ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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Formerly: Eczacılık Bülteni / Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal GÜVEN

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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:

Title Page

Abstract

Keywords

Introduction (without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of Interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

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

The title should be concise and informative. Avoid abbreviations and formulae, where possible. The title page should include full title, author names and affiliations, present addresses, corresponding author, and ORCID numbers for every author. Also, the full manuscript should include a full title page.

Abstracts should not be separated into categories; it should be written in a paragraph format.

Keywords: Max. 5

Graphics may be included with both in the text and uploaded as separate files.

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

Table and figure titles should not be abbreviated exp. fig. is not acceptable. It should be written as; Table 1. .... Figure 1. .....

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.

Titles: Number tables consecutively by their appearance in the text and place any table notes below the table body. Table captions should be written on the top.

References in the text should be identified using Arabic numerals. 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. References should conform to Vancouver style and be numbered consecutively in the order in which they are cited in the text.

*Obligatory files are manuscript main document, title page and copyright form 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 studies 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 Research Articles.

Research 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.

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

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 consent of all coauthors must be obtained prior to submission of manuscripts. If an author is removed after submission, the submitting author must have the removed author consent to the change by e-mail or faxed letter to the assigned editor.

1.5.2 Plagiarism

Manuscripts must be original with respect to concept, content, and writing. It is not appropriate for an author to reuse wording from other publications, including one's own previous publications, whether or not that publication is cited. Suspected plagiarism should be reported immediately to the editorial office. Report should specifically indicate the plagiarized material within the manuscripts. Acta Pharmaceutica Sciencia uses iThenticate or Turnitin software to screen submitted manuscripts for similarity to published material. 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 au-

thors. Studies submitted for publication approval must present evidence that the described experimental activities have undergone local institutional review assessing safety and humane usage of study subject animals. In the case of human subjects, authors must also provide a statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed 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 describing the Material and Methods utilized in the studies.

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.

1.6 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 for suggestions.

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. A standard list of Abbreviations, Acronyms, and Symbols is in section 5.

It is best to use the font “Times New Roman”. Other fonts, particularly those that do not come bundled with the system software, may not translate properly. 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 title page, 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.

2.1.1 Articles of All Kind

Use page size A4. Vertically orient all pages. Articles of all kind must be double-spaced including text, references, tables, and legends. This applies to figures, schemes, and tables as well as text. They do not have page limitations but should be kept to a minimum length. The experimental procedures for all experimental steps must be clearly and fully included in the experimental section of the manuscripts.

2.1.2 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/).

2.1.3 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 Ara-

bic 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.

2.1.4 Trademark Names

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

2.1.5 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

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. Minimal use of nonfunctional words is encouraged. 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.

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 (*).

2.2.2 Abstract and Keywords

Articles of all types must have an abstract following the title page. 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 sum-

marized. Code numbers may be used once in the abstract. 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 searching and indexing of the manuscripts.

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 the 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 introductory 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.

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. (See section 4 for more abbreviations)

2.2.5 Results and Discussion

This section could include preparation, isolation, synthetic schemes and tables of biological and statistical data. The discussions should be descriptive. Authors should discuss the analysis of the data together with the significance of results and conclusions. An optional conclusions section is not required.

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 email addresses for each of the designated corresponding authors.

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

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

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

Abbreviations Used: Provide a list of nonstandard abbreviations and acronyms used in the paper, e.g., YFP, yellow fluorescent protein. Do not include compound code numbers in this list. It is not necessary to include abbreviations and acronyms from the Standard Abbreviations and Acronyms listed in section 4.

2.2.7 References and Notes

Vancouver style is used in the reference list and citations. List manuscripts as "in press" only accepted for publication. Manuscripts available on Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished work" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. In-text citations should be given superscript numbers (see examples) according to order in the manuscript.

References

Please check with your faculty for any specific referencing or formatting requirements.

- References are listed in numerical order, and in the same order in which they are cited in text. The reference list appears at the end of the paper.
- Begin your reference list on a new page and title it 'References'.
- The reference list should include all and only those references you have cited in the text. (However, do not include unpublished items such as correspondence.)
- Use Arabic numerals (1, 2, 3, 4, 5, 6, 7, 8, 9) as a superscripts.
- 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.
- Use of DOI URL at the end of reference is strongly advised.

Examples

For printed articles

• Article with 1-6 authors:

Author AA, Author BB, Author CC, Author DD. Title of article. Abbreviated title of journal, Date of publication YYYY;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. https://doi.org/10.1016/j.bmc.2018.02.048.

• Article with more than 6 authors:

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

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 [cited YYYY Mon DD];volume number(issue number):page numbers. Available from: URL DOI

For books and book chapters

Book: a.) Print book OR b.) Electronic book

a.) Author AA. Title of book. # edition [if not first]. Place of Publication: Publisher; Year of publication. Pagination.

b.) Author AA. Title of web page [Internet]. Place of Publication: Sponsor of Website/Publisher; Year published [cited YYYY Mon DD]. Number of pages. Available from: URL DOI: (if available)

2.2.8 Tables

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. Footnotes in tables should be given italic lowercase 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. 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.

2.2.9 Figures, Schemes/Structures, and Charts

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 preferences. 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). If submitting individual graphic files in addition to them being embedded in a Word document, ensure the files are named based on graphic function (i.e., Scheme 1, Figure 2, Chart 3), not the scientific name. Labeling of all figure parts should be present and the parts should be assembled into a single graphic.

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.

2.2.10 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 "Create Account" button on the registration screen and the fill the opening form with real information. Some of the information required in form is absolutely necessary and the registration will not work if these fields are not completely filled.

After the registration, a "Welcome" mail is sent to the user by the system automatically reminding user name and password. Authors are expected to return to the entry screen and log on 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 mailed to your e-mail in a short while.

3.3 Submitting a New Article

The main page of author module consists of various parts showing the situation of manuscripts in process. By clicking the New Manuscript button, authors create the beginning of new submission, a process with a total of 9 consecutive levels. In 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 move the new submission to "Partially Submitted Manuscripts" part and the transaction can be continued from here.

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 automatically assigned as "Partially Submitted Manuscripts".

