magnesium stearate were purchased from CentralChem s.r.o (Bratislava, Slovakia). Potato starch was from Lyckeby Amylex, a.s. (Horažďovice, Czech Republic). Lactose was from Lyckeby Culinar a.s, (Hražďovice, Czech Republic). Aerosil 200 was purchased from Chemex (Prague, Czecg Republic). The purified water was prepared by distillation apparatus Kavalier (Prague, Czech Republic).

Preparation of pellets

The powdered components of the mixture, which are ACV and a special filler, Specicel® 140, containing microcrystalline cellulose, lactose monohydrate, and croscarmellose sodium salt, were homogenized together and then wetted with a binder solution. 2% w/w chitosan solution acidified with a 3% w/w acetic acid solution was used as a binder. The wet mixture was then extruded using a Pharma extruder DE-120 (Gabler Engineering GmbH, Malsch, Germany) with 0.8 mm diameter holes, while being set in motion by augers rotating at 40 rpm. The extrudate was broken and pelletized in the spheronizer R-600 (Gabler Engineering GmbH, Malsch, Germany) at 1200 rpm. The entire process took 3 minutes. The resulting pellets were dried in a fluidized bed dryer (Glatt GPCG2 Lab System, Binzen, Germany) at 50°C until the moisture content was less than 3%. The moisture content was continuously monitored using a halogen moisture analyzer (Mettler-Toledo Halogen Moisture Analyzer HG63, Greifensee, Switzerland).

Preparation of MUPS tablets

The prepared pellets were mixed with one of the tested fillers (potato starch, lactose, or Avicel®) in a 1:1 ratio (w/w). Magnesium stearate (0.6%; w/w) as an anti-adhesive agent and Aerosil (0.3%; w/w) as a lubricant was added to their mixtures. The mixture was homogenized for 15 min in a homogenizing device (Turbula, Basel, Switzerland). The flow rate of the prepared mixtures was then compared by pouring 10 g of material freely into a closed glass funnel with a hopper diameter of 6.4 cm and outlet diameter of 0.6 cm, measuring the time taken for the entire volume of material to flow through the funnel until it was completely emptied. From the prepared mixtures, tablets weighing 0.500 g were compressed using single tablet press machine (Korsch, Berlin, Germany) at uniform pressure (120 MPa). The cross-sectional images of the MUPS tablets were taken with a Canon 2000D (Canon Inc., Tokyo, Japan) camera in macro mode. The differences in tablet quality due to the type of filler were evaluated by the following pharmacopeial test.

Quality assessment of pellets and MUPS tablets

Weight Variation Test: 20 randomly selected tablets were weighed to three decimal places by analytical scale HZY A200 (Libra, Bratislava, Slovakia) to see if their weights were within the permitted limits. For tablets weighing more than 250 mg, the Ph. Eur. 10[17] permits a deviation of 5% percent. A maximum of two tablets may differ by more than the permitted variation, but this vari-

ation must not exceed twice the permitted variation. At the same time, the dimensions of the tablet (height, diameter) were verified by a digital caliper, type 14016458 KS (Somet, Hradec Králové, Czech Republic).

Hardness: 10 tablets were inserted radially between the jaws of the hardness tester (Schleuniger 2E, Solothurn, Switzerland). The force required to crush the tablets was measured by the device, indicating the tablet hardness in Newton (N)[18]

Friability: 10 tablets were dusted off on a 250 μm sieve. Afterward, they were weighed and placed into a rotating drum with an internal diameter of 286 mm and a width of 39 mm, which is made of translucent synthetic polymer (Tablet Friability Tester from Erweka GmbH, Heusenstamm, Germany). The tablets were then rotated 100 times. Following the rotations, the tablets were dusted off and weighed again. The percentage weight loss of 10 tablets corresponds to their friability[18].

Disintegration: Six tablets were placed in the basket of the disintegration tester - apparatus type A (PIS SPOFA, n.p. VVZ, Kroměříž, Czech Republic), which was then immersed in a container filled with 800 mL of purified water at a temperature of 37 ± 1°C. The basket was continuously moved up and down for 15 minutes at a rate of 20 oscillations per minute. The time taken for all tablets to disintegrate was recorded, and the test was repeated three times for each formulation[19].

Dissolution test

The *in vitro* dissolution testing was conducted using Erweka DT 6 basket-type dissolution tester (Erweka GmbH, Langen, Germany). Dissolution medium contained either simulated gastric or simulated intestinal fluid. Simulated gastric juice (1 L) was made of 2 g of sodium chloride, 7 mL of hydrochloric acid, and the remaining volume of purified water (pH approximately 1.2). Simulated intestinal juice (1 L) was prepared by dissolving potassium dihydrogen phosphate in water. To this solution, 0.2 M sodium hydroxide solution was added, and purified water was added to make up to 500 mL. After adjusting the pH to 6.8, water was added up to 1000 mL. For dissolution testing, the MUPS tablet was placed in the basket, which was then dipped into the dissolution medium (900 mL) and rotated at a speed of 50 rpm. The testing was carried out for 6 hours at a constant temperature of 37 ± 0.5°C. Samples from the dissolution containers were taken at specific time intervals (0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 hrs). The amount of ACV released in the solutions was measured spectrophotometrically either at 255 nm (testing in the simulated

gastric fluid) or 272 nm (testing in the simulated intestinal fluid) [13] using a Genesys™ 10S UV-Visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA) against a blank (the dissolution medium). A linear regression analysis was performed to obtain the equation, which was used for subsequent concentration determinations: c = (A-0.0149)/0.065 (dissolution in the simulated gastric juice); c = (A-0.0756)/0.0615 (dissolution in the simulated intestinal fluid), where c is concentration (μg/mL) and A is absorbance.

Kinetic models

The *in vitro* drug release data was fitted to various kinetic models to determine the drug release mechanism: Zero order kinetics ($Q=k_0t$), First order kinetics ($\ln(100-Q)=-k_1t$), Higuchi model ($Q=k_ht^{1/2}$), and Weibull model ($\log[-\ln(1-Q/100)]=\beta \log(t-Ti) - \log(\alpha)$)[20]. The best fitted model was selected based on the highest correlation coefficient (R^2).

Statistical analysis

The data was analyzed using Microsoft Excel 2016 (Microsoft Corporation, Washington, U.S.) for statistical processing. The results were presented as mean ± standard deviation (SD). Data differences were assessed for significance using one-way ANOVA with Daniel's XL Toolbox add-in. The graph shows significant (*) or non-significant (NS) differences.

RESULTS and DISCUSSION

Pellets are often formulated to release drugs at specific rates, providing a more controlled and sustained release of the drug[21]. Due to pellets are easier to dose-divide via their compression into a tablet than by dosing into a capsule, our primary objective was to formulate MUPS tablets and investigate the impact of the choice of tablet filler on the quality parameters of MUPS tablets, as well as the drug release from them. In our study, we utilized ACV as the model drug, which belongs to BCS class III exhibiting adequate water solubility but low intestinal permeability[22]. The drug-containing pellets were prepared using the traditional extrusion and spheronization method, resulting in spherical pellets[13]. Through sieve analysis, the average pellet size was found to be 0.6 mm. Hamman et al.[23] conducted a study on the compression of pellets into tablets, finding all size fractions from 0.2 to 2.5 mm to be suitable.

Flow rate of pressing material

Before actual compression of the material, we compared the flow rate of the pellets mixed with filler and lubricant/anti-adhesive agent to the flow rate of pure pellets (reference sample). Figure 1 shows that adding filler reduced the material flow rate by 49.3 to 58.1%.

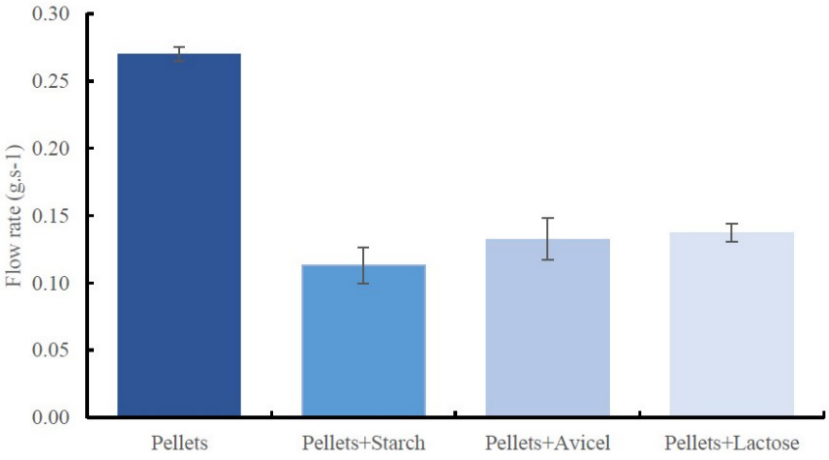


Figure 1. The influence of the filler on flow rate of the pellets; pellets without filler, pellets and starch (1:1, w/w), pellets and Avicel® (1:1, w/w), pellets and lactose (1:1, w/w).

Quality assessment of pellets and MUPS tablets

The fillers perform several functions in MUPS tablets. The filler particles have to occupy the space around the pellets, function as a cushioning agent to mitigate the impact of the compressive force during the compression, and ensure a uniform blend with the particles. Furthermore, even under relatively low compression force, the inert excipient must yield a sufficiently firm tablet, characterized by rapid disintegration time and no influence on the release of the API from the particles[24].

The basic physical characteristics of MUPS tablets are summarized in Table 1. The weights of the MUPS tablets are near the average value and do not exceed the permitted 5% deviation (Figure 2). The lowest variability in weights was observed when lactose was used as a filler in the MUPS tablets, but the RSD values were also low using the other two types of fillers. The size (height and diameter) of the MUPS tablets remains consistent due to the low RSD. The hardness of the tablets is significantly influenced by the choice of filler. Tablets containing Avicel® exhibited the highest hardness at 85 ± 11.3 N, while those containing lactose had the lowest strength. The differences in tablet hardness were also evident in their mechanical resistance when tested for friability. Tablets with a strength ranging from 50 to 90 N are considered to have optimal quality. The results from the friability and disintegration tests of MUPS tablets correlate with the hardness and disintegration test. Stronger tablets are more mechanically resistant and take a longer time to disintegrate in the dissolution medium. Only MUPS tablets containing Avicel® meet the pharmaceutical

requirement for friability when the result is rounded to one decimal place. All three types of MUPS tablets disintegrated in water within 15 minutes, meeting the required time limit for uncoated tablets as per the pharmacopoeia.

The MUPS tablets containing Avicel® showed the longest disintegration time at 10 minutes and 57 seconds. It was observed that these tablets were the only ones that met the required pharmacopoeial limits for tests predicting the general quality parameters of uncoated tablets.

Table 1. Physical characteristics of MUPS tablets

	F1 (with starch)	F2 (with Avicel®)	F3 (with lactose)	Note
Mass (g)	0.495 ± 0.002	0.502 ± 0.002	0.500 ± 0.002	Mean ± SD; n=20
RSD (Mass variability)	0.005	0.004	0.003	
Height (mm)	4.050 ± 0.015	4.028 ± 0.016	4.047 ± 0.012	Mean ± SD; n=20
Diameter (mm)	11.986 ± 0.013	12.012 ± 0.013	12.018 ± 0.006	Mean ± SD; n=20
Hardness (N)	53.4 ± 9.7	85.0 ± 11.3	33.9 ± 5.1	Mean ± SD; n=10
Friability (%)	7.73	1.02	19.96	10 tablets
Disintegration time (s)	164 ± 34	657 ± 23	109 ± 4	Mean ± SD; n=3

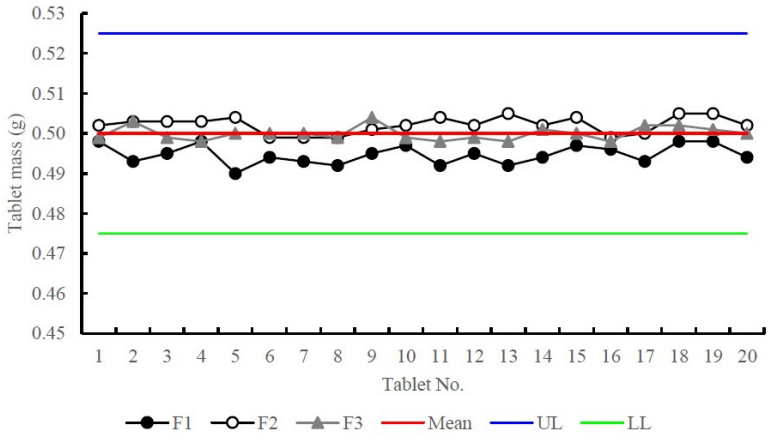
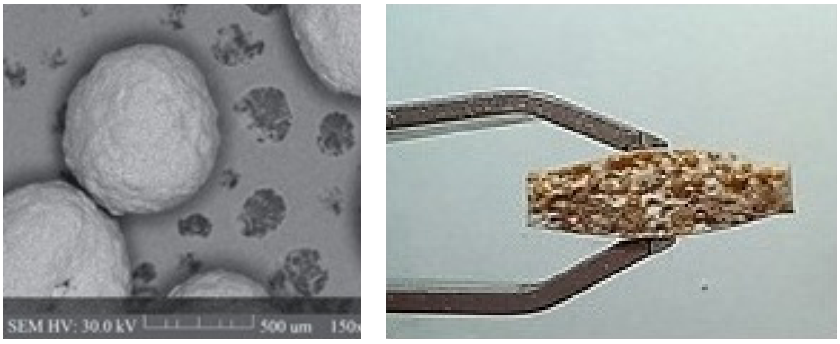


Figure 2. The weight variance of MUPS tablets: F1 with starch, F2 with Avicel®, F3 with lactose (UL illustrates upper limit, LL illustrates lower limit).

The pellets were physically characterized through several tests, and their morphology was analyzed using SEM. The results are part of another scientific study[13]. In Figure 3A, a pellet is shown by SEM at 150x magnification. Figure 3B illustrates a cross section of the MUPS tablet (F2), which was created using a scalpel. F1 and F3 were too fragile to make a uniform cut. Although this technique may not be the most convenient, as it can lead to empty voids at the cut site after some pellets have fallen out, it does allow for the direct assessment of the tablets' mechanical resistance. It is evident that F2 appears the most compact and least damaged even after incision.



A) B)

Figure 3. A) SEM image of the pellets at 150-fold magnification; B) Photographic image of a cross-section of MUPS tablet (F2).

Avicel® is frequently employed as an excipient in both tablet compression and pelletizing processes[24]. Tablets containing Avicel® generally have higher mechanical strength and better disintegration characteristics[25]. Certain Avicel® grades, like PH-113 and CE-15, can enhance the organoleptic properties of tablets and provide a smoother, creamier mouthfeel. This is advantageous mainly for chewable tablets and orally disintegrating tablets.

An important consideration when manufacturing MUPS tablets is the particle size distribution of the filler and the pellets themselves. As a result, we conducted a sieve analysis to evaluate the particle size distribution of the individual fillers. The potato starch used was found to be the finest material among the evaluated fillers, with an average particle size of d=40.95 μm (calculated according to[26]). Avicel® had a slightly higher average particle size (d=73.46 μm), and lactose had a particle size almost three times higher (d=116.68 μm) compared to starch. The strength of the MUPS tablets was optimized by the presence of starch. This is because the small powder particles effectively fill the

spaces between the individual pellets during the compression, and they also contribute to the rapid disintegration of the tablet in the dissolution medium, reverting it back to its original microparticles.

It is clear that the filler is crucial in the formulation of MUPS tablets. This is highlighted by the fact that attempting to compact MUPS tablets directly from the pellets under the same pressing conditions was unsuccessful. The resulting tablets were found to be brittle and crumbly.

The filler particle size can impact tablet processing. For instance, Carpin et al.[27] found that smaller lactose particles led to increased moisture sorption and caking tendency compared to larger ones.

Dissolution test and drug release kinetics

The rate and extent of drug release from tablets and its passage into the biological tissue impacts the drug's bioavailability and therapeutic effect. The dissociation test simulates physiological conditions in order to predict the efficacy and safety of the drug.

As the Figure 4 indicates, the course of drug dissolution is greatly influenced by dissolution medium. While in acidic medium almost 80% drug release was achieved after 15 minutes, neutral pH slowed down the drug release. This phenomenon may be due to the use of chitosan in the pellets as a binder.

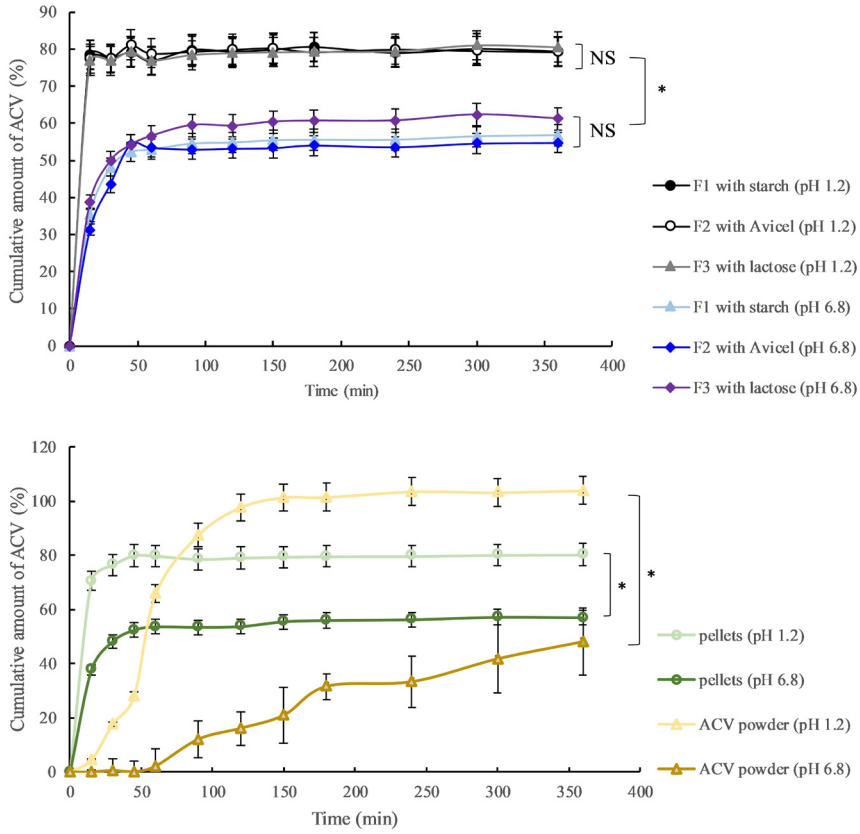


Figure 4. Dissolution profiles of ACV from MUPS tablets, pellets and capsules in different media, simulated gastric fluid (pH 1.2) vs. simulated intestinal fluid (pH 6.8) indicating significant (*) and non-significant (NS) differences.

Chitosan exhibits excellent swelling properties in acidic environments. It contains primary amino groups ($-NH_2$) along its backbone. In acidic conditions, these amino groups become protonated ($-NH_3^+$), resulting in an increase in the positive charge density of the polymer. Further, acidic media can disrupt the intramolecular and intermolecular hydrogen bonds within the chitosan structure, allowing for greater water penetration and swelling[28,29]. When the pellets come into contact with an aqueous medium, water diffuses into the interior of the particle, causing the drug to dissolve and diffuse out. Drug release rate in artificial gastric juice can vary significantly depending on the formulation design and the specific mechanisms governing drug release, such as polymer swelling, pH-responsiveness, and gastric emptying kinetics.

In our case, there was no significant effect of the filler in MUPS tablets on the rate of drug release in an acidic environment. The dissolution profiles are almost identical. Non-significant differences were also confirmed by statistical analysis.

Chitosan is a weak base, and in neutral and basic environments, the chitosan molecules will lose their charge. As Ferrari et al.[30] refer, chitosan is ineffective as an absorption enhancer at these higher pH values when the chitosan molecules exist in a more coiled conformation.

The MUPS tablets formulated in this study are designed primarily for controlled release. The use of specific binders, such as chitosan, aims to modulate the drug release profile over an extended period, thus enhancing therapeutic efficacy. To provide a comprehensive understanding of how tablet formation affects drug release, we include data on the dissolution profiles of un-compressed pellets. The data, illustrated in Figure 4, demonstrate that an acidic pH significantly enhances the release of ACV across all formulations (ACV powder, pellets, MUPS tablets). In contrast, a pH of 6.8 slowed down the drug release. For the dissolution tests, we utilized a basket apparatus to maintain consistency in our comparison. Therefore, we weighed the ACV powder into a gelatin capsule to ensure that it does not fall through the basket to the bottom of the container. The packaging formed by the gelatin capsule caused the delay of the drug's actual release until the capsule dissolved. As the capsule swelled, it became more permeable to the drug, resulting in an increase in the cumulative amount of drug released after one hour. In the acidic medium, nearly all the drug was released within two hours. However, in the dissolution medium at pH 6.8, only 48.13 ± 12.43% of the drug was released after six hours. The standard deviations indicate that the variability in ACV release from the capsule is significant, suggesting a less predictable and non-uniform drug release pattern. This highlights a major advantage of pellet formulations, particularly MUPS tablets, over conventional powders, or granules. With pellets, it is possible to achieve a consistent and sustained release of the drug at a steady rate over an extended period. Based on the dissolution profiles it can be concluded that the pellets compression into the MUPS tablets has no significant effect on drug release. Additionally, presented data of the dissolution profile of ACV in its powder form serve as a baseline for evaluating the effects of formulation changes. This comparison clarifies how processing into MUPS tablets alters the dissolution characteristics relative to raw ACV.

Drug release kinetics is a crucial aspect of pharmaceutical science that describes the rate and pattern of drug release from a dosage form. Understand-

ing the drug release kinetics helps to ensure the desired therapeutic effect, improve bioavailability, and minimize side effects. Table 2 records the coefficient of determination (R^2) values and rate release constant (c) for the zero order, first order, Higuchi, and Weibull kinetic models of the drug release from the MUPS tablets (F1, F2, and F3) either in acidic or near-neutral environments. The Weibull model provide the best fit for all three formulations regardless of the pH environment, with R^2 values around 0.96. This finding is consistent with other research papers, e.g., Dévay et al.[31] declared that theophylline was released from the pellets.

Table 2. The coefficient of determination (R2) and rate release constant (c) for the kinetic models

	pH 1.2			**pH 6.8**		
Model	**F1**	**F2**	**F3**	**F1**	**F2**	**F3**
Zero Order						
R^2	0.6305	0.6627	0.6627	0.6653	0.6743	0.6748
k_0	1.7	1.7	1.7	1.7	1.7	1.7
First Order						
R^2	0.6494	0.6748	0.6748	0.6743	0.6743	0.6748
k_1	0.0079	0.0080	0.0080	0.0080	0.0080	0.0080
Higuchi						
R^2	0.8621	0.8787	0.8787	0.8803	0.8803	0.8787
k_H	26.41	26.68	26.68	26.76	26.76	26.68
Weibull						
R^2	0.9582	0.9652	0.9652	0.9663	0.9663	0.9652
α	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
β	1.5	1.5	1.5	1.5	1.5	1.5

The Weibull model is an empirical model that can fit a variety of release patterns, including monophasic, biphasic, triphasic and complex multiphasic profiles. MUPS tablets composed of uncoated pellets exhibit complex release kinetics that the flexible Weibull model is well-suited to describe. It uses two parameters - α (scale parameter) and β (shape parameter)[33]. The β value provides information about the release mechanism. Lower β values (<0.75) point to diffusion-controlled release, while higher values indicate more complex release kinetics involving swelling, erosion, or wear-out phenomena[34].

The drug release profiles are similar across the three formulations, with the percentage of drug released ranging from around 77-81% in acidic environment over the 6-hour time period studied. There is an initial burst release of the drug within the first 0.5 hours, followed by a more gradual release over the remaining time.

When comparing the drug release in artificial gastric fluid *versus* artificial intestinal fluid, the data shows a higher percentage of drug released under acidic conditions for all three formulations. A similar observation was made by Partheniadis et al.[32] studying the release of piroxicam from chitosan pellets, who explain this phenomenon through extensive dissolution/erosion of the gel matrix observed at pH 1.2 but not at pH 5.6.

This would mean that this dosage form formulated from pellets in which the function of the binder is performed by chitosan, subsequently pressed into MUPS tablets, is suitable for drugs that are to be released immediately in the stomach, where they are to act therapeutically, and after passage into the intestine, the drug release from pellets will be slowed down due to the higher pH, i.e., the drug will be released outside the stomach to a minimal extent, which is facilitated by the pH dependence of the solubility of the chitosan. However, the pellets must also have sufficient density (at least 2 g/cm^3)[11] to remain in the stomach as long as possible after the MUPS tablet disintegrates into subunits.

The decrease in MUPS tablet weight during the dissolution test at time t=0h *vs*. the pellets weight at time t=6h was variable depending on the filler used in MUPS tablets. The MUPS tablets containing lactose (F3) showed the lowest pellet residues after disintegration, with minimal impact of the pH. At pH 1.2, the residue was 31.33 ± 2.20% and at pH 6.8, it was 29.98 ± 7.10%. This result aligns with the high solubility of lactose compared to other fillers. For MUPS tablets with starch (F1), there was a noticeable pH effect. The residue at pH 1.2 was 74.12 ± 1.95%, while at pH 6.8, it was 44.49 ± 4.41%. This is consistent with the lower solubility of starch in acidic environments. Microcrystalline cel-

lulose (MCC) in F2 caused the least weight loss after dissolution, the residue at pH 1.2 was 75.22 ± 4.40% and at pH 6.8 was 77.23 ± 2.13%, as MCC is insoluble in both acidic and neutral mediums. Despite the loss on weight, the pellets retained their spherical shape during the dissolution testing in most cases.

There are numerous factors that affect drug release, absorption, and stability. Apart from the physical and chemical properties of an API, pH, viscosity, ionic strength, and the hydrophilic-lipophilic balance of digestive juices in specific parts of the GIT have a significant impact.

Variables such as the volume of these juices, passage time in specific segments of the GIT, and the intensity of stomach and bowel movements must be considered[35].

ACV due to its pKa (2.27) is soluble in acidic environment therefore, is preferable absorbed in an upper GIT[36]. Bejgum et al.[37] analyzed the degradation products of acyclovir in an acidic environment. In addition to the previously identified guanine, they also found other degradation products such as methyl acetal ethylene glycol, formaldehyde, ethylene glycol, ACV-formaldehyde adduct, and guanine-formaldehyde adduct. This implies that the determined concentrations of ACV released during dissolution into gastric juice are at their highest level and that the remaining portion consists of these degradation products. Due to the unstable nature of ACV in acidic environments, it is recommended to incorporate the drug in a more basic micro-environment with basic excipients. Additionally, MCC (Avicel®) properties depend on pH. At lower pH values, the electrostatic repulsion force between MCC particles decreases[38], which may also contribute to the higher amount of drug released in the acidic environment.

MCC is a common cushioning excipient used in MUPS tablets. The results of our study confirm its effectiveness and indispensability in this role. Generally, cushioning material protect the pellets from compaction-induced damage[39]. Traditional cushioning material include MCC, starch, lactose, dicalcium phosphate and mannitol. They reduce mechanical stress on the pellets, maintaining their stability and efficacy, enhance the overall mechanical strength of the tablet and ensure consistent and reliable tablet production by minimizing defects during compression[24]. In order to avoid processing problems caused by the large dispersion of particle size between the cushioning excipients and the pellets themselves, cushioning pellets have also started to be formulated[39,40] to prevent a risk of segregation during production. These are often formulated from MCC.

According to the obtained results from our experiment, following summary and recommendations can be postulated:

- The choice of filler in MUPS tablets had a significant effect on the quality parameters. The best results were found for MUPS tablets containing Avicel®.
- The type of filler in MUPS tablets did not significantly affect the dissolution of ACV from them.
- ACV is better released in acidic environments, possibly due to the use of chitosan as a binder in the pellets, which dissolves well in acidic environments.

The choice of filler significantly affected the mechanical properties and drug release profiles of the formulated MUPS tablets containing ACV. Tablets with Avicel® exhibited the highest hardness and longest disintegration time, while those containing lactose had the lowest strength. Only the MUPS tablets with Avicel® met the required limits of all pharmacopoeial tests. The fillers in the MUPS tablets did not have a significant effect on the dissolution profiles. However, there were notable differences in the dissolution profiles due to the pH of the dissolution medium. ACV was released more quickly in the acidic medium, and in all cases, the release followed Weibull kinetics. The findings underscore the importance of selecting appropriate excipients in the formulation of MUPS tablets to achieve desired therapeutic outcomes. This research provides valuable guidance for the pharmaceutical industry in developing robust, patient-friendly oral drug delivery systems.

STATEMENT OF ETHICS

This study does not require ethical permission to be carried out.

CONFLICT OF INTEREST STATEMENT

The author declares that there are no conflicts of interest regarding the publication of this manuscript.

AUTHOR CONTRIBUTIONS

The study was designed by Martina Papadakos and Miroslava Špaglová. Data were acquired by Martina Papadakos and Miroslava Špaglová. Miroslava Špaglová and Dominika Žigeayová performed the data analysis. The manuscript was drafted by Miroslava Špaglová and Dominika Žigrayová. Juraj Piešťanský and Martina Papadakos contributed to the critical revision of the manuscript. Statistical analysis was carried out by Miroslava Špaglová. Technical or financial support was provided by Juraj Piešťanský and Dominika Žigrayová. The study was supervised by Juraj Piešťanský.