3.3.2 Institutions Authors should give their institutional information during submission.

3.3.3 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.

3.3.4 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.

3.3.5 Abstract The summary of the article should be entered to Abstract window at this level. 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 by 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 arbitrators; 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.

3.3.6 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).

3.3.7 Cover Letter If the submitting article was published as thesis and/or presented in a congress or elsewhere, all information of thesis, presented congress or elsewhere should be delivered to the editor and must be mentioned by the "Cover Letter" field.

3.4 Adding Article

This process consists of four different steps beginning with the loading of the article in to system. Browse button is used to reach the article file, under the Choose a file to upload tab. After finding the article you may click to Choose File and file will be attached.

Second step is to select the 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 third step and the last step is submitting the prepared article into the system. Therefore, Download button under the Send your file by clicking on download button tab is clicked.

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 previously defined format. After all required files were added, Continue button should be clicked. All details and features of the article might be reached from the Article Information page.

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

3.4.1 Your Files After adding the article you may find all information related to 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 the file to be opened in valid site and the second one is to ensure to download submitted file into the computer.

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

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

3.4.2 Sending Article Last level is submitting the article and the files into the system. Before continuing the transaction, Article Information window must be controlled where it is possible to return back; by using Previous button and required corrections can be made. If not, clicking the Send the Article button completes transaction.

3.4.3 Page to Follow the Article The Main Page of 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.

3.4.3.1 Articles Not Completed to be Sent 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. Here you can 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, if the article is not considered to send into the system.

3.4.3.2 Articles that Require Revision Articles, which were evaluated by the referee and accepted by the editor with revision, continues to Waiting for Revision table.

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 one by one. All changes must be made in the registered file, and this changed file must be resent. Author's most efficacious replies relating to the changes must be typed in "Cover Letter" part.

If the is transaction is discontinued, the system move the revised article to Submitted Manuscripts part and the transaction can be continued from here.

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

3.4.4 Submitted Manuscripts Information related to articles can be followed through the Submitted Manuscripts line. 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.

3.5 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 resubmit (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 appropriate.

Studies found appropriate after language-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 referee comments will be asked.

Authors should consider that this time 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 referees, submits his/her comment and suggestion to the editor-in-chief. In this way, the article takes one of the acceptance, rejection, or revision decisions. In the case of revision, after the author revises, 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 journal homepage.

EDITORIAL

Opinions of Ottoman physicians on the effect of coffee as a medicine

Editorial Article

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Coffee, which ranks first in the world as a beverage, is a medicinal plant with important effects. As it is known, coffee is the fruit seed of a tree from the *Coffea* genus of the Rubiaceae family, which grows in tropical hot climates.

The emergence of coffee worldwide and its use by the masses began in Yemen in the 1450s. Common ideas of historians who study the history of coffee based on the documents of that time; Coffee was known by religious Sufi groups in Yemen since the 15th century and was drunk due to its "stimulating" effect. In addition, when it was realized that it gave lightness and vigor to the body and eliminated lethargy and laziness, it became frequently consumed in Hejaz, Egypt, and Damascus in twenty years.

The Ottoman Empire ruled these geographies at that time. Those who went on pilgrimage brought the coffee in these lands to Ottoman cities and made them popular. Due to demand, coffee was brought to Istanbul by ships and sold by merchants. In the 1555s, two coffeehouses were opened in Tahtakale, Istanbul, and as their customers increased, many coffeehouses were established in other neighborhoods.

Europe became familiar with coffee from the Ottomans in the 17th century. The first coffeehouse was opened in Venice in 1615, and soon coffeehouses were opened in Marseille, London, Vienna and Paris, and coffee became a sought-after beverage in a short time.

When coffee began to be loved and consumed widely by the Ottomans, questions began to be asked whether this new drink was harmful to the body and, if so, what it was. Of course, the doctors of the time had to answer these questions.

Since coffee was a new drink, there was no information in the medical books of the time. Ömer Eş-Şâzelî, one of the Islamic physicians, stated that it is a cure for every ailment, like Zamzam, and that it works for whatever purpose it is consumed, in

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1418. Although Şihabeddin et-Tanbedavi said that coffee has no intoxicating properties, it has relaxing and reinforcing properties; Ottoman physicians had to say the last word.

The most comprehensive report on this subject was written by al-Kusuni, the chief physician of Suleiman the Magnificent, in 1566; “I have not found any information about coffee in any of the medical books I have read or seen. What I will say now is based on my own experience”. He and other physicians summarized the benefits of coffee as follows; its biggest benefit is that it is stimulant, promotes vigor and comfort to the body, relieves anxiety, and please the heart. Due to its drying effect, it dries the moisture in the body, such as cold and sputum, and removes excess moisture from the stomach. It treats diseases caused by excess blood and sputum fluids. It prevents excess fluid accumulated in the body from going bad, dries it, strengthens the stomach, and increases urination. It releases the intestinal gases, prevents vomiting, moderates the temperament, increases blood flow, increases appetite and facilitates digestion.

But one thing that was well known was not to overdo it in drinking coffee. Ottoman physicians drew attention to two things; Do not roast the coffee too much and drink the appropriate amount.

Ottoman physicians wrote that coffee had also harmful effects due to its medicinal properties and this problem could be solved with some precautions, in their medical books. According to the medical rules of the time, each medicinal plant had “hot, cold, dry, moist” properties, and its harmful effects were corrected by balancing its dominant feature with its opposite. On this basis, coffee had a “cold and dry” feature. For this reason, some measures had to be taken to balance this effect.

They reported that the “dry” feature of coffee could be balanced with a glass of water, and the “cold” feature could be balanced by eating a piece of sugary food with it. This situation was explained in medical books as follows; A person who wants to drink coffee should either eat dessert before or along with it, and add sugar or honey in it. A person who wants to drink coffee to feel fit, to get rid of laziness and for the other benefits mentioned earlier, should eat plenty of desserts and drink peanut oil or liquid oil. Thus, offering sugar and Turkish delight along with coffee became in line with the doctors’ regulations.

In response to a question about whether it is healthy to drink coffee immediately after a meal, chief physician Bedreddin el-Kûsûnî said that anything drunk during a meal disrupts the digestion of the food, and the best time to drink coffee is when the meal is complete and digestion begins.