FUNDING SOURCES

This work was supported by the Slovak Research and Development Agency under the Contract no. APVV-23-0508, and Grant of Faculty of Pharmacy Comenius University in Bratislava FaF/1/2024.

ACKNOWLEDGMENTS

The authors would like to thank to Dominika Soláriková for collaboration in carrying out the experiment.

REFERENCES

1. Das S, Kaur S, Rai VK. Gastro-retentive drug delivery systems: a recent update on clinical pertinence and drug delivery. Drug Deliv Transl Res, 2021;11(5):1849-1877. Doi: 10.1007/s13346-020-00875-5

2. Kállai-Szabó N, Lengyel M, Farkas D, et al. Review on starter pellets: inert and functional cores. Pharmaceutics, 2022;14(6):1299. Doi: 10.3390/pharmaceutics14061299

3. Piret J, Boivin G. Antiviral resistance in herpes simplex virus and varicella-zoster virus infections: diagnosis and management. Curr Opin Infect Dis, 2016;29(6):654-662. Doi: 10.1097/QCO.0000000000000288

4. Shin S, Kim TH, Lee DY, et al. Development of a population pharmacokinetics-based *in vitro-in vivo* correlation model for drugs with site-dependent absorption: the acyclovir case study. AAPS J, 2020;22(2):27. Doi: 10.1208/s12248-019-0382-2

5. Arnal J, Gonzalez-Alvarez I, Bermejo Sanz M, et al. Biowaiver monographs for immediate release solid oral dosage forms: aciclovir. J Pharm Sci, 2008;97:5061-5073. Doi: 10.1002/jps.21392

6. Muley S, Nandgude T, Poddar S. Extrusion–spheronization a promising pelletization technique: in-depth review. Asian J Pharm Sci, 2016;11(6):684-699. Doi: 10.1016/j.ajps.2016.08.001

7. Patel NG, Patel SA, Joshi AB. Multiple Unit Pellet System (Mups Technology) for development of modified release fast disintegrating tablets: a review. J Pharm Sci Innov, 2017;6(3):50-56. Doi: 10.7897/2277-4572.06352

8. Thio DR, Heng PWS, Chan LW. MUPS tableting—comparison between crospovidone and microcrystalline cellulose core pellets. Pharmaceutics, 2022;14(12):2812. Doi: 10.3390/pharmaceutics14122812

9. Novikova A, Carstensen JM, Rades T, et al. UV imaging of multiple unit pellet system (MUPS) tablets: a case study of acetylsalicylic acid stability. Eur J Pharm Biopharm, 2017;119:447-453. Doi: 10.1016/j.ejpb.2017.08.002

10. Aulton ME, Taylor K. Aulton's pharmaceutics: the design and manufacture of medicines. Elsevier Health Sciences; 2013.

11. Panda S, Parida KR, Roy H, Talwar P, Ravanan P. A current technology for modified release drug delivery system: Multiple-Unit Pellet System (MUPS). Int J Pharm Sci Health Care, 2013;6:51-63.

12. Mikušová V, Mikuš P. Advances in chitosan-based nanoparticles for drug delivery. Int J Mol Sci, 2021;22(17):9652. Doi: 10.3390/ijms22179652

13. Čierna M, Mučaji P, Špaglová M, et al. Chitosan and sodium alginate implementation as pharmaceutical excipients in multiple-unit particulate systems. Polymers, 2022;14:2822. Doi:10.3390/polym14142822

14. Charoenthai N, Kleinebudde P, Puttipipatkhachorn S. Influence of chitosan type on the properties of extruded pellets with low amount of microcrystalline cellulose. AAPS PharmSciTech, 2007;8(3):E99–E109. Doi:10.1208/pt0803064

15. Chatchawalsaisin J, Podczeck F, Newton J. The influence of chitosan and sodium alginate and formulation variables on the formation and drug release from pellets prepared by extrusion/spheronisation. Int J Pharm, 2004;275:41-60. Doi: 10.1016/j.ijpharm.2004.01.025

16. Santos H, Veiga F, Pina M, et al. Physical properties of chitosan pellets produced by extrusion-spheronisation: influence of formulation variables. Int J Pharm, 2002;246(1-2):153-169. Doi: 10.1016/s0378-5173(02)00376-9

17. European Pharmacopoeia. Uniformity of mass of single-dose preparations. 10th ed. Strasbourg: Council of Europe; 2019.

18. Bartoníková K, Špaglová M, Papadakos M, et al. Mucoadhesive vaginal tablets containing metronidazole: screening of optimal natural polymer in the composition. Sci Pharm, 2024;92(1):10. Doi: 10.3390/scipharm92010010

19. European Pharmacopoeia. Supplement 10.6, disintegration of tablets and capsules. 10th ed. Strasbourg: Council of Europe; 2022.

20. Bruschi ML. Mathematical models of drug release. In: Strategies to modify the drug release from pharmaceutical systems. 5th ed. Woodhead Publishing; 2015. p. 63-86. Doi: 10.1016/B978-0-08-100092-2.00005-9

21. Handattu MS, Thirumaleshwar S, Prakash GM, et al. A comprehensive review on pellets as a dosage form in pharmaceuticals. Curr Drug Targets, 2021;22(10):1183-1195. Doi: 10.2174/1389450122999210120204248

22. Ates M, Kaynak MS, Sahin S. Effect of permeability enhancers on paracellular permeability of acyclovir. J Pharm Pharmacol, 2016;68(6):781-790. Doi: 10.1111/jphp.12551

23. Hamman H, Hamman J, Wessels A, et al. Development of multiple-unit pellet system tablets by employing the SeDeM expert diagram system I: pellets with different sizes. Pharm Dev Technol, 2018;23(7):706-714. Doi: 10.1080/10837450.2017.1342657

24. Sántha K, Kállai-Szabó N, Fülöp V, et al. Comparative evaluation of pellet cushioning agents by various imaging techniques and dissolution studies. AAPS PharmSciTech, 2021;22(1). Doi: 10.1208/s12249-020-01902-x

25. Yasmin R, Shoaib MH, Ahmed FR, et al. Aceclofenac fast dispersible tablet formulations: effect of different concentration levels of Avicel PH102 on the compactional, mechanical and drug release characteristics. PLoS One, 2020;15(2):e0223201. Doi: 10.1371/journal.pone.0223201

26. Franc A, Dvořáčková K, Muselík J, et al. Formulation of cores for the controlled release of glucose for prevention of hypoglycemia in diabetes patients. Čes Slov Farm, 2014;63:206-212.

27. Carpin M, Bertelsen H, Dalberg A, et al. How does particle size influence caking in lactose powder? J Food Eng, 2017;209:61-67. Doi: 10.1016/j.jfoodeng.2017.04.006

28. Sharkawy A, Barreiro MF, Rodrigues AE. Chitosan-based Pickering emulsions and their applications: A review. Carbohydr Polym, 2020;250:116885. Doi: 10.1016/j.carbpol.2020.116885

29. Aranaz I, Alcántara AR, Civera MC, et al. Chitosan: an overview of its properties and applications. Polymers, 2021;13(19):3256. Doi: 10.3390/polym13193256

30. Ferrari PC, Souza FM, Giorgetti L, et al. Development and *in vitro* evaluation of coated pellets containing chitosan to potential colonic drug delivery. Carbohydr Polym, 2013;91(1):244-252. Doi: 10.1016/j.carbpol.2012.08.044

31. Dévay A, Mayer K, Pál S, et al. Investigation on drug dissolution and particle characteristics of pellets related to manufacturing process variables of high-shear granulation. J Biochem Biophys Methods, 2006;69(1):197-205. Doi: 10.1016/j.jbbm.2006.03.006

32. Partheniadis I, Gkogkou, Kantiranis N, et al. Modulation of the release of a non-interacting low solubility drug from chitosan pellets using different pellet size, composition and numerical optimization. Pharmaceutics, 2019;11(4):175. Doi: 10.3390/pharmaceutics11040175

33. Pourtalebi Jahromi L, Ghazali M, Ashrafi H, et al. A comparison of models for the analysis of the kinetics of drug release from PLGA-based nanoparticles. Heliyon, 2020;6(2):e03451. Doi: 10.1016/j.heliyon.2020.e03451

34. Papadopoulou V, Kosmidis K, Vlachou M, et al. On the use of the Weibull function for the discernment of drug release mechanisms. Int J Pharm, 2006;309(1-2):44-50. Doi: 10.1016/j.ijpharm.2005.10.044

35. Franc A, Dvořáčková K, Kejdušová M, et al. Physiological factors with impact on the drug behaviour in the gastrointestinal tract. Čes Slov Farm, 2013;62:243-248.

36. Bhosale U, Kusum DV, Jain N. Formulation and optimization of mucoadhesive nanodrug delivery system of acyclovir. J Young Pharm, 2011;3(4):275-283. Doi: 10.4103/0975-1483.90236

37. Bejgum BC, Johnson PR, Stagner WC. Acyclovir chemical kinetics with the discovery and identification of newly reported degradants and degradation pathways involving formaldehyde as a degradant and reactant intermediate. Int J Pharm, 2018;535(1):172-179. Doi: 10.1016/j.ijpharm.2017.10.034

38. Yaginuma Y, Kijima T. Effect of pH on rheological properties of microcrystalline cellulose dispersions. J Dispers Sci Technol, 2006;27:365-370. Doi: 10.1080/01932690500359483

39. Elsergany RN, Chan LW, Heng PWS. Influence of the porosity of cushioning excipients on the compaction of coated multi-particulates. Eur J Pharm Biopharm, 2020;152:218-228. Doi: 10.1016/j.ejpb.2020.05.015

40. Elsergany RN, Chan LW, Heng PWS. Cushioning pellets based on microcrystalline cellulose – crospovidone blends for MUPS tableting. Int J Pharm, 2020;586:119573. Doi: 10.1016/j.ijpharm.2020.119573

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6344

Comparison of the polyamine content of white, whole wheat and rye bread

Ayse Lamia OZTURK[1*], Nihal BUYUKUSLU[2], Cuneyd PARLAYAN[3]

1 Istanbul Medipol University, Graduate School of Health Sciences, Istanbul, Türkiye
2 Istanbul Medipol University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Istanbul, Türkiye
3 Bahcesehir University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Istanbul, Türkiye

ABSTRACT

Polyamines participate in many biological processes, predominantly in cell growth and proliferation. Body polyamine pool is provided through de novo biosynthesis, diet, and microbiota. However, data on polyamines in bread are limited. Therefore, we aimed to ascertain the polyamine levels of different bread species and whether it change after a time. Thirty bread samples of white, whole wheat, and rye bread were analysed using HPLC at day of procurement and after four days. Crumb/crust ratio was calculated to measure polyamine content in a single serving of bread. Total polyamine content increased in order of white, whole wheat, and rye bread in both crumb and crust. The polyamine level varied as follows: spermidine > spermine > putrescine, except for whole wheat. The difference in polyamine levels in the crumb and crust was significant. After four-day, the difference in total polyamine content of crumb was found significant. Total polyamine content (nmol) of a single serving of white, whole wheat and rye bread is 1170.32, 3496.72, and 3850.84 respectively. The polyamine content of bread varied according to both type and regions of the bread. Storage at 20-24°C led to elevation of the polyamine level

Keywords: polyamines, bread types, putrescine, spermidine, spermine

* Corresponding author: Ayse Lamia OZTURK
E-mail: lamiaozturk@gmail.com
ORCIDs:
Ayse Lamia OZTURK: 0000-0003-1986-5939
Nihal BUYUKUSLU: 0000-0003-1420-0989
Cuneyd PARLAYAN: 0000-0002-6183-9489
(Received 17 Aug 2024, Accepted 12 Mar 2025)

INTRODUCTION

Polyamines (putrescine, spermidine and, spermine) are aliphatic polycationic molecules present in all cell types. They are involved in apoptosis, cell division and differentiation, cell proliferation, DNA and protein synthesis and gene expression[1]. The polyamine pool is derived from three primary sources: de novo synthesis, dietary intake, and microbiota[2], however, the diet provides much more polyamines than does de novo biosynthesis. As dietary polyamines are completely absorbed, diet might be an effective source[3]. Polyamines play a vital role in rapidly dividing cells such as immune system and digestive system. Since polyamines take part in cell proliferation, they are also involved in carcinogenesis. Therefore, studies investigate whether a diet low in polyamines has a beneficial effect on cancer[4-6]. Additionally, various pathological conditions, including inflammation, renal failure, stroke and diabetes are associated with the polyamine levels[7].

Polyamines found in both animal and vegetable are crucial exogenous sources. Since diet provides a larger quantity of polyamines than the endogenous biosynthesis, dietary polyamines are important for health. A diet of an adult provides a daily supply of several micromoles of polyamines. The effects of dietary polyamines might be detrimental, neutral or beneficial depending on individual's health condition. Increasing dietary polyamines is beneficial in rapid growth such as during the neonatal period, wound healing and after surgery while cancer patients are advised to reduce dietary polyamines for a better quality of life[8].

Grain-based foods supply much of the individuals' energy and nutrient requirements, providing 25-50% of energy in Western diets and over half of the energy intake of world population. Moreover, they are significant sources of carbohydrates, dietary fibre, micronutrients and plant-based protein[9]. Bread is a staple food and contributes at least 10% of energy requirements. Europeans consume on average 160 g per day (4-5 slices/day)[10]. As for Türkiye, it is estimated that 39.5% of daily energy intake is derived from bread and grains. On average, men consume 227 g of bread per day, while women consume 134 g[11]. It has been established that bread plays significant role in spermine intake, and the wheat products are identified as the primary source of spermidine in Türkiye[2].

In addition to macro- and micronutrients, we propose monitoring daily polyamine intake to evaluate nutritional status. Polyamine contents in foods vary widely between and even within food types due to origin, processing, storage conditions, seasonal variation and different methodological applications of foods[12]. In general, meat is rich in spermine, plant-based foods contain mostly putrescine and spermidine, dairy products include mainly putrescine and sper-

midine, and among them, cheese have higher polyamine values depending on fermentation conditions. The polyamine-rich foods consumption increases blood polyamine levels. The bread polyamine content is associated with flour source, fermentation and, baking conditions[12,13].

Although many studies have revealed the health effects of polyamines, the number of studies on the polyamine content of foods is limited. Furthermore, none of the studies have investigated the polyamine content of bread crumb and crust separately and how it changes after a storage period. It is acknowledged that dietary polyamines have significant impacts, and that the consumption of bread in the world is widespread. The objective of this study was to establish the polyamine concentration in the crust and crumb of three distinct bread types and to ascertain the alteration of polyamine level after four days of storage.

METHODOLOGY

General procedure

As seen at Figure 1, a total of ten samples of three kinds of bread were purchased from ten distinct bakeries. Then, crust and crumb were weighed in the laboratory. Crumb/crust ratio was calculated. The crumb and the crust were then analysed separately. The breads were also analysed once more after four days of storage.

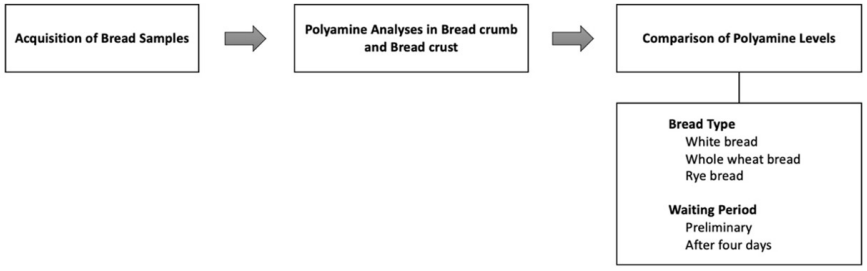


Figure 1. Schematic presentation of analysis stages

Acquisition of bread samples

The breads were collected from 10 bakeries located within the Uskudar district of Istanbul. The samples consisted of 10 loaves of each of the following bread types: white, whole wheat, and rye. The bread samples were purchased in the morning and promptly transported to the laboratory within a few hours to minimise temperature and humidity fluctuations. The experimental phase of the study was conducted in the laboratory of the Regenerative and Restorative Medicine Research Centre at Istanbul Medipol University, Türkiye, in Decem-

ber 2017. The laboratory conditions were maintained at a relative humidity of 50-60% and a temperature range of 20-24°C. The bread samples were analysed on the day of acquisition and four days after storage in the laboratory at 20-24°C and 50-60% humidity. Due to the variations observed in the products formed on the crumb and crust during the baking process, crumb and crust portions of bread samples were analysed separately.

Preparation of samples for analysis

The midpoint of the bread loaves was measured, and slices with a thickness of 2 cm were subsequently cut from this point. A second slice of the same thickness was also cut and stored in the laboratory for analysis conducted four days later. Subsequently, crumb and crust portions were separated. Samples weighing 5.0 g each from the crumb and crust portions were prepared and weighed in 50 mL Falcon tubes. The weighing of samples was performed using a precision scale (Shimadzu ATX224). To each tube, 25 mL of 1.5 M $HClO_4$ was added, and the mixture was vortexed at approximately 5-minute intervals (BioSan, V-1 Plus).

Subsequently, sonication was applied for 5 minutes in a cold ultrasonic bath (Bandelin-Sonorex, RK510). The homogenized bread sample solution underwent centrifugation at 5000 g for 10 minutes at 4 (Biocen, 22R). After centrifugation, 100 of the supernatant was transferred to a new 1.5 mL Eppendorf tube. To this, 100 of cold 1.5 M $HClO_4$ was added and mixed at moderate speed at 25°C for 30 seconds (Benchmark, H4000-HSE). Then, 100 µL of cold 2 M K_2CO_3 was added, and due to the rapid gas formation observed during this process, the procedure was completed as quickly as possible, followed by closing the cap and mixing for 10 seconds. Subsequently, the tube cap was opened, and evaporation was carried out under vacuum (Eppendorf, AG 22331). After this step, the tube caps were closed and mixed at room temperature for 30 seconds. To release any excess gas, the tube cap was opened and closed after a few seconds. The tube was then centrifuged at 15000 g for 10 minutes at 4°C (Biocen, 22R). Following this stage, the sample preparation step for High Performance Liquid Chromatography (HPLC) was initiated. The initial analysis included ten samples, while the subsequent analysis encompassed five samples.

Preparation of solutions

Mobile Phase Solution A: (0.1 M sodium acetate, pH 7.2): 27.3 g of sodium acetate (trihydrate) (Merck) and 96 µL of 6 N HCl (Merck) were dissolved in distilled water. To this solution, 180 mL methanol (Sigma Aldrich) and 10 mL of tetrahydrofuran (Sigma Aldrich) were added. The final volume was adjusted to 2 L.

Mobile Phase Solution B: A solution consisting of 100% HPLC-grade acetonitrile (Merck).

6 N HCl Solution: 49.1 mL of concentrated HCl (37%) was slowly added to 50.9 mL of distilled water and stirred.

1.5 M $HClO_4$ (perchloric acid) Solution: 32.2 mL of 70% $HCLO_4$ (Sigma Aldrich) was diluted to 250 mL with distilled water.

2 M K_2CO_3 Solution: 69.11 g of K_2CO_3 (Merck) was dissolved in 250 mL of distilled water.

1.2% (w/v) Benzoic Acid Solution: 8.4 g of benzoic acid (Sigma Aldrich) was dissolved in 525 mL of distilled water. Then, 175 mL of saturated $K_2B_4O_7$ (potassium tetraborate tetrahydrate) (Sigma Aldrich) solution was added.

40 mM Sodium Borate Buffer Solution (pH 9.5): 30.51 g of $Na_2B_4O_7.10H_2O$ (sodium tetraborate decahydrate – borax) (Merck) was dissolved in distilled water and made up to 2 litres.

Polyamine standard solutions

The solutions were prepared in water of high purity using HPLC. Polyamine standards prepared with ultrapure water in plastic tubes were stored at -80°C for no longer than 6 months.

20 mM Putrescine Solution: A 20 mM putrescine solution was prepared by dissolving 16.12 mg of putrescine.2HCl (Sigma Aldrich) (molecular weight 161.1 g/mol) in 5 mL of water. From the prepared solution, 50 µL was taken and mixed with 950 µL of ultrapure water to obtain a 1 mM putrescine standard solution.

20 mM Spermidine Solution: 25.5 mg of spermidine.3HCl (Sigma Aldrich) (molecular weight: 254.6 g/mol) were dissolved in 5 mL of water. Subsequently, 50 µL were aliquoted from the prepared solution and mixed with 950 µL of distilled water to obtain a 1 mM spermidine standard solution.

20 mM Spermine Solution: 34.9 mg of spermine.4HCl (Sigma Aldrich) (molecular weight: 348.2 g/mol) were dissolved in 5 mL of water. Subsequently, 50 µL were aliquoted from the prepared solution and mixed with 950 µL of distilled water to obtain a 1 mM spermine standard solution.

Standard mixture solution of 100 nmol/mL: 100 µL of each of the three prepared standard solutions were pipetted into an HPLC vial, followed by the addition of 700 µL distilled water.

Standard mixture solution of 10 nmol/mL: 100 μL of the 100 nmol/mL standard mixture solution was taken, followed by the addition of 900 μL of distilled water.

Analysis of the samples

For the HPLC analysis, the standard and sample solutions were prepared in vials. The standard solution was prepared by adding 750 μL of water and 50 μL of 1.2% (w/v) benzoic acid to a 2 mL plastic vial. Then, 50 μL of the standard solution was added on top. For the bread sample, 750 μL of water and 50 μL of 1.2% (w/v) benzoic acid were sequentially added to 2 mL vials. Subsequently, 200 μL of the sample was added on top. The vials were mixed for a period of 10 seconds at room temperature and then introduced into the instrument for injection. The injection volume was set to 10 μL, the injection time to 30 minutes, and the flow rate to 1.0 mL/min. Two injections were made from each sample, and the mean of the two results was calculated.

The analysis of the samples was performed using a Waters Alliance e2695 HPLC instrument equipped with a Waters 2475 FLR detector. The operating wavelengths were set to 450 nm (emission) and 340 nm (extraction). The samples were applied to a Waters WAT086344 column packed with Waters Nova-Pak C18 (150 mm length, 3.9 mm inner diameter, 4.0 μm particle size). The column temperature was maintained at 25°C.

Statistical analysis

The IBM SPSS version 22 software package (Statistical Package for Social Sciences) was employed for statistical analysis. Descriptive statistics, normality checks, and examinations through graphical and analytical methods were conducted. The dependent samples t-test, one way ANOVA, and Kruskal-Wallis analyses were employed. The results were evaluated at a significance level of 5%.

RESULTS and DISCUSSION

Comparison of polyamine level in the crumb and the crust

The level of polyamine presents in crumb and crust regions of bread (except for putrescine) was found to decrease in the following order: rye, whole wheat, and white bread, respectively. The quantities of all kinds of polyamine present in whole wheat bread were found to be significantly higher than those in white bread ($p<0.01$). Similarly, the polyamine content of rye bread was also found to be greater than that of in white bread ($p<0.01$). The spermine level in rye bread was found to be higher than that in whole wheat bread ($p<0.01$) (Table 1). It is estimated that rye bread contained the highest level of total polyamine.

Table 1. Polyamine amounts in bread crumb

Polyamines (nmol/g)	Bread Type (n=10)	Mean	SS	F/Chi* Square	p
Putrescine*	White	4.64	3.53	18.263	p<0.01 (1-2, 1-3)
	Whole wheat	26.16	24.41		
	Rye	16.16	4.31		
Spermidine*	White	16.16	12.54	18.418	p<0.01 (1-2,1-3)
	Whole wheat	46.29	17.02		
	Rye	57.43	14.17		
Spermine*	White	5.30	5.32	19.564	p<0.01 (1-2, 1-3, 2-3)
	Whole wheat	20.42	22.61		
	Rye	29.19	8.56		
Total**	White	26.11	20.64	20.201	p<0.01[a] (1-2, 1-3)
	Whole wheat	92.88	60.43		
	Rye	102.78	25.11		

1. White bread, 2. Whole wheat bread, 3. Rye bread. * Anova, ** Kruskal Wallis [a] $p<0.05$

Regarding the crust, it was observed that the levels of each kind of polyamines in rye bread were higher than those in the white bread ($p<0.01$). Moreover, the putrescine level in white bread was lower than in whole wheat ($p<0.01$). The spermine level of the rye bread was found to be significantly higher than that in the whole wheat bread ($p<0.05$) (Table 2).

Table 2. Polyamine amounts in bread crust

Polyamines (nmol/g)	Bread Type (n=10)	Mean	SS	F/Chi* Square	p[a]
Putrescine*	White	3.78	1.15	7.115	0.003 (1-2, 1-3)
	Whole wheat	7.52	3.93		
	Rye	7.38	1.49		
Spermidine*	White	9.07	4.17	11.42968	0.003 (1-3)
	Whole wheat	13.89	6.70		
	Rye	20.55	7.66		
Spermine*	White	4.79	2.37	12.01548	0.002 (1-3, 2-3)
	Whole wheat	6.56	4.38		
	Rye	12.77	5.61		
Total**	White	17.64	6.87	9.825	0.002 (1-3)
	Whole wheat	27.97	12.77		
	Rye	40.71	14.05		

1.White bread, 2. Whole bread, 3. Rye bread, * Anova, ** Kruskal Wallis, [a] $p<0.05$

The polyamine level after a four-day waiting period

This was the first study investigated the impact of staling on the polyamine content of bread. Although each polyamine varieties were predominantly found in whole bread, the differences among breads were determined to be statistically insignificant. However, the total polyamine content in whole wheat bread was found to be significantly greater than that of white bread ($p<0.05$) (Table 3).

Table 3. Polyamine amount in bread crumb after a four-day waiting period

Polyamines (nmol/g)	Bread Type (n=5)	Mean	SS	F/Chi* Square	p
Putrescine*	White	11.81	2.67	2.283	0.144
	Whole wheat	24.56	16.22		
	Rye	22.47	6.05		
Spermidine*	White	46.90	9.91	3.225	0.076
	Whole wheat	68.72	15.62		
	Rye	56.43	14.63		
Spermine*	White	24.91	4.33	3.740	0.055
	Whole wheat	35.69	9.24		
	Rye	34.75	6.22		
Total**	White	83.63	14.31	3.802	0.042[a] (1-2)
	Whole wheat	128.97	36.09		
	Rye	113.65	24.30		

1.White bread, 2. Whole wheat bread, 3. Rye bread, *Anova **Kruskal Wallis, [a]p<0.05

The results demonstrated that the variations in the levels of putrescine, spermidine, spermine, and total polyamines in the crust of bread, following a four-day waiting period, were statistically insignificant (Table 4).

Table 4. Polyamine amount in bread crust after a four-day waiting period

Polyamines (nmol/g)	Bread Type (n=5)	Mean	SS	F/Chi* Square	p
Putrescine*	White	5.37	0.31	0.527	0.603
	Whole wheat	6.51	2.59		
	Rye	6.55	2.47		
Spermidine*	White	11.64	1.90	0.757	0.490
	Whole wheat	13.78	5.91		
	Rye	9.94	5.90		
Spermine*	White	8.59	2.46	0.763	0.488
	Whole wheat	12.01	5.21		
	Rye	10.11	4.95		
Total**	White	25.59	4.13	0.600	0.564
	Whole wheat	32.31	11.76		
	Rye	26.61	13.11		

The polyamine amount of a single serving of bread

As a result, the polyamine content of 1 g of bread crumb and crust was quantified. Subsequently, the entire bread sample was weighed separately for its crumb and crust portions, enabling the calculation of the crumb-to-crust ratio. The crumb/crust ratio (%) for white, whole wheat and rye bread was estimated 68.16/31.84, 64.66/35.34 and, 58.50/41.50, respectively.

Total polyamine content of a single serving (50 g) of the rye, whole wheat and white bread were 3850.84, 3496.72 and, 1170.32 nmol of polyamines (Figure 2). Therefore, a single portion of rye bread was found to contain the highest level of polyamines. White bread was found to contain nearly three times lower than rye bread. Furthermore, it was determined that spermidine was the most abundant polyamine species in the bread.

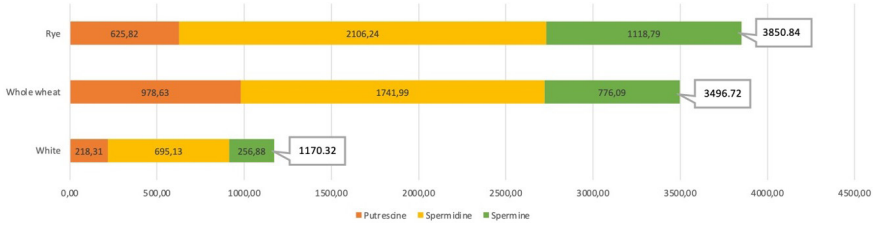


Figure 2. Polyamine content of a single portion (50 g) of bread (nmol)

Since weight and polyamine content of crumb was higher than crust, the polyamine content is greater in the crumb. It is evident that the polyamine level of the rye and whole wheat bread crumb were markedly higher in comparison to white bread. Regarding the crust, the polyamine level in the rye bread is the highest (Figure 3).