This information functioned as rules followed by Ottoman society. Serving Turkish coffee with a glass of water and a piece of sugar, especially Turkish delight, became a tradition which shaped by the directives of Ottoman physicians. This tradition continues to this day.

REVIEW ARTICLE

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Review article
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Anthraquinone and coumarin as potential natural products against human coronavirus strain

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ABSTRACT

The COVID-19 pandemic has prompted researchers to look for novel compounds with SARS-CoV-2 antiviral potential. Numerous studies have demonstrated that natural compounds have potent antiviral properties. This review suggests potential natural products and their isolates for treating human coronaviruses based on effective herbal treatments for coronavirus infections. Given the effects of human coronavirus strains, finding alternative isolated compounds from plants (like anthraquinones and coumarin) that could prevent viral infection will aid in hastening the medication discovery process. It is known that anthraquinone derivatives have immune-stimulating, anti-inflammatory, and antiviral properties. Coumarin, a naturally occurring compound, is also a promising therapeutic candidate because of its solubility, stability, and low toxicity. These natural substances may interfere with target-specific proteins to stop viral replication in the host. Natural remedies not only stop viruses from attaching to the host body but also stop them from reproducing and strengthen the host's immune system.

Keywords: antiviral, human coronavirus, COVID-19, natural products, protease

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INTRODUCTION

Coronaviruses (CoVs) are a family of viruses that can cause illness in humans and animals. They belong to the subfamily Coronavirinae in the family Coronaviridae, and they derive their name from their crown-like appearance when viewed under a microscope. The four coronavirus genera are α-, β-, γ-, and -δ, each contain a positive-sense single-stranded RNA genome[1]. Due to CoVs' tendency for mutation and recombination during replication, coronaviruses are more diverse than they otherwise could be. Human Coronaviruses (HCoVs) are recognized respiratory infections linked to a variety of respiratory outcomes. The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 and severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002-2003 highlighted the significance of human coronaviruses and their potential for high pathogenicity. These outbreaks prompted increased attention and research focused on understanding and mitigating the impact of HCoVs[2,3].

The primary pharmacologically active targets of severe acute respiratory syndrome coronavirus (SARS-CoV-2) are 3-chymotrypsin-like protease (3CLpro), papain-like protease (PLpro), RNA-dependent RNA polymerase, and spike (S) proteins. There are currently no proven antiviral treatments or preventative coronavirus vaccinations available[4]. However, several synthetic drugs have shown promise, such as hydroxychloroquine and chloroquine phosphate, which function by various mechanisms, including the alkalization of the phagolysosomes of the host cell[5]. Several more recent antiviral drugs are also promising, including lopinavir, remdesivir, and arbidol. Along with these, lopinavir/ritonavir, nucleoside analogs, neuraminidase inhibitors, and the peptide EK1 are also recommended as possible treatments[6].

To prevent coronavirus disease (COVID-19) infection, natural products, and herbal remedies are employed since they potentially impact medication discovery[7]. Drug development for several illnesses is already based on plant, fungal, and marine sources of natural compounds. These exhibit antiviral activity against human CoV-2, contributing to the creation of a high-quality medication for the treatment of COVID-19. These herbal remedies operate by preventing the S protein from interacting with the angiotensin-converting enzyme-2 (ACE-2) receptors of host cells[8]. Many of these boost the immune system's ability to fight the virus, inflammation, oxidative stress, and fibrosis caused by COVID-19[9].

To prioritize the screening of compounds against SARS-CoV-2 in the quest for innovative therapeutic candidates to treat or prevent COVID-19, a compre-

hensive assessment of the antiviral activity attributed to well-known natural products is a helpful place to start[10]. The present study claims that the natural products anthraquinone and coumarin have strong anti-SARS-CoV-2 efficacy.

METHODOLOGY

SARS-CoV and natural products

The SARS-CoV-2 family of enveloped RNA viruses infects people and causes severe pneumonia and life-threatening respiratory illnesses. The spike protein binds to ACE-2 receptors and facilitates the fusion of the virus and the host membrane, which allows the coronavirus (CoV) to enter host cells (pulmonary and bronchial epithelial cells)[11,12,13,14]. Viral variety and SARS-CoV capacity for fast mutation, even during an epidemic, have impeded the creation of potent antiviral medicines with a broad range of action. Therefore, it is crucial to create antiviral medications that may either greatly lessen the signs and symptoms of SARS-CoV infection or safely and effectively prevent the transmission of SARS-CoV. It would be crucial to focus on creating straightforward, tiny molecules that are affordable to create and administer. Recently, numerous promising treatments against SARS-CoV-2 virus, including remdesivir, infliximab, and imatinib, have been discovered. Remdesivir has been approved for use in urgent situations and exhibits strong antiviral activity[15,16]. To stop the illness from spreading, finding new medication leads that are more generally effective against coronavirus is crucial.

Natural products may be used to treat COVID-19 by preventing some stages of the virus' life cycle, such as C3en the virion binds to the appropriate receptor on the cell surface. Transmembrane protease serine 2 is a 3CLpro that is important for cleaving viral peptides into virulence-enhancing functional units. It can activate spike proteins and is involved in the infection of host cells by SARS-CoV-2. Because excessive soluble versions of the ACE-2 are implicated at this stage, utilizing ACE-2 inhibitors as a therapy option is a possibility[3]. Coronavirus replication-transcription complex enzymes involved in transcription, translation, virion assembly, budding, and release include reverse transcriptase and RNA-dependent RNA polymerase (RdRP). Many of the aforementioned mechanisms have been observed for the action of natural coumarin compounds[17]. Therefore, it is expected that blocking the SARS CoV-2's main protease, non-structural proteins (NSP10/NSP16) methyltransferase, phosphatase, and endoribonuclease by a possible inhibitor molecule may significantly increase the coronavirus's ability to develop and infect its host as shown in Figure 1.