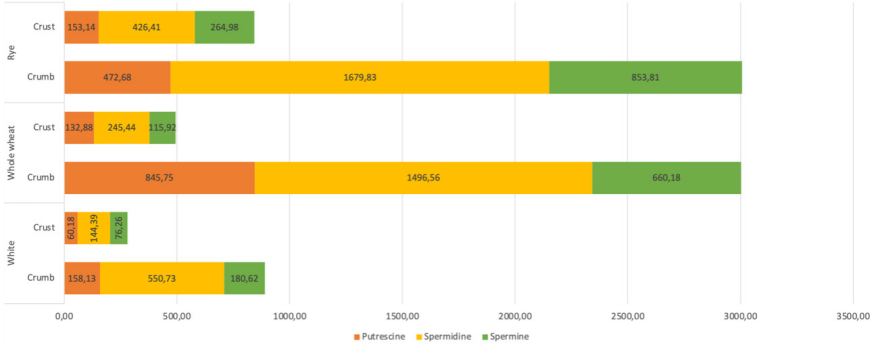


Figure 3. Polyamine content of diverse parts of bread (nmol/1 portion)

Our study was the first to perform separate polyamine analyses in bread crumb and crust, and to investigate how polyamine levels alter after storage. The study revealed that the highest level of polyamine found in both crumb and crust was spermidine, and the lowest level of polyamine was putrescine (except for whole wheat).

Baking is a complex process that involves heat and mass transfer, physical, chemical and biochemical in a product such as volume expansion, evaporation of water, formation of a porous structure, denaturation of protein, gelatinization of starch, crust formation and browning reaction. A dramatic change of physical and chemical property of dough takes place during baking process[14]. There are several ways in which crumb and crust differ from each other. The organoleptic characteristics that result from the baking process are primarily influenced by the geographical origin of the flour and the temperature at which

it is baked. The characteristics of high temperatures of the baking process result in accelerated water evaporation across the surface, leading to a reduction in water content (<20% wet basis) relative to the core[15]. In the crust, the volatile fraction is formed by thermal reactions occurring during baking process. The chemical modifications such as starch gelatinization, gluten coagulation, Maillard reactions and caramelisation of sugars affect the crust setting[16] and ingredients. We hypothesised that the polyamine content of crumb and crust differs from each other. Our results demonstrated that total polyamine levels were higher in crumb compared to crust. It could be because of insulation of the interior part by surrounding dough layers from high temperatures during the baking process. The outer parts of the dough are affected from heat more than interior parts. This causes the proofing process in the centre to continue for a while longer. The centre temperature increases independently of the oven temperature and approaches the boiling point[17]. However, the impact of the baking on polyamine content of bread has not been studied before. To date, only three studies have investigated the various cooking methods except for baking. They all found a reduction of the polyamine level. Muñoz-Esparza et al. revealed that boiling and grilling caused a significant reduction, in contrast to the effects of microwave and sous-vide methods. In the boiling, the reduction may arise from polyamine transfer to the boiling water, while high temperatures during grilling (180°C) may favour the Maillard reaction by the interaction of the primary amino groups of polyamines with reducing sugars[12]. According to Dadáková et al., stewing rabbit saddle caused 20-25% spermidine and spermine losses, while roasting and pan-roasting without oil led to 50% decrease[18]. Similarly, boiling and stewing mutton legs caused 40% losses, while roasting led to 60% reduction[19]. Consequently, high temperatures could be the driving force behind the loss of polyamines in the bread crust.

We hypothesised that the polyamine content varies between the three types of bread, since their content is different. We found that the polyamine content of whole wheat bread was greater than that in the white bread. However, the studies on the polyamine content of the bread species are strictly limited. These studies[20,21] analysed the polyamine content of the bread, however they did not include different kinds of bread. The results of Muñoz-Esparza et al.[12] and Cipolla et al.[22] are similar to our results. We also found that the level of spermidine was the highest. Similar results were reported by other studies[20,22,23]. Muñoz-Esparza et al. indicated the highest level of spermidine in wheat germ (440.6 mg/kg) among the polyamine content in cereal and derivatives. Polyamines can act as growth factors, having an important role for germination[24]. Indeed, white bread is made from the refined flour, which

constitutes 80% of the endosperm, while whole wheat flour contains the bran and the germ, which represents about 3% of the grain[12]. The higher amount of bran and the germ may lead the higher spermidine level in whole wheat bread compared to white bread. Nordlund et al. demonstrated that bread prepared with refined, whole wheat and rye flour exhibited different composition and texture characteristics. Whole wheat and rye breads contain higher level of dietary fibre compared to white bread[25]. According to Constantinescu, replacing 15% of wheat flour with rye increased the dietary fibre content 10% in rye bread[26]. Although we did not analyse the fibre in the bread, it appears that the amount of polyamine rises in accordance with the fibre content of the bread. The separation of the germ and bran from the endosperm in white bread may result in a reduction in polyamine content compared to that observed in whole wheat bread. Two studies have resulted as we suggested. Nishimura et al. reported that higher polyamine levels present in the rice bran than in the rice[21]. Karayigit et al. demonstrated that the polyamine content of whole wheat flour is higher than that of white flour[27]. However, the association between the fibre and the polyamine remains to be elucidated.

In addition to the natural presence of polyamines in foods, putrescine can also be produced through microbial[12]. Del Rio et al. found that Lactobacillus rossiae strain isolated from sourdough produces putrescine from arginine[28]. Sourdough bread is a fermented product; however, we analysed bakery-made bread produced from straight dough. The relationship between putrescine levels in bread and the yeast products used remains to be elucidated. Kobayashi et al. reported that the selection of the starter cultures with high spermidine productivity improved polyamine levels in natto, a traditional Japanese fermented soy food[29].

Our present study is the one of the first to explore the alteration of polyamine levels in bread after storage. We hypothesised that the polyamine content would alter after storage, as the quality of bread is disrupted after the production. The changes that occur aside from those influenced by microorganisms are referred to as staleness. During this process, the hardness of the bread increases, and its crumbliness is enhanced, while its water retention capacity is diminished, along with the amount of soluble starch within the bread. Crust and crumb of bread exhibit distinct behaviours. It is hypothesised that a substantial part of alterations occurs within the bread when it is taken out of oven are result of the diffusion of moisture from the surrounding air and the water present within the bread into the crust[30]. Our study revealed that the total polyamine content in both crumb and crust increased after stored four days at

20-24°C (except for rye breadcrust). However, only two studies have examined storage impact. The first showed a decline in spermine and spermidine levels in red meat and offal after eight months at -18°C and 15-20% decrease after 9 days at +2°C[18]. The second found about 20% loss of spermidine and 50% of spermine mutton loins stored at -18°C for 6 months with significant losses aerobic, vacuum-packaged or in a modified atmosphere condition at +2°C[19]. However, the storage conditions and foodstuff in our study are quite different, making comparisons difficult.

Dietary polyamines have been linked to various health benefits, including improved health and reduced mortality[31], benefits in dementia and Alzheimer's disease[32], cardiovascular health[33,34]. They may also enhance gut barrier function in short bowel syndrome[35], reduce inflammation[36], age-associated DNA methylation alterations, and tumorigenesis[37], and attenuates ischemia/reperfusion injury[38,39]. However, their role in cancer is controversial. Some studies suggest benefits for colon health[40] and oral cancer[41], while others highlight their involvement in cancer development[42-44]. Spermidine and putrescine are linked to a lower risk of type 2 diabetes, while spermine is linked to a higher risk[45]. Spermidine promotes wound healing[46], improves mitochondrial respiration and cognitive function[47], supports pulmonary system[48], offers cardioprotective effects by increasing cardiac autophagy and mitophagy, decreases blood pressure[49], and reduces lipid accumulation and core formation in atherosclerotic plaques[50]. Spermine promotes bone formation[51], and putrescine benefits the female reproductive system[52,53]. Therefore, dietary intake of polyamine is crucial for maintaining overall health. The diet provides more polyamines than the de novo biosynthesis pathway[3]. According to the recommendations of EFSA, 45-65% of energy intake should come from carbohydrates[54], richest source of which is bread and cereals. Bread is considered a staple food, contributing no less than 10% of energy requirements in the global population[10]. EU citizens are estimated to consume on average 160 g of bread per person per day, corresponding to 4-5 slices of bread[55]. In Türkiye, 39.5% of daily energy intake comes from bread and grains[11], and daily bread consumption is 134 g for women and 227 g for men[11]. Therefore, cereals and bread could contribute significantly to daily polyamine intake. In Türkiye, the daily polyamine intake is 135.899 nmol/day/person, with 12.75% coming from wheat products and 1.6–5.3% from bread[2].

This study is subject to several limitations that must be acknowledged. The utilisation of industrially produced breads rendered the ingredients unknown, and consequently, the bread production methods, such as the temperature and

duration of baking, were not considered. The genetic and cultivar characteristics of each grain were not incorporated into the study, even though they can exert a substantial influence on the dietary fibre content, as well as endosperm and germ composition of grains. The sample size may not be sufficient to capture all variations, and geographical limitations should be considered, as only bread from Istanbul was included, potentially differing from other regions. The consistency of the temperature and humidity during the storage and the separate analysis of the crumb and crust further reinforced the results. Future studies could benefit from analysing breads from different geographical region and including a wider variety of bread types. Furthermore, increasing the sample size would enhance the reliability of the findings.

In terms of human health, an increased intake of the dietary polyamine is crucial in certain health conditions. Bread plays a significant role in the diet, making it an important source of polyamines. The total polyamine content was found to be higher in the crumb than in the crust. Spermidine levels were found to be the highest across all three types of bread. The total polyamine content was estimated to increase in the following order: white, whole wheat, and rye bread, in both the crumb and crust. Regarding storage, the polyamine levels in both the crumb and crust increased after four days at 20-24°C except for rye breadcrust.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Ayse Lamia Ozturk: Design study, practical performance, data analysis, preparation manuscript; Nihal Buyukuslu: Design study, data analysis, preparation manuscript, critical review manuscript; Cuneyd Parlayan: Practical performance, data analysis.

FUNDING SOURCES

The authors declare no funding for the study.

ACKNOWLEDGMENTS

We would like to thank Orhan Çakan for his technical expertise at laboratory process. Also, we are grateful to Dr. Pakize Yiğit for her support at statistics.

REFERENCES

1. Handa AK, Fatima T, Mattoo AK. Polyamines: bio-molecules with diverse functions in plant and human health and disease. Front Chem, 2018;6:1-18. Doi: 10.3389/fchem.2018.00010

2. Buyukuslu N, Hizli H, Esin K, Garipagaoglu M. A cross-sectional study: nutritional polyamines in frequently consumed foods of the Turkish population. Foods, 2014;3(4):541-557. Doi: 10.3390/foods3040541

3. Kalač P. Health effects and occurrence of dietary polyamines: a review for the period 2005–mid 2013. Food Chem, 2014;161:27-39. Doi: 10.1016/j.foodchem.2014.03.102

4. Ramani D, De Bandt JP, Cynober L. Aliphatic polyamines in physiology and diseases. Clin Nutr, 2014;33(1):14-22. Doi: 10.1016/j.clnu.2013.09.019

5. Cipolla B, Guillé F, Moulinoux JP. Polyamine-reduced diet in metastatic hormone-refractory prostate cancer (HRPC) patients. Biochem Soc Trans, 2003;31(2):384-387. Doi: 10.1042/bst0310384

6. Cipolla B, Bansard JY, Ecalard JP, Moulinoux JP. Treating metastatic castration-resistant prostate cancer with novel polyamine-free oral nutritional supplementation: phase I study. BioMedicine, 2013;3(3):114-119. Doi: 10.1016/j.biomed.2013.04.005

7. Büyükuslu N. Dietary polyamines and diseases: reducing polyamine intake can be beneficial in cancer treatment. J Nutrients, 2015;2(2):27-38. Doi: 10.18488/journal.87/ 2015.2.2/87.2.27.38

8. Soda K. Overview of polyamines as nutrients for human healthy long life and effect of increased polyamine intake on DNA methylation. Cells, 2022;11(1):164. Doi: 10.3390/cells11010164

9. Jones JM, García CG, Braun HJ. Perspective: whole and refined grains and health—evidence supporting "make half your grains whole." Adv Nutr, 2019;11(3):492-506. Doi: 10.1093/advances/nmz114

10. Ribet L, Kassis A, Jacquier E, Monnet C, Durand-Dubief M, Bosco N. The nutritional contribution and relationship with health of bread consumption: a narrative review. Crit Rev Food Sci Nutr, 2024;1-28. Doi: 10.1080/10408398.2024.2428593

11. Ministry of Health of Turkey, General Directorate of Public Health. Turkey Nutrition and Health Survey (TNHS). Publication No: 1132. Ankara: Ministry of Health; 2019.

12. Muñoz-Esparza NC, Costa-Catala J, Comas-Basté O, Toro-Funes N, Latorre-Moratalla ML, Veciana-Nogués MT, et al. Occurrence of polyamines in foods and the influence of cooking processes. Foods, 2021;10(8):1752. Doi: 10.3390/foods10081752

13. Binh PNT, Soda K, Maruyama C, Kawakami M. Relationship between food polyamines and gross domestic product in association with longevity in Asian countries. Health, 2010;2(12):1390-1396. Doi: 10.4236/health.2010.212206

14. Dessev T, Lalanne V, Keramat J, Jury V, Prost C, Le-Bail A. Influence of baking conditions on bread characteristics and acrylamide concentration. J Food Sci Nutr Res, 2020;3(4):291-310. Doi: 10.26502/jfsnr.2642-11000056

15. Vanin FM, Lucas T, Trystram G. Crust formation and its role during bread baking. Trends Food Sci Technol, 2009;20(8):333-343. Doi: 10.1016/j.tifs.2009.04.001

16. Cho IH, Peterson DG. Chemistry of bread aroma: a review. Food Sci Biotechnol, 2010;19(3):575-582. Doi: 10.1007/s10068-010-0081-3

17. Cauvain SP. Breadmaking: an overview. In: Cauvain SP, editor. Breadmaking: improving quality. 2nd ed. Cambridge: Woodhead Publishing; 2012. p. 8-28. Doi: 10.1533/9781855737129.8

18. Dadáková E, Pelikánová T, Kalač P. Concentration of biologically active polyamines in rabbit meat, liver and kidney after slaughter and their changes during meat storage and cooking. Meat Sci, 2012;90(3):796-800. Doi: 10.1016/j.meatsci.2011.11.017

19. Dadáková E, Pelikánová T, Kalač P. Concentration of biologically active polyamines in meat and liver of sheep and lambs after slaughter and their changes in mutton during storage and cooking. Meat Sci, 2011;87(2):119-124. Doi: 10.1016/j.meatsci.2010.09.009

20. Nishibori N, Fujihara S, Akatuki T. Amounts of polyamines in foods in Japan and intake by Japanese. Food Chem, 2007;100(2):491-497. Doi: 10.1016/j.foodchem.2005.09.070