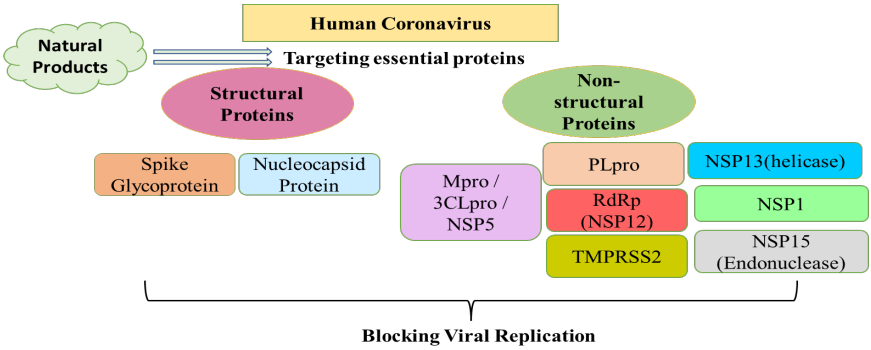


Figure 1. Overview of inhibition of human coronavirus protein

RESULTS and DISCUSSION

Anthraquinone against human coronavirus

Anthraquinone

Anthraquinones (AQs) are a class of chemicals derived from a variety of herbal remedies (Figure 2), including Senna species, that are used in both traditional Chinese medicine and the Ayurvedic medical system to treat a variety of infectious and non-infectious disorders[18]. Additionally, anthraquinone derivatives have been linked to antiviral[19], anti-inflammatory[20], and immunological boosting properties[21]. Therefore, it may be advantageous in the COVID-19 infection if the bioactive is shown to have anti-viral, anti-inflammatory, and immune-stimulating characteristics. This may be proven using the idea of network pharmacology or a polypharmacological approach.

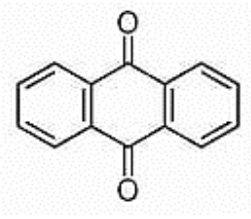


Figure 2. Structure of anthraquinone

Bioactives of anthraquinone

About 700 compounds associated with AQs have been identified. 200 of these were extracted from plants, while the remaining were found in diverse marine and terrestrial sources like lichens, bacteria, fungi, marine invertebrates, and

sponges[22]. For instance, the most well-known and well-studied AQs, emodin, chrysophanol, and physcion, were found in marine fungi such as *Aspergillus*, *Penicillium*, and *Microsporum sp*., as well as in terrestrial sources (plant endophytic fungi). Amrubicin, daunorubicin, diacerein, doxorubicin, epirubicin, idarubicin, mitoxantrone, and valrubicin are examples of commercially available medications that contain anthraquinone scaffold[23].

Bioactive compounds of anthraquinone against human coronaviruses and their antiviral mechanism

Numerous investigations revealed that anthraquinones might be used to prevent the entrance, replication, and release of numerous viruses, including coronaviruses as shown in Table 1. Aloe-emodin, anthrarufin, alizarine, dantron, and emodin are some of the anthraquinones of *R. emodi* that have been shown to bind to SARS-CoV-2 in the active sites of the RNA binding domain of nucleocapsid phosphoprotein using *in silico* studies. The compounds might be completely evaluated and released as possible therapeutic candidates to treat COVID-19 due to their ability to bind to all three active sites with binding energies ranging from -25.45 kcal/mol to -45.48 kcal/mol[24].

Various research indicates that emodin exhibits significant antiviral activities against coxsackie B virus, herpes simplex viruses, hepatitis B virus, human cytomegalovirus, Japanese encephalitis virus, Cypridine herpesvirus 3 virus, influenza A virus, Zika virus, poliovirus, and several viral diseases. Therefore, it has been stated that emodin has antiviral properties that help to prevent or lessen SARS-CoV infection[25]. According to Hsiang and colleagues, emodin can prevent spike protein-pseudotyped retrovirus infectivity and SARS-CoV spike protein interactions with ACE-2 in Vero E6 cells[26]. Researchers also demonstrated that emodin can prevent the release of SARS-CoV from infected cells as well as the 3A ion channel of the coronavirus[27]. Blind molecular docking experiments revealed that certain naturally occurring antiviral anthraquinones could work well as COVID-19 main protease (Mpro) inhibitors because they bind close to the active site that contains the catalytic dyad HIS41 and CYS145 through non-covalent forces[28].

Hepatitis B virus replication was suppressed by Rhein from *Rheum palmatum* ethanol extract[29]. Rhein is a promising therapeutic drug for the treatment of SARS-CoV-2 because it inhibits the interaction between the S protein and ACE-2[30]. The S protein-ACE-2 interaction was reduced by pre-incubating rhein with biotinylated S protein. The influence of rhein on the activity of the human liver enzyme cathepsin B within endosomes is a crucial stage in SARS-CoV-2

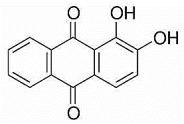
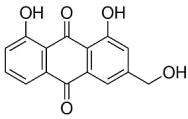
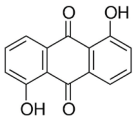
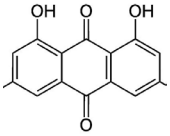
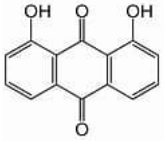
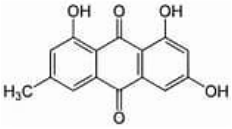
infections, as described by Savarino et al. in 2007[31]. It has been demonstrated that rhein is also capable of docking the spike proteins including PLpro, Mpro, and RdRP active sites. Rhein is favorable as a SARS-CoV-2 inhibitor as a result of all these discoveries. Furthermore, the scientists demonstrated that rhein from Polygonaceae had a minor suppression of the interaction of enzymes with S proteins[30]. Rhein has demonstrated its capacity to prevent several human coronaviruses (HCoV-229E, HCoV-OC43, and SARS-CoV-2) from infecting and replicating in research[32].

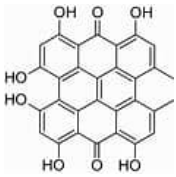
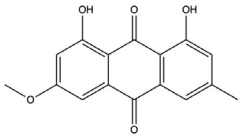
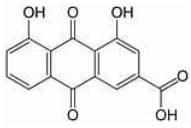
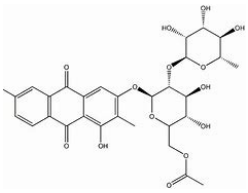
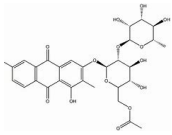
A powerful inhibitor of SARS-CoV-2 Mpro is the antitumoral valrubicin[33]. Our research revealed that valrubicin has a strong affinity for Mpro, PLpro, and RdRp, as well as for spike proteins, allowing us to hypothesize that valrubicin may be a viable inhibitor of SARS-CoV-2. It has been demonstrated that idarubicin has a multi-target anti-SARS-CoV-2 impact due to its ability to bind to spike proteins as well as Mpro, PLpro, and RdRp. Idarubicin showed a significant affinity for endoribonuclease[34]. According to Jin et al., daunorubicin was claimed to be a possible SARS-CoV-2 Mpro inhibitor[35]. According to the current study, daunorubicin shows a strong affinity for PLpro, RdRp, and spike proteins. Doxorubicin, an anticancer medication with previous antiviral efficacy, might be employed to beat SARS-CoV-2 by elevating cellular methylglyoxal content to virucidal levels[36]. Patients with COVID-19 may benefit from very brief therapy with doxorubicin that increases cellular MG. Human cytomegalovirus inhibition by emodin, rhein, and hypericin was reported[17], and the antiviral activity of emodin, aloe-emodin, chrysophanol, physcion, and rhein, against the Japanese encephalitis virus, was also reported[37]. Aloe-emodin, emodin, and chrysophanol were also shown to inhibit the SARS-CoV 3CLpro[38].

With an IC_{50} value of 366 μM, aloe-emodin has been shown to have anti-viral properties against SARS-CoV-2 by reducing the cleavage activity of 3CLpro in a dose-dependent manner[38]. The results of docking research demonstrated that several anthraquinones and their derivatives like emodin 8-glucoside, and chrysophanol 8-O-D-glucoside may bind to SARS-CoV-2 proteins, including the spike protein, papain-like protease, and 3CLpro. 1,3,6-trihydroxy-2-methyl-9,10-anthraquinone-3-O-(6′-O-acetyl)—d-xylopyranosyl-(1->2)-d-glucopyranoside and Torososide B were identified as the top hits[39]. The viral polypeptides are incorporated into the ubiquitin system by 3CLpro, which also interferes with the homeostatic action of functional proteins[40], and was primarily targeted by torososide B. Additionally, PLpro modifies the role of protein phosphatases 1A and 1B as replicase proteins to modify the viral life cycle, which is mostly blocked by torososide B[41].

Similar to how ACE-2 serves as a receptor for spike proteins to enter the host cell, this process is primarily controlled by 1,3,6-trihydroxy-2-methyl-9,10-anthraquinone-3-O-(6′-O-acetyl)—D-xylopyranosyl-(1->2)—D-glucopyranoside[42]. These findings suggest the likelihood that COVID-19 will be resistant to the antiviral effects of anthraquinone derivatives.

Table 1. Anthraquinones involved in the inhibition of target proteins of coronavirus

Bioactive compounds	Structure	Source(s)	Target Inhibitor	References
Alizarine		*Rheum emodi*	binds to SARS-CoV-2 in the RNA binding domain of the nucleocapsid phosphoprotein active sites	[24]
Aloe-emodin		*Aloe vera, Rheum emodi, Rheum officinale, Rheum palmatum*	inhibits the SARS-CoV 3CLpro	[38]
Anthrarufin		*Rheum emodi*	binds to SARS-CoV-2 in the RNA binding domain of the nucleocapsid phosphoprotein active sites.	[24]
Chrysophanol		*Aloe vera, Rheum emodi, Rheum officinale, Dianella longifolia*	binds to SARS-CoV-2 proteins, including 3CLpro, PLpro and spike protein	[39]
Dantron		*Rheum emodi*	binds to SARS-CoV-2 in the RNA binding domain of the nucleocapsid phosphoprotein active sites	[24]
Emodin		*Aloe vera, Cassia occidentalis, Cassia obtusifolia, Rheum palmatum, Polygonum multiflorum, Rumex chalepensis, Scutellaria baicalensis*	prevents SARS-CoV spike protein interactions with ACE-2	[26]

Hypericin		*Hypericum perforatum*	binds to the membrane envelope of SARS-CoV-2 with a strong affinity	[43]
Physcion		*Rheum officinale, Rheum palmatum*	binds to SARS-CoV-2 proteins	[37]
Rhein		*Rheum officinale, Rheum palmatum*	reduces S protein-ACE-2 interaction	[30]
Torososide B		*Cassia torosa*	inhibits 3CLpro and PLpro	[39]
1,3,6-trihydroxy-2-methyl-9,10-anthraquinone-3-O-(6'-O-acetyl)—D-xylopyranosyl-(1 2)—D-glucopyranoside		*Morinda citrifolia*	prevents SARS-CoV spike protein interactions with spike protein and ACE-2	[39]

Coumarin against human coronavirus

Coumarin

The naturally occurring heterocyclic compounds known as coumarins (2H-1-benzopyran-2-ones) are made up of fused benzene and pyrone rings (Figure 3). They are O-hydroxycinnamic acid lactone derivatives. The term "coumarou", which is the French name for the tonka bean (*Dipteryx odorata*, Fabaceae), from which coumarin was initially isolated in 1820, served as the basis for the name coumarin[44]. A group of naturally occurring or artificially altered chemicals of natural origin known as coumarin derivatives demonstrate a wide range of biological properties, including antibacterial, anticancer, antioxidant, anti-HIV, and antiviral activities[45,46,47,48]. Given that coumarin derivatives and pharmaceuticals share a similar structural makeup, it is reasonable to assume that this class of chemicals may also be active against SARS-CoV-2.