21. Nishimura K, Shiina R, Kashiwagi K, Igarashi K. Decrease in polyamines with aging and their ingestion from food and drink. J Biochem, 2006;139(1):81-90. Doi: 10.1093/jb/mvj003

22. Cipolla BG, Havouis R, Moulinoux JP. Polyamine contents in current foods: a basis for polyamine reduced diet and a study of its long term observance and tolerance in prostate carcinoma patients. Amino Acids, 2007;33(2):203-212. Doi: 10.1007/s00726-007-0524-1

23. Eliassen KA, Reistad R, Risoen U, Ronning HF. Dietary polyamines. Food Chem, 2002;78(3):273-280. Doi: 10.1016/S0308-8146(01)00405-8

24. Chen D, Shao Q, Yin L, Younis A, Zheng B. Polyamine function in plants: metabolism, regulation on development, and roles in abiotic stress responses. Front Plant Sci, 2019;9:429326. Doi: 10.3389/fpls.2018.01945

25. Nordlund E, Katina K, Mykkänen H, Poutanen K. Distinct characteristics of rye and wheat breads impact on their *in vitro* gastric disintegration and *in vivo* glucose and insulin responses. Foods, 2016;5(2):24. Doi: 10.3390/foods5020024

26. Pop G. Quality of high-fiber wheat bread improved with rye flour. J Agroaliment Process Technol, 2009;15(2):287-292.

27. Karayigit B, Colak N, Ozogul F, Gundogdu A, Inceer H, Bilgiçli N, et al. The biogenic amine and mineral contents of different milling fractions of bread and durum wheat (*Triticum* L.) cultivars. Food Biosci, 2020;37:100676. Doi: 10.1016/j.fbio.2020.100676

28. Del Rio B, Alvarez-Sieiro P, Redruello B, Martin MC, Fernandez M, Ladero V, et al. *Lactobacillus rossiae* strain isolated from sourdough produces putrescine from arginine. Sci Rep, 2018;8:3989. Doi: 10.1038/s41598-018-22309-6

29. Kobayashi K, Shimojo S, Watanabe S. Contribution of a fermentation process using *Bacillus subtilis* (natto) to high polyamine contents of natto, a traditional Japanese fermented soy food. Food Sci Technol Res, 2016;22(1):153-157. Doi: 10.3136/fstr.22.153

30. Gerçekaslan KE, Kotancılar HG, Karaoğlu MM, Ertugay MF. Ekmek bayatlaması ve bayatlama derecesini ölçmede kullanılan yöntemler: II. Gıda, 2008;33(1):27-34.

31. Madeo F, Hofer SJ, Pendl T, Bauer MA, Eisenberg T, Carmona-Gutierrez D, et al. Nutritional aspects of spermidine. Annu Rev Nutr, 2020;40:135-159. Doi: 10.1146/annurev-nutr-120419-015419

32. Qian M, Zhang N, Zhang R, Liu M, Wu Y, Lu Y, et al. Non-linear association of dietary polyamines with the risk of incident dementia: results from population-based cohort of the UK Biobank. Nutrients, 2024;16(16):2774. Doi: 10.3390/nu16162774

33. Han S, Qian M, Zhang N, Zhang R, Liu M, Wang J, et al. The association of dietary polyamines with mortality and the risk of cardiovascular disease: a prospective study in UK Biobank. Nutrients, 2024;16(24):4335. Doi: 10.3390/nu16244335

34. Soda K, Kano Y, Chiba F. Food polyamine and cardiovascular disease – an epidemiological study. Glob J Health Sci, 2012;4(6):170-178. Doi: 10.5539/gjhs.v4n6p170

35. Kasahara N, Teratani T, Yokota S, Sakuma Y, Sasanuma H, Fujimoto Y, et al. Dietary polyamines promote intestinal adaptation in an experimental model of short bowel syndrome. Sci Rep, 2024;14(1):4605. Doi: 10.1038/s41598-024-55258-4

36. Soda K, Uemura T, Sanayama H, Igarashi K, Fukui T. Polyamine-rich diet elevates blood spermine levels and inhibits pro-inflammatory status: an interventional study. Med Sci (Basel), 2021;9(2):22. Doi: 10.3390/medsci9020022

37. Soda K, Kano Y, Chiba F, Koizumi K, Miyaki Y. Increased polyamine intake inhibits age-associated alteration in global DNA methylation and 1,2-dimethylhydrazine-induced tumorigenesis. PLoS One, 2013;8(5):e64357. Doi: 10.1371/journal.pone.0064357

38. Doi J, Fujimoto Y, Teratani T, Kasahara N, Maeda M, Tsuruyama T, et al. Bolus administration of polyamines boosts effects on hepatic ischemia-reperfusion injury and regeneration in rats. Eur Surg Res, 2019;60(1-2):63-73. Doi: 10.1159/000497434

39. Okumura S, Teratani T, Fujimoto Y, Zhao X, Tsuruyama T, Masano Y, et al. Oral administration of polyamines ameliorates liver ischemia/reperfusion injury and promotes liver regeneration in rats. Liver Transplant, 2016;22(9):1231-1244. Doi: 10.1002/lt.24471

40. Vargas AJ, Ashbeck EL, Wertheim BC, Wallace RB, Neuhouser ML, Thomson CA, et al. Dietary polyamine intake and colorectal cancer risk in postmenopausal women. Am J Clin Nutr, 2015;102(2):411-419. Doi: 10.3945/ajcn.114.103895

41. Coeli-Lacchini FB, da Silva G, Belentani M, Alves JSF, Ushida TR, Lunardelli GT, et al. Spermidine suppresses oral carcinogenesis through autophagy induction, DNA damage repair, and oxidative stress reduction. Am J Pathol, 2023;193(12):2172-2181. Doi: 10.1016/j.ajpath.2023.09.005

42. Vargas AJ, Wertheim BC, Gerner EW, Thomson CA, Rock CL, Thompson PA. Dietary polyamine intake and risk of colorectal adenomatous polyps. Am J Clin Nutr, 2012;96(1):133-141. Doi: 10.3945/ajcn.111.030353

43. Huang CY, Fang YJ, Abulimiti A, Yang X, Li L, Liu KY, et al. Dietary polyamines intake and risk of colorectal cancer: a case-control study. Nutrients, 2020;12(11):3575. Doi: 10.3390/nu12113575

44. Arisan ED, Çoker A, Palavan-Ünsal N. Polyamine depletion enhances the roscovitine-induced apoptosis through the activation of mitochondria in HCT116 colon carcinoma cells. Amino Acids, 2012;42(2-3):655-665. Doi: 10.1007/s00726-011-1040-x

45. Zhang X, Qian M, Liu M, He M, Li FR, Zheng L. The associations of dietary polyamines with incident type 2 diabetes mellitus: a large prospective cohort study. Nutrients, 2025;17(1):186. Doi: 10.3390/nu17010186

46. Ito D, Ito H, Ideta T, Kanbe A, Ninomiya S, Shimizu M. Systemic and topical administration of spermidine accelerates skin wound healing. Cell Commun Signal, 2021;19(1):36. Doi: 10.1186/s12964-021-00717-y

47. Schroeder S, Hofer SJ, Zimmermann A, Pechlaner R, Dammbrueck C, Pendl T, et al. Dietary spermidine improves cognitive function. Cell Rep, 2021;35(2):108985. Doi: 10.1016/j.celrep.2021.108985

48. Chen YJ, Li HF, Zhao FR, Yu M, Pan SY, Sun WZ, et al. Spermidine attenuates monocrotaline-induced pulmonary arterial hypertension in rats by inhibiting purine metabolism and polyamine synthesis-associated vascular remodeling. Int Immunopharmacol, 2024;132:111946. Doi: 10.1016/j.intimp.2024.111946

49. Eisenberg T, Abdellatif M, Zimmermann A, Schroeder S, Pendl T, Harger A, et al. Dietary spermidine for lowering high blood pressure. Autophagy, 2017;13(4):767-769. Doi: 10.1080/15548627.2017.1280225

50. Michiels CF, Kurdi A, Timmermans JP, De Meyer GRY, Martinet W. Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques via induction of autophagy. Atherosclerosis, 2016;251:319-327. Doi: 10.1016/j.atherosclerosis.2016.07.899

51. Jiang RX, Hu N, Deng YW, Hu LW, Gu H, Luo N, et al. Potential therapeutic role of spermine via Rac1 in osteoporosis: Insights from zebrafish and mice. Zool Res, 2024;45(2):367-380. Doi: 10.24272/j.issn.2095-8137.2023.371

52. Tao Y, Tartia A, Lawson M, Zelinski MB, Wu W, Liu JY, et al. Can peri-ovulatory putrescine supplementation improve egg quality in older infertile women? J Assist Reprod Genet, 2019;36(3):395-402. Doi: 10.1007/s10815-018-1327-x

53. Liu D, Mo G, Tao Y, Wang H, Liu XJ. Putrescine supplementation during *in vitro* maturation of aged mouse oocytes improves the quality of blastocysts. Reprod Fertil Dev, 2017;29(7):1392-1400. Doi: 10.1071/RD16061

54. European Food Safety Authority. EFSA sets European dietary reference values for nutrient intakes. *EFSA* [Internet]. European Food Safety Authority; 2020 [Feb 24, 2025]. Available from: https://www.efsa.europa.eu/en/press/news/nda100326

55. Association Internationale de la Boulangerie Industrielle (AIBI). AIBI bread market report 2013. AIBI; 2015.

Acta Pharm. Sci. Vol 63:(3), 2025
Original article
DOI: 10.23893/1307-2080.APS6345

Content analysis of pictograms used in pharmaceutical leaflets in Turkey

Fikriye YILMAZ[1*], Sema DOKME YAGAR[2], Deniz GUNGOR OZCAN[1], Zelal OZYILDIZ[1]

1 Baskent University, Faculty of Health Sciences, Department of Healthcare Management, Ankara, Türkiye

2 Kahramanmaras Sutcu Imam University, Faculty of Health Sciences, Department of Healthcare Management, Kahramanmaras, Türkiye

ABSTRACT

This study investigates the use of pictograms in pharmaceutical leaflets in Turkey as a means to enhance patient understanding and compliance with medication instructions. The research examines the frequency, characteristics, and effectiveness of pictograms in drug instructions licensed by the Turkish Medicines and Medical Devices Agency. A cross-sectional, exploratory, and descriptive approach was used to analyze 17,709 drugs, with a focus on 1,959 drugs that feature at least one pictogram. Findings indicated that 11.1% (n=1,959) of licensed drugs utilized pictograms, mainly for sensory organ and respiratory system medications requiring specific administration. The study found most pictograms to be simple, averaging under seven images. Notably, 55.95% (n=1,095) included human figures, yet only 32.8% (n=359) depicted full faces or bodies. Pictograms with accompanying text were infrequent, but 96.3% (n=428) of the included text was readable. Consequently, promoting the widespread use of a standardized pictogram set within the Turkish pharmaceutical sector is crucial.

Keywords: health communication, medication adherence, pharmaceutical leaflets, pharmaceutical pictograms, Turkey

* Corresponding author: Fikriye YILMAZ
E-mail: fyilmaz@baskent.edu.tr
ORCIDs:
Fikriye YILMAZ: 0000-0002-4884-3803
Sema DOKME YAGAR: 0000-0003-0298-7534
Deniz GUNGOR OZCAN: 0000-0003-2693-4576
Zelal OZYILDIZ: 0000-0002-6561-6864
(Received 17 Mar 2025, Accepted 5 May 2025)

INTRODUCTION

Medicines, defined as chemicals or compounds used for the treatment, prevention, and management of diseases, are the most common intervention in healthcare[1]. Although thousands of medicines have been developed to combat diseases today, medication adherence is the cornerstone of treatment effectiveness and success[2]. According to the World Health Organization, medication adherence is defined as "the extent to which a person's behavior coincides with the recommendations of the healthcare provider"[3]. Medication non-adherence, a global health issue, is viewed as the failure to translate decades of enormous financial and human capital investment into the development of proven treatments that improve clinical outcomes[4]. Despite more than half a century of dedicated work and interventions, it has been reported that global medication adherence still does not exceed the 50% reported twenty years ago[5] and this continues to negatively affect the quality of life, life expectancy, health outcomes, and healthcare costs worldwide[6,7].

Medication non-adherence can be caused by various factors related to treatment, the patient, or the healthcare system[8]. However, especially in outpatient settings, it is the patient's responsibility to apply the information provided about medical treatment appropriately[9]. Understanding medication therapy is critical for adherence and the safe and effective use of medicines. One of the risk factors for medication non-adherence is the inability to retain verbal information[10]. Studies show that 40-80% of the information provided by healthcare professionals is quickly forgotten, as patients tend to focus more on clinical diagnosis information and often cannot recall information about medication therapy[3,11,12]. This situation worsens when health literacy is low[13,14]. Moreover, it is recognized that text-based instructions, which are the most accessible and frequently consulted source of information for patients, are difficult to understand even for literate individuals due to their complex designs, layouts, technical terms, and language[12,15]. This challenge is even greater for immigrants and tourists due to language barriers[9].

One way to facilitate patients' understanding of prescribed pharmacotherapy is by supplementing labels and instructions with visual tools such as pharmaceutical pictograms[9,16]. Pictograms represent actions (e.g., putting drops in the eye) graphically to ensure that the underlying meaning is understood independently of the patient's literacy skills[17]. A pictogram is defined as a two-dimensional figurative/ metaphorical drawing designed to attract attention and convey information or express an idea[18]. Studies have shown that pictograms improve patients' understanding of the correct use of medicines and, consequently, their adherence[9,15,19,20,21,22].

The use of pictograms has gained increasing attention in recent years, likely due to growing awareness among healthcare professionals of the need to provide sufficient information to patients who have difficulty understanding their treatments, such as the elderly, children, and those with low literacy levels[23-26]. In pharmacy, two reference systems are commonly used for pictograms: The United States Pharmacopeia (USP) and the International Pharmaceutical Federation (FIP). The USP offers 82 pictograms that are available for free download after accepting the license agreement[27]. USP pictograms are standardized graphic images designed to help communicate drug instructions, precautions, and/or warnings to patients and consumers, and they are widely used in Western countries. However, studies on their availability and comprehensibility in countries like South Africa have highlighted potential limitations[6]. In contrast, FIP pictograms, developed in June 2009, have been pre-tested in different populations and were most recently updated in February 2017 to address comprehensibility issues[28-29].