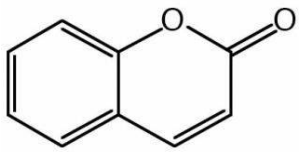


Figure 3. Structure of coumarin

Bioactives of coumarin

More than 1300 coumarins have been identified from a variety of natural sources, including fungi, bacteria, and plants. About 150 distinct plant species from over 30 different families, including Asteraceae, Apiaceae, Caprifoliaceae, Calophyllaceae, Fabaceae, Guttiferae, Moraceae, Nyctaginaceae, Oleaceae, Rutaceae, and Thymelaeaceae, have been documented to contain coumarins[49]. The numerous plants contain coumarin and its derivatives, which are referred to as "natural coumarins". Examples include floroselin, frutinone A, and rutamarin, which were derived from the respective plants *Ruta graveolens*, *Polygal fruticose*, and *Seseli sessiliflorum*[50]. Simple coumarins, furanocoumarins, pyranocoumarins, phenylcoumarins, and diocoumarins are the different types of natural coumarins.

Bioactive compounds of coumarin against human coronaviruses and their antiviral mechanism

The most highly recommended plant-derived drugs that may have considerable inhibitory effects on COVID-19's primary protease were bergapten, heraclenin, heraclenol, imperatorin, oxypeucedanin, psoralen, and saxalin[51]. Numerous studies have looked into how different classes of naturally occurring coumarin phytochemicals affect the activity of viral proteins like protease, integrase, reverse transcriptase, and DNA polymerase, as well as how they affect the blocking of viral entry against a variety of human viruses like the influenza virus, human immunodeficiency virus, herpes simplex virus, and hepatitis B and C[52]. Researchers have demonstrated the *in silico* screening of coumarin derivatives against methyltransferase NSP10/NSP16, protease, ADP-ribose of phosphatase NSP3, and NSP15 endonuclease of SARS-CoV-2[53].

The findings demonstrated the identification of eight compounds from coumarin phytochemicals (licopyranocoumarin, glycycoumarin, inophyllum, oxypeucedanin hydrate, mesuol, and wedelolactone) with a significant inhibitory potential against the SARS coronavirus. All of these drugs docked at the active site and interacted with 3CLpro proteins catalytic dyad (Cys-His) in a manner reminiscent of ritonavir and lopinavir[54].

Bavacoumestan A (C-97) and 6-O-D-Glucopyranosyl-5-hydroxyangelicin (C-88) were two of the substances with the highest docking scores in the main protease active site. Daphnorin (C-49) and glycycoumarin (AV-29), which were tested against methyl transferase, produced the best results. In comparison to other studied compounds, daphnorin has good effects when docked in the active site of RBD-COV-2-S proteins. Finally, the most effective coumarin was inophyllum G2 (AV-35) against human ACE-2[55].

To compare the binding scores of 17 coumarin derivatives with those of the model inhibitors like favipiravir, warfarin, hydroxychloroquine, and remdesivir, molecular docking studies were performed against MPro, Spike with ACE-2, NSP12 with RNA, NSP15, and NSP16. All receptors were found to be sensitive to the coumarin series; however, NSP12 or NSP12/RNA received the highest ratings[56].

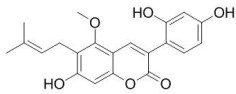
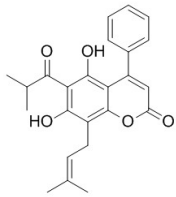
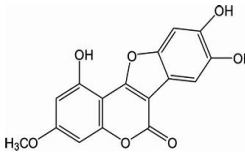
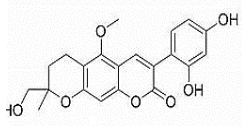
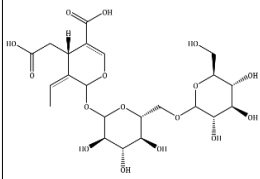
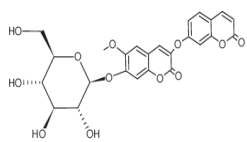
All three of the developed derivatives of hymecromone, psoralen, and phenprocoumon showed strong interactions and high binding affinities with the drug target. The high binding attractions were caused by the compound's -OH groups, His41, a catalytic dyad in Mpro, and the quantity and distance of hydrogen bond interactions with the SARS-CoV-2 targets[57]. Aerial portions of *Artemisia glauca* were used to extract a novel dicoumarin, jusan coumarin. Jusan coumarin and X77, the co-crystallized ligand of Mpro, showed a lot of similarities. Four ligand-based computational, molecular similarity, fingerprinting, Density Functional Theory (DFT,) studies, and pharmacophore studies were used to confirm the similarities. The molecular docking tests of jusan coumarin against Mpro demonstrated that it binds perfectly, with a binding energy of about -18.45 kcal/mol[58].

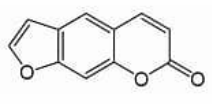
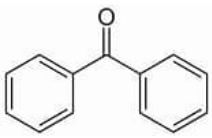
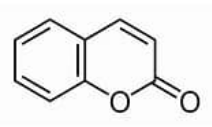
The primary viral protein 3-CLpro, was the target of *in silico* research that demonstrated the antiviral potential of coumarin and benzophenone derivatives with favourable docking scores[59]. In a recent study, Singh and co-workers suggested benzophenone-coumarin derivatives (BCDs) as effective inhibitors of the SARS-CoV-2 virus's major target RdRp, which is important in the replication of the viral genome[60,61].

According to the findings of the molecular docking investigation, the binding affinities of the complexes L1 ((3-(1-((3-chlorophenyl)amino)ethylidene)-chroman-2,4-dione), L2 ((3-(1-((4-chlorophenyl)amino) ethylidene)-chroman-2,4-dione), and L3 ((3-(1-(phenylamino)ethylidene)-chroman-2,4-dione)) and their corresponding palladium complexes were higher than those of chloroquine and cinanserin. All of the compounds were attached to the protein's active site near the His41-Cys145 catalytic dyad[62].

According to the molecular dynamics simulation results, coumarin-triazole-thiophene hybrid4-(((4-ethyl-5-(thiophene-2-yl)-4H-1,2,4-triazol-3-yl)thio) methyl)-6,7-dimethyl-2H-chromen-2-one forms stable complexes with PLpro, Mpro, and NSP3 (range 207-379-AMP and 207-379-MES)[63].

Table 2. Coumarins involved in the inhibition of target proteins of coronavirus

Bioactive compounds	Structure	Source(s)	Target Inhibitors	References
Glycycoumarin		Vegetable roots	interacted catalytically with His41, and inhibited 3CLpro via hydrogen bonding with Cys44 and Asp48	[54]
Mesuol		Stem Bark of *Mesua borneensis* L.	inhibit the SARS-CoV CLPro	[54]
Wedelolactone		*Eclipta alba* (false daisy) and in *Wedelia calendulacea*	inhibit the SARS-CoV CLPro	[54]
Licopyrano coumarin		*Glycyrrhiza uralensis*	inhibit the SARS-CoV CLPro	[54]
6-O-D-Glucopyranosyl-5-hydroxyangelicin (C-88)		*Ficus ruficaulis*	inhibit the SARS-CoV MPro	[55]
Daphnorin (C-49)		-	inhibit the SARS-CoV methyltransferase	[55]

Psoralen		*Psoralea corylifolia* seeds, celery, common fig, parsley, West Indian satinwood, and in all citrus fruits	inhibit the SARS-CoV MPro	[57]
Benzophenone-coumarin derivatives (BCDs)		-	inhibit the SARS-CoV RdRp	[60]
Jusan coumarin		*Artemisia glauca*	inhibit the SARS-CoV MPro	[58]

The current study discusses the possibilities for treating COVID-19 and the role of natural compounds like anthraquinone and coumarin that have shown promise as anti-CoV medicines. To fully understand the underlying cellular and molecular mechanisms, however, extensive *in vivo* investigations on relevant animal models are required because various research regarding the antiviral effects of these drugs is still in the preliminary stages. There aren't many pharmacokinetic studies on these compounds, but they ought to be done to develop a pharmacokinetic profile. Clinical trials are necessary to examine the effectiveness and security of their anti-CoV medications on human beings. More significantly, research should be done to examine any possible interactions between available natural antivirals and anti-CoV effects.

STATEMENT OF ETHICS

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest relevant to this article.

AUTHOR CONTRIBUTIONS

All authors contribute the work equally throughout.

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REFERENCES

1. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. Nat Rev Microbiol, 2021;19(3):155-170. Doi: 10.1038/s41579-020-00468-6

2. Rabaan AA, Mutair AA, Alawi ZA, Alhumaid S, Mohaini MA, Aldali J, et al. Comparative pathology, molecular pathogenicity, immunological features, and genetic characterization of three highly pathogenic human coronaviruses (MERS-CoV, SARS-CoV, and SARS-CoV-2). Eur Rev Med Pharmacol Sci, 2021;25(22):7162-7184. Doi: 10.26355/eurrev_202111_27270

3. Shaikh Z, Sundarrajan P, Bhagtaney L, Zehra S, Kamal FZ, Boubakeur B, et al. Applicability of vitamins in the management of COVID-19: an overview. Ann Phytomed, 2021;10(1):65-76. Doi: 10.21276/ap.covid19.2021.10.1.7

4. Orooji Y, Sohrabi H, Hemmat N, Oroojalian F, Baradaran B, Mokhtarzadeh A, et al. An overview on SARS-CoV-2 (COVID-19) and other human coronaviruses and their detection capability via amplification assay, chemical sensing, biosensing, immunosensing, and clinical assays. Nanomicro Lett, 2021;13(1):18. Doi: 10.1007/s40820-020-00533-y

5. Islam MT, Sarkar C, El-Kersh DM, Jamaddar S, Uddin SJ, Shilpi JA, et al. Natural products and their derivatives against coronavirus: a review of the non-clinical and pre-clinical data. Phytother Res, 2020;34(10):2471-2492. Doi: 10.1002/ptr.6700

6. Qomara WF, Primanissa DN, Amalia SH, Purwadi FV, Zakiyah N. Effectiveness of Remdesivir, Lopinavir/Ritonavir, and Favipiravir for COVID-19 treatment: a systematic review. Int J Gen Med, 2021;23(14):8557-8571. Doi: 10.2147/IJGM.S332458

7. Bogan-Brown K, Nkrumah-Elie Y, Ishtiaq Y, Redpath P, Shao A. Potential efficacy of nutrient supplements for treatment or prevention of COVID-19. J Diet Suppl, 2021;19(3):336-365. Doi: 10.1080/19390211.2021.1881686

8. Yang J, Petitjean SJL, Koehler M, Zhang Q, Dumitru AC, Chen W, et al. Molecular interaction and inhibition of SARS-CoV-2 binding to the ACE2 receptor. Nat Commun, 2020;11(1):4541. Doi: 10.1038/s41467-020-18319-6

9. Huang Y, Yang C, Xu XF, Xu W, Liu SW. Structural and functional properties of SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19. Acta Pharmacol Sin, 2020;41(9):1141-1149. Doi: 10.1038/s41401-020-0485-4

10. Bharadwaj S, Dubey A, Yadava U, Mishra SK, Kang SG, Dwivedi VD. Exploration of natural compounds with anti-SARS-CoV-2 activity via inhibition of SARS-CoV-2 Mpro. Brief Bioinform, 2021;22(2):1361-1377. Doi: 10.1093/bib/bbaa382

11. Attia YA, El-Saadony MT, Swelum AA, Qattan SYA, Al-Qurashi AD, Asiry KA, et al. COVID-19: pathogenesis, advances in treatment and vaccine development and environmental impact-an updated review. Environ Sci Pollut Res Int, 2021;28(18):22241-22264. Doi: 10.1007/s11356-021-13018-1

12. Bhat EA, Khan J, Sajjad N, Ali A, Aldakeel FM, Mateen A, et al. SARS-CoV-2: insight in genome structure, pathogenesis and viral receptor binding analysis - an updated review. Int Immunopharmacol, 2021;95:107493. Doi: 10.1016/j.intimp.2021.107493

13. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell, 2020;181(2):271-280.e8. Doi: 10.1016/j.cell.2020.02.052

14. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. Cell, 2020;181(2):281-292.e6. Doi: 10.1016/j.cell.2020.02.058

15. Awadasseid A, Wu Y, Tanaka Y, Zhang W. Effective drugs used to combat SARS-CoV-2 infection and the current status of vaccines. Biomed Pharmacother, 2021;137:111330. Doi: 10.1016/j.biopha.2021.111330