In Turkey, the information that must be included in the packaging and instructions for use of licensed or authorized human medicinal products to ensure their correct use for the health and safety of individuals is regulated by Law No. 30048, dated April 25, 2017, which states that symbols and pictorial diagrams that are useful to users and other information consistent with the summary of product characteristics may be included[30]. Thus, there is no standard or requirement for the use of pictograms. To date, no research has been found in the literature that evaluates the use of pictograms in drug instructions in Turkey. Considering current legal regulations and the literature, it is necessary to determine the characteristics of pictograms used in licensed drugs in Turkey. This exploratory and descriptive study aims to determine the frequency of pictogram use in the instructions for use of licensed drugs in Turkey and to reveal the qualitative and quantitative characteristics of the pictograms used. Considering that medication compliance plays a vital role in achieving positive health outcomes, easily understandable and consistent pictograms can greatly enhance patients' ability to follow their medication instructions accurately. Consequently, this research holds the potential to highlight areas for improvement in medication adherence through the strategic implementation of standardized visual cues.

METHODOLOGY

This cross-sectional exploratory and descriptive study was conducted to determine the frequency of pictogram use in the patient information leaflets of the drugs licensed by the Turkish Medicines and Medical Devices Agency and to examine the characteristics of the pictograms used[31]. The population of the study consists of 17,709 drugs licensed by the Turkish Medicines and Medical Devices

Agency and listed in the summary of product characteristics/patient information leaflets list on the agency's website as of January 2024. In the first stage of the study, no sampling was conducted, and the patient information leaflets of all drugs in the list were examined. Content analysis was performed for 1,959 drugs with pictograms in their patient information leaflets. These pictograms are manufacturer-generated as no standard pictogram set exists in Turkey for pharmaceuticals. Content analysis, originally developed for analyzing written and verbal texts, was used in this study based on its definition as "the systematic, objective, and quantitative analysis of message characteristics"[32]. Visual content analysis involves methodologically examining and analyzing a series of images for the presence of common visual elements and the frequency of repeated visual elements[32]. The visual content analysis followed four steps: 1) defining the criteria for selecting pictograms, 2) developing categories for coding, 3) coding the images, and 4) analyzing the results[33]. The inclusion criterion for pictogram selection was that the images in the patient information leaflets should consist of at least two images, or if there was only one symbol/image, it should have a reference/meaning related to the application/use. There is no standard or tool for evaluating the appropriateness of pharmaceutical pictograms. Based on the literature, the following categories were determined for the content analysis of the pictograms, allowing for a more objective assessment[34]:

1. Concrete or abstract nature of the pictogram: Pictograms depicting real objects were considered concrete, while design elements that did not pictorially represent objects (e.g., arrows, lines, shapes, and letters) were considered abstract.

2. Drawing/real image status.

3. Use of a human face/body in the pictogram.

4. Complexity or simplicity of the pictogram: Pictograms with more visual elements and details were considered complex, while those with fewer visual elements and details were considered simple, and the number of visuals in the pictogram was used as a measure.

5. Clarity of the pictogram: Assessed based on whether the pictogram was clear or blurry.

6. Use of text in the pictogram: Assessed based on the presence of text in the pictogram and the readability of the text.

Python programming language was used for coding the pictograms and for visual content analysis. The images were uploaded to the Colab editor and opened with the Pillow library. Tesseract OCR (Optical Character Recognition) was used to

separate and analyze the texts. Features such as clarity and the number of shapes in the images were evaluated using the OpenCV library. The results obtained were divided into separate rows using the Pandas library and saved as an Excel document. The results were also manually checked by comparing them with the images.

In addition to visual content analysis, some pharmaceutical information about the drugs was obtained from the Turkish Medicines and Medical Devices Agency database:

- The pharmaceutical form of the drug.
- The drug's ATC code.
- Prescription type.
- Status on the essential/children's essential drug list.

All variable data for the 1,959 drugs included in the study were combined into an Excel file. Descriptive statistics were used to evaluate the analysis results.

RESULTS and DISCUSSION

As of January 2024, it was determined that 11.1% (n=1,959) of the 17,709 drugs licensed by the Turkish Medicines and Medical Devices Agency included at least one pictogram in their patient information leaflets, while 88.9% (n=15,750) did not. Figure 1 shows the distribution of pictogram use by ATC code. As seen in Figure 1, among 17,709 licensed drugs, pictograms were used in 222 (63%) of total 350 drugs for sensory organs and 529 (30%) of total 1,735 drugs for the respiratory system. Pictogram use was lowest for 31 cardiovascular system drugs (1% of total 2,067 drugs) and 29 musculoskeletal system drugs (2% of total 1,161 drugs).

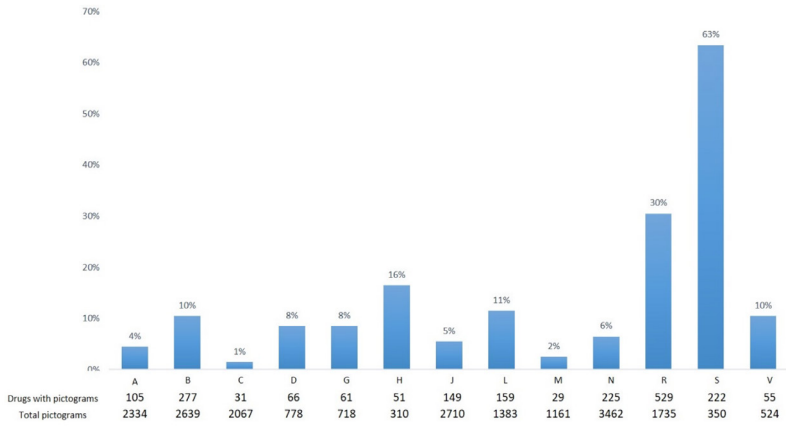


Figure 1. Pictogram use in 17,709 licensed drugs in Turkey by ATC codes (%)

The pharmaceutical characteristics of the 1,959 drugs that included at least one pictogram in their package leaflets are shown in Table 1.

Table 1. Some pharmaceutical characteristics of drugs with pictograms in the patient information leaflets

ATC Code	Number (n)	Percent (%)
A: Gastrointestinal tract and metabolism	105	5.36
B: Blood and blood-forming organs	277	14.4
C: Cardiovascular system	31	1.58
D: Dermatologicals	66	3.37
G: Genitourinary system and sex hormones	61	3.11
H: Systemic hormonal preparations excluding sex hormones and insulins	51	2.60
J: Anti-infective for systemic use	149	7.61
L: Antineoplastic and immunomodulation agent.	159	8.12
M: Musculoskeletal system	29	1.48
N: Nervous system	225	11.49
R: Respiratory system	529	27.00
S: Sensory organs	222	11.33
V: Various	55	2.81
Total	1,959	100
Form of the drug*		
Solid Dosage Forms I	102	5.21
Solid Dosage Forms II	49	2.50
Solid Dosage Forms III	64	3.27
Solid Dosage Forms Pre-Dissolved or Dispersed in Water	67	3.42
Liquid Formulations	108	5.51
Parenteral Preparations I	42	2.14
Parenteral Preparations II	84	4.29
Parenteral Preparations III	543	27.72
Sterile Eye/Ear/Nose Preparations	322	16.44
Locally Acting Semi-Solid Preparations	55	2.81
Locally Effective Liquid Preparations	34	1.74

Preparations applied by different routes (rectal and vaginal)	18	0.92
Powder-Containing Inhaler Preparations	243	12.40
Metered Dose Inhalers and Nebulization Solutions	228	11.64
Total	1,959	100
Prescription Status**		
Normal	1,816	92.70
Purple	58	2.96
Green	16	0.82
Orange	64	3.27
Red	5	0.26
Total	1,959	100
Essential Medicines List Status		
0: Medicines not on the WHO list	1168	59.62
1: Medicines that are fully compatible with the WHO list in terms of active substance, dose and formulation.	418	21.34
2: Medicines that are on the WHO list as active substance but are not compatible with this list in terms of dosage and formulation.	373	19.04
Total	1,959	21.34
Child Essential Medicines List Status		
0: Medicines not on the WHO list	1,468	74.94
1: Medicines that are fully compatible with the WHO list in terms of active substance, dose and formulation.	261	13.32
2: Medicines that are on the WHO list as active substance but are not compatible with this list in terms of dosage and formulation.	230	11.74
Total	1,959	100

*Solid Dosage Forms I; Tablet, Film-Coated Tablet, Sugar-Coated Tablet (Drage), Chewable Tablet Buccal Tablets, Sublingual Tablets.

Solid Dosage Forms II; Capsules, Soft Gelatin Capsule, Hard Gelatin Capsule.

Solid Dosage Forms III; Orodispersible Tablets.

Solid Dosage Forms Pre-Dissolved or Dispersed in Water; Granules, Effervescent Granules Coated Granules, Gastro resistant Granules Powders, Effervescent Powders.

Liquid Formulations; Oral Solution, Oral Drops Syrup, Emulsion Suspension.

Parenteral Preparations I; Ampoule/Vial Containing Solution Ampoule/Vial Containing Suspension, Ampoule/Vial Containing Powder + Solvent Ampoule.

Parenteral Preparations II; Solution for Infusion Vial/Ampoule Concentrate Solution for Infusion Vial/Ampoule Powder for Infusion.

Parenteral Preparations III; Lyophilized Powder/Suspension/Solution in Ready-to-Use

Syringe/Syringe Cartridge Solution/Suspension in Injection Pen.

Sterile Eye/Ear/Nose Preparations; Solution, Suspension, Emulsion, Ointment.

Locally Acting Semi-Solid Preparations; Gel, Cream/Lotion, Ointment.

Locally Effective Liquid Preparations; Solution, Suspension, Emulsion.

Preparations applied by different routes (rectal and vaginal); Enema, Rectal Foam, Suppositories, Pessaries.

Powder-Containing Inhaler Preparations; Capsules/Blister/Inhaler Containing Powder.

Metered Dose Inhalers and Nebulization Solutions; Nebulization Solution (Inhalation Solution) Metered Dose Inhaler / Inhalation Aerosol.

** Color codes[35]:

Normal: Non-controlled drugs are given with a white prescription.

Green: Prescription issued for drugs with a potential for addiction and abuse.

Red: Prescriptions written for drugs containing internationally controlled agents such as opioids and cocaine.

Purple: Prescriptions issued for blood and blood products.

Orange: Prescriptions issued for some blood products for hemophilia patients.

As seen in Table 1, the drug groups in which pictograms were used the most were determined as respiratory system drugs with 27%, and drugs for blood and blood-forming organs with 14.1%. The drug forms in which pictograms were commonly used were Parenteral preparations III with 27.7% (example Figure 2A), and Sterile Eye/Ear/Nose Preparations with 16% (example Figure 2B). 92.7% of the drugs with pictograms in the patient information leaflets are used with a normal prescription. 59.6% of the drugs with pictograms in the patient information leaflets are not included in the essential drugs list, and 74.9% are not included in the essential drugs list for children. Table 2 shows the content analysis results of the pictograms of 1,959 drugs with pictograms in the patient information leaflets.

Table 2. Content analysis results of pictograms in patient information leaflets

Pictogram usage	Number (n)	Percent (%)
Used in more than one leaflet for different doses/forms	1,438	73.40
Used in only one patient information leaflet	521	26.60
Total	1,959	100
Number of images		
<7 image	1,355	69.16
≥7 image	604	30.83
Total	1,959	100
Drawing/actual situation		
Drawing	1,864	95.15
Real image	95	4.85
Total	1,959	100
Clarity of the pictogram		
Clear	1,665	84.99
Blurry	294	15.01
Total	1,959	100
The presence of a human face in a pictogram		
None	864	44.05
Available	1,095	55.95
Total	1,959	100
Integrity of the human face		
Not Complete	736	67.2
Complete	359	32.8
Total	1,095	100
Use of text in pictograms		
None	1,515	77.34
Available	444	22.66
Total	1,959	100
Readability of text in pictogram		
Readable	428	96.3
Unreadable	16	3.60
Total	444	100

As seen in Table 2, 73.4% of the drugs with pictograms in patient information leaflets are also used in the same drug in different doses and forms, while 26.6% are used in only one drug. It was determined that the pictograms in the patient information leaflets with pictograms consisted of an average of 6.55 ± 4.46 images. Less than 7 images were used in 69.1% of the pictograms, while 30.83% had 7 or more images. 84.99% of the pictograms were clear, and 15.01% were blurry (example Figure 2C). It was determined that real images were used in only 4.8% of the pictograms (example Figure 2D). Human faces/bodies were used in 55.95% of pictograms, but only 32.8% of these showed full faces/bodies. (example Figure 2E). It was determined that text was used in 22.66% of the pictograms, and of these, 96.3% of the text was readable (example Figure 2F). Figure 2 shows some examples of pictograms found on drugs licensed in Turkey.

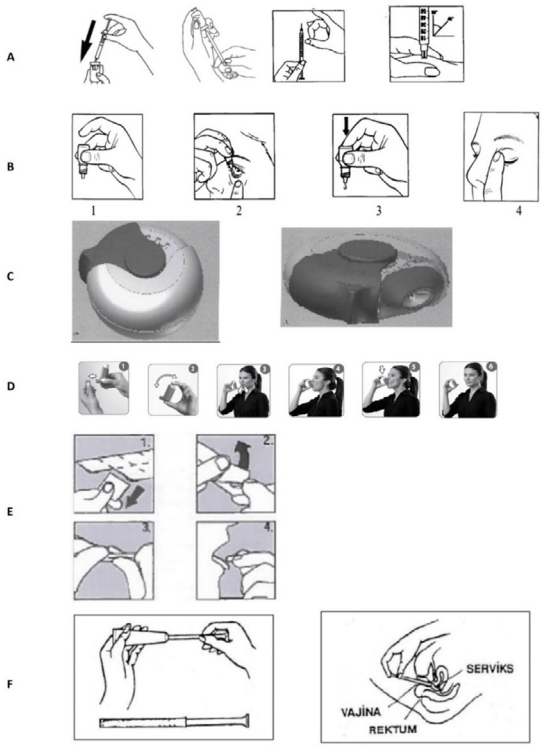


Figure 2. Some examples of pictograms used in patient information leaflets of drugs in Turkey:

2A. An example pictogram for Parenteral preparations III
2B. An example pictogram for Sterile Eye/Ear/Nose Preparations
2C. An example pictogram for blurry pictogram
2D. An example pictogram with real image
2E. An example pictogram with human face
2F. An example pictogram with readable text

Copyright/license: The images have been adapted from patient information leaflets available on the Turkish Medicines and Medical Devices Agency (TITCK) website[31], which provides publicly accessible content.

In this study, conducted to determine the frequency and qualitative and quantitative characteristics of pictograms used in the instructions for licensed medicines in Turkey, the patient information leaflets for 17,709 drugs were examined, and visual content analysis was performed on 1,959 drugs that included pictograms. The most important finding of the study is that the frequency of pictogram use in the leaflets for licensed drugs in Turkey is quite low, at 11.1%. The low use of pictograms at this level can be explained by the absence of a nationally developed standard pictogram set, the fact that internationally developed pictograms such as those by USP and FIP have not yet been validated for the Turkish population, and the lack of legal regulations by the public authority to encourage the use of pictograms. A study conducted by Pires et al. (2015) in Portugal, which examined the leaflets for cardiovascular, nervous system, and musculoskeletal system drugs, also concluded that visual elements were used very little, and the design of the leaflets needed to be carefully reviewed[36].

The second important result of the study is that pictograms are most frequently used in the leaflets for sensory and respiratory system drugs. It is thought that the forms of these drugs, which require more local application than other drug types, such as creams, sprays, drops, and ointments, and their typically more tangible nature, facilitate the use of pictograms. This observation aligns with findings from Dowse and Ehlers (2005), who highlight the potential of pictograms for complex or locally applied medications[25]. Similarly, a scoping review by Sedeh et al. (2022) on communicating with patients through pictograms and pictures in dermatological treatments (a field with frequent topical applications) likely supports the efficacy of visual communication in enhancing patient understanding and adherence for locally applied medications[37].

The third significant result of the study is that pictograms are mostly prepared to explain the use of injectors/syringes/cartridges containing powder/solution/suspension. It is not surprising that pharmaceutical companies use pictograms to facilitate the usage of these forms, which are more challenging to use, take more time, and are the patient's sole responsibility. Research indicates that clear instructions are crucial for minimizing self-injection errors, suggesting that visual aids like pictograms play a vital role in improving patient understanding and reducing mistakes with these challenging drug delivery methods[38].

In the visual content analysis, the average number of images/shapes used in a pictogram, representing its complexity, was found to be 6.55. It can be stated that 69% of the pictograms use fewer images than the average, and the overall complexity is not high. Dowse and Ehlers (1998), in their review of pictograms in pharmacy, recommended using simple, realistic images with limited content, using full body images as references for body parts, and minimizing the use of abstract symbols[24].

Another significant result of the study is that "drawings" are predominantly preferred in pictograms. The use of "real images" in pictograms is highly debated. While some studies argue that real images increase tangibility[24,39], others suggest that details in real images can distract the user and move focus away from the main point[40,41,42]. Another finding from the visual content analysis is that the criterion often emphasized in the literature, the use of full face/body for pictograms related to drug use, is met in only 32% of the instructions. It is evident that the use of the full face in the instructions for sensory organ drugs will prevent confusion and make usage easier.

Finally, the use of text in the pictogram for information such as dosage/time/action/description can make it possible for the drug to be used solely by understanding the pictogram. Indeed, Levie and Lentz's (1982) systematic review also suggested that the use of captions alongside images would make understanding easier, especially among people with low literacy skills[43]. Houts et al. (2006) also stated that when using a series of images, the sequence should be explained with simple words because people with low literacy skills might not see the connection between sequential images[12]. However, the critical point here is that the text must be legible in terms of size, character, and other attributes. It was found that 22.6% of the licensed drugs contain text in the pictogram, and 96% of these texts are legible. Houts et al. (2006) emphasized that if the text in the image is unclear, the meaning of the images might also be unclear, but if the accompanying text is clear, the images will be easier to understand[12].

It is appropriate to evaluate the study results with some strengths and limitations. The greatest strength of this study is the large sample size, which made it possible to examine the characteristics of pictograms. While the uniqueness of the study lies in the fact that no prior research has been found in the literature evaluating the presence of visual elements, including pictograms, in the patient leaflets for licensed drugs in Turkey, this also creates a limitation in assessing the results. The visual content analysis process followed in the study is time-consuming, and due to the limited studies that provide guidance on this subject, the analysis was conducted with a small number of categories to ensure objectivity. It

is believed that future studies with smaller samples that include more subjective evaluations, and more categories could be beneficial for the development of new pharmacological pictograms specific to Turkish society.

Determining the frequency of pictograms and their characteristics in the leaflets for licensed drugs in Turkey is of great importance for rational drug use and health literacy intervention programs. Considering the study results and the literature review, it is believed that some micro and macro interventions for the use of pictograms in licensed drugs could result in significant gains in terms of medication adherence and health literacy. Micro interventions involve individual and small-scale efforts such as developing user-centered pictogram designs and educating healthcare professionals on their effective use; macro interventions encompass system-wide changes like public authorities encouraging pictogram use and establishing a standardized pictogram set for pharmaceutical companies. Given the potential benefits of well-designed pictograms for the pharmaceutical industry and healthcare system, there is a strong need for initiatives aiming to promote the widespread use of a standardized pictogram set in the pharmaceutical sector in Turkey. For this purpose, it is recommended to develop a comprehensive set of pictograms that pharmaceutical companies can use, with a focus on user-centered designs, in coordination with the fields of graphic design, health communication, and pharmacy. The public authority should also encourage the use of pictograms, and pharmaceutical companies should employ them in the instructions for different forms and treatment groups.

STATEMENTS OF ETHICS

No ethical approvals are required for this study.

CONFLICT OF INTEREST STATEMENT

The authors claim no conflicts of interest.

AUTHOR CONTRIBUTIONS

F.Y. designed and planned the study, analyzed and interpreted the data, and wrote the manuscript; S.D.Y. collected and analyzed the data; D.G.O. and Z.O. revised the manuscript.

FUNDING SOURCES

The authors declare that there are no funding sources.

ACKNOWLEDGMENTS

We thank Zeynep Bade Aydin for her assistance in obtaining the data.

REFERENCES

1. National Institute for Health and Care Excellence. Medicines optimisation: the safe and effective use of medicines to enable the best possible outcomes [Internet]. UK: NICE Medicines and Prescribing Centre; 2015 [Sept 15, 2024]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305021/

2. Jimmy B, Jose J. Patient medication adherence: measures in daily practice. Oman Med J, 2011;26(3):155-159. Doi: 10.5001/omj.2011.38

3. World Health Organization. Adherence to long-term therapies: evidence for action [Internet]. World Health Organization; 2003 [Sept 15, 2024]. Available from: https://iris.who.int/handle/10665/42682

4. Fang Y, Jiang Z, Han Z, Dong F, Chen D, Xiang X, et al. Barriers and facilitators to medication adherence in patients after PCI: a mixed-methods systematic review. Authorea, 2024. Doi: 10.22541/au.171156534.47703668/v1

5. Brown MT, Bussell JK. Medication adherence: WHO cares? Mayo Clin Proc, 2011;86(4):304-314. Doi: 10.4065/mcp.2010.0575

6. Kanji L, Xu S, Cavaco A. Assessing the understanding of pharmaceutical pictograms among cultural minorities: the example of Hindu individuals communicating in European Portuguese. Pharmacy, 2018;6(1):22. Doi: 10.3390/pharmacy6010022

7. Al Meslamani AZ. Challenges in health economics research: insights from real-world examples. J Med Econ, 2024;27:215-218. Doi: 10.1080/13696998.2024.2310466

8. Fallis BA, Dhalla IA, Klemensberg J, Bell CM. Primary medication non-adherence after discharge from a general internal medicine service. PLoS ONE, 2013;8:e61735. Doi: 10.1371/journal.pone.0061735

9. Reijnen E, Laasner Vogt L, Kühne SJ, Fiechter JP. Do pictograms on medication packages cause people to consult package inserts less often? If so, with what consequences? Behav Sci, 2023;13(8):696. Doi: 10.3390/bs13080696

10. Hughes CM. Medication non-adherence in the elderly: how big is the problem? Drugs Aging, 2004;21:793-811. Doi: 10.2165/00002512-200421120-00004

11. Kessels RPC. Patients' memory for medical information. J R Soc Med, 2003;96(5):219-222. Doi: 10.1258/jrsm.96.5.219

12. Houts PS, Doak CC, Doak LG, Loscalzo MZ. The role of pictures in improving health communication: a review of research on attention, comprehension, recall, and adherence. Patient Educ Couns, 2006;61(2):173-190. Doi: 10.1016/j.pec.2005.05.004

13. Davis TC, Wolf MS, Bass III PF, Thompson JA, Tilson HH, Neuberger M, et al. Literacy and misunderstanding prescription drug labels. Ann Intern Med, 2006;145(12):887-894. Doi: 10.7326/0003-4819-145-12-200612190-00144

14. Braich PS, Almeida DR, Hollands S, Coleman MT. Effects of pictograms in educating 3 distinct low-literacy populations on the use of postoperative cataract medication. Can J Ophthalmol, 2011;46(3):276-281. Doi: 10.1016/j.jcjo.2011.05.004

15. Mohamed L, Idris K. Impact of newly designed, culturally sensitive pharmaceutical pictograms on medication information and use. World J Pharm Res, 2018;7(8):56-98. Doi: 10.20959/wjpr20188-11889

16. Lindquist LA, Go L, Fleisher J, Jain N, Friesema E, Baker DW. Relationship of health literacy to intentional and unintentional non-adherence of hospital discharge medications. J Gen Intern Med, 2012;27:173-178. Doi: 10.1007/s11606-011-1886-3

17. Kolers PA. Some formal characteristics of pictograms. Am Sci, 1969;57:348-363.

18. Sorfleet C, Vaillancourt R, Groves S, Dawson J. Design, development and evaluation of pictographic instructions for medications used during humanitarian missions. Can Pharm J, 2009;142(2):82-88. Doi: 10.3821/1913-701X-142.2.8

19. Barros IM, Alcântara TS, Mesquita AR, Santos ACO, Paixão FP, Lyra Jr DP. The use of pictograms in the health care: a literature review. Res Social Adm Pharm, 2014;10(5):704-719. Doi: 10.1016/j.sapharm.2013.11.002

20. Sletvold H, Sagmo LAB, Torheim EA. Impact of pictograms on medication adherence: a systematic literature review. Patient Educ Couns, 2020;103(6):1095-1103. Doi: 10.1016/j.pec.2019.12.018

21. Sletvold H, Dowse R. Pictograms in medicine information and counselling. Norsk Farm Tidsskr, 2022;8:42-46.

22. Abdu-Aguye SN, Sadiq AM, Shehu A, Mohammed EN. Guessability of standard pharmaceutical pictograms in members of the Nigerian public. Explor Res Clin Soc Pharm, 2023;9:100240. Doi: 10.1016/j.rcsop.2023.100240

23. Dowse R, Ramela T, Browne SH. An illustrated leaflet containing antiretroviral information targeted for low-literate readers: development and evaluation. Patient Educ Couns, 2011;85(3):508-515. Doi: 10.1016/j.pec.2011.01.013

24. Dowse R, Ehlers MS. Pictograms in pharmacy. Int J Pharm Pract, 1998;6(2):109-118. Doi: 10.1111/j.2042-7174.1998.tb00924.x

25. Dowse R, Ehlers M. Medicine labels incorporating pictograms: do they influence understanding and adherence? Patient Educ Couns, 2005;58(1):63-70. Doi: 10.1016/j.pec.2004.06.012

26. Vaillancourt R, Grenier S. Development of pictograms to enhance medication safetypractices of health care workers and international preferences. Can J Hosp Pharm, 2018;71(4):243-257.

27. The United States Pharmacopeial Convention. USP Pictograms. 1997.

28. International Pharmaceutical Federation. World Health pictograms-instructions for use [Internet]. International Pharmaceutical Federation; 2009 [Sept 15, 2024]. Available from: https://www.fip.org/files/fip/MEPS/PictogramsInstructions2009-07.pdf

29. Kheir N, Awaisu A, Radoui A, El Badawi A, Jean L, Dowse R. Development and evaluation of pictograms on medication labels for patients with limited literacy skills in a culturally diverse multiethnic population. Res Social Adm Pharm, 2014;10(5):720-730. Doi: 10.1016/j.sapharm.2013.11.003

30. Turkish Medicines and Medical Devices Agency. Beşeri tıbbi ürünlerin ambalaj bilgileri, kullanma talimatı ve takibi yönetmeliği [Internet]. Ankara: Resmi Gazete; 25 Apr 2017 [Sept 13, 2024]. Available from: https://www.mevzuat.gov.tr/mevzuat?MevzuatNo=23536&MevzuatTur=7&MevzuatTertip=5

31. Turkish Medicines and Medical Devices Agency. Production information/patient information leaflets lists (in Turkish) [Internet]. Turkish Medicines and Medical Devices Agency; 2024 [Sept 15, 2024]. Available from: https://www.titck.gov.tr/kubkt

32. Margolis E, Pauwels L, editors. The sage handbook of visual research methods. New York, USA: Sage Publications; 2011.

33. Rose G, editor. Visual methodologies: an introduction to researching with visual materials. New York, USA: Sage Publications; 2012.

34. Park MH. The study of signage: pictograms. Int J Image, 2013;3(4):33-40. Doi: 10.18848/2154-8560/CGP/v03i04/44097

35. Ministry of Health, Turkish Medicines and Medical Devices Agency. Coloured prescription system circular (in Turkish) [Internet]. Ministry of Health, Turkish Medicines and Medical Devices Agency; 2017 [Sept 15, 2024]. Available from: https://titck.gov.tr/storage/legislation/Genelge%202017-1.pdf

36. Pires C, Vigário M, Cavaco A. Graphical content of medicinal package inserts: an exploratory study to evaluate potential legibility issues. Health Info Libr J, 2016;33(2):121-139. Doi: 10.1111/hir.12128

37. Sedeh FB, Henning AS, Mortensen OS, Jemec GBE, Ibler KS. Communicating with patients through pictograms and pictures – a scoping review. J Dermatol Treat, 2022;33(6):2730-2737. Doi: 10.1080/09546634.2022.2068790

38. Smith MY, Wallace LS. Reducing drug self-injection errors: a randomized trial comparing a "standard" versus "plain language" version of patient instructions for use. Res Social Adm Pharm, 2013;9(5):621-625. Doi: 10.1016/j.sapharm.2012.10.007

39. Targoutzidis A. A guide for fixed safety pictogram signage in workplaces based on human factors approach. IETI Trans Ergon Saf, 2023;7(1):28-48. Doi: 10.6722/TES.202304_7(1).0004

40. Moll JM. Doctor-patient communication in rheumatology: studies of visual and verbal perception using educational booklets and other graphic material. Ann Rheum Dis, 1986;45(3):198-209. Doi: 10.1136/ard.45.3.198

41. Readance JE, Moore DW. A meta-analytic review of the effect of adjunct pictures on reading comprehension. Psychol Sch, 1981;18(2):218-224. Doi: 10.1002/1520-6807(198104)18:2<218:AID-PITS2310180219>3.0.CO;2-1

42. Jones D, Moran S, Sanchez J, Latham A, Vu KP. Users' interpretation of *pictograms* and *pictures* for conveying instructions and warnings on pharmaceutical labels. In: Yamamoto S, Mori H, editors. Human interface and the management of information: visual information and knowledge management. HCII 2019. Cham: Springer; 2019. (Lecture Notes in Computer Science; vol. 11569). Available from: https://doi.org/10.1007/978-3-030-22660-2_2

43. Levie WH, Lentz R. Effects of text illustrations: a review of research. ECTJ, 1982;30:195-232.