16. Pruijssers AJ, George AS, Schäfer A, Leist SR, Gralinksi LE, Dinnon KH, Yount BL, et al. Remdesivir potently inhibits SARS-CoV-2 in human lung cells and chimeric SARS-CoV expressing the SARS-CoV-2 RNA polymerase in mice. bioRxiv Preprint, 2020.04.27.064279. Doi: 10.1101/2020.04.27.064279

17. Barnard DL, Huffman JH, Morris JL, Wood SG, Hughes BG, Sidwell RW. Evaluation of the antiviral activity of anthraquinones, anthrones and anthraquinone derivatives against human cytomegalovirus. Antiviral Res, 1992;17(1):63-77. Doi: 10.1016/0166-3542(92)90032-z

18. Malik EM, Müller CE. Anthraquinones as pharmacological tools and drugs. Med Res Rev, 2016;36(4):705-748. Doi: 10.1002/med.21391

19. Andersen DO, Weber ND, Wood SG, Hughes BG, Murray BK, North JA. *In vitro* virucidal activity of selected anthraquinones and anthraquinone derivatives. Antiviral Res, 1991;16(2):185-196. Doi: 10.1016/0166-3542(91)90024-l

20. Park JG, Kim SC, Kim YH, Yang WS, Kim Y, Hong S, et al. Anti-Inflammatory and antinociceptive activities of anthraquinone-2-carboxylic acid. Mediators Inflamm, 2016;2016:1903849. Doi: 10.1155/2016/1903849

21. Panigrahi GK, Yadav A, Srivastava A, Tripathi A, Raisuddin S, Das M. Mechanism of rhein-induced apoptosis in rat primary hepatocytes: beneficial effect of cyclosporine A. Chem Res Toxicol, 2015;28(6):1133-1143. Doi: 10.1021/acs.chemrestox.5b00063

22. Diaz-Muñoz G, Miranda IL, Sartori SK, de Rezende DC, Diaz MAN. Anthraquinones: an overview. In Studies in Natural Products Chemistry; Elsevier: Amsterdam, The Netherlands, 2018;58:313-338. Available from: 10.1016/B978-0-444-64056-7.00011-8

23. Fouillaud M, Venkatachalam M, Girard-Valenciennes E, Caro Y, Dufossé L. Anthraquinones and derivatives from marine-derived fungi: structural diversity and selected biological activities. Mar Drugs, 2016;14(4):64. Doi: 10.3390/md14040064

24. Rolta R, Salaria D, Kumar V, Patel CN, Sourirajan A, Baumler DJ, et al. Molecular docking studies of phytocompounds of *Rheum emodi* Wall with proteins responsible for antibiotic resistance in bacterial and fungal pathogens: *in silico* approach to enhance the bio-availability of antibiotics. J Biomol Struct Dyn, 2022;40(8):3789-3803. Doi: 10.1080/07391102.2020.1850364

25. Xiong HR, Luo J, Hou W, Xiao H, Yang ZQ. The effect of emodin, an anthraquinone derivative extracted from the roots of *Rheum tanguticum*, against herpes simplex virus *in vitro* and *in vivo*. J Ethnopharmacol, 2011;133(2):718-723. Doi: 10.1016/j.jep.2010.10.059

26. Hsiang CY, Ho TY. Emodin is a novel alkaline nuclease inhibitor that suppresses herpes simplex virus type 1 yield in cell cultures. Br J Pharmacol, 2008;155(2):227-235. Doi: 10.1038/bjp.2008.242

27. Schwarz S, Wang K, Yu W, Sun B, Schwarz W. Emodin inhibits current through SARS-associated coronavirus 3a protein. Antiviral Res, 2011;90(1):64-69. Doi: 10.1016/j.antiviral.2011.02.008

28. Das S, Singh A, Samanta SK, Singha Roy A. Naturally occurring anthraquinones as potential inhibitors of SARS-CoV-2 main protease: an integrated computational study. Biologia (Bratisl), 2022;77(4):1121-1134. Doi: 10.1007/s11756-021-01004-4

29. Li Z, Li LJ, Sun Y, Li J. Identification of natural compounds with anti-hepatitis B virus activity from *Rheum palmatum* L. ethanol extract. Chemotherapy, 2007;53(5):320-326. Doi: 10.1159/000107690

30. Ho TY, Wu SL, Chen JC, Li CC, Hsiang CY. Emodin blocks the SARS coronavirus spike protein and angiotensin-converting enzyme 2 interaction. Antiviral Res, 2007;74(2):92-101. Doi: 10.1016/j.antiviral.2006.04.014

31. Savarino L, Fioravanti A, Leo G, Aloisi R, Mian M. Anthraquinone-2,6-disulfonic acid as a disease-modifying osteoarthritis drug: an *in vitro* and *in vivo* study. Clin Orthop Relat Res, 2007;461:231-237

32. Zannella C, Rinaldi L, Boccia G, Chianese A, Sasso FC, De Caro F, et al. Regulation of m6A methylation as a new therapeutic option against COVID-19. Pharmaceuticals (Basel), 2021;14(11):1135. Doi: 10.3390/ph14111135

33. Wang L, He W, Yu X, Hu D, Bao M, Liu H, et al. Coronavirus disease 2019 in elderly patients: characteristics and prognostic factors based on 4-week follow-up. J Infect, 2020;80(6):639-645. Doi: 10.1016/j.jinf.2020.03.019

34. Chandra A, Gurjar V, Qamar I, Singh N. Identification of potential inhibitors of SARS-COV-2 endoribonuclease (EndoU) from FDA approved drugs: a drug repurposing approach to find therapeutics for COVID-19. J Biomol Struct Dyn, 2021;39(12):4201-4211. Doi: 10.1080/07391102.2020.1775127

35. Jin Z, Du X, Xu Y, Deng Y, Liu M, Zhao Y, et al. Structure of M^{pro} from SARS-CoV-2 and discovery of its inhibitors. Nature, 2020;582(7811):289-293. Doi: 10.1038/s41586-020-2223-y

36. Al-Motawa MS, Abbas H, Wijten P, de la Fuente A, Xue M, Rabbani N, et al. Vulnerabilities of the SARS-CoV-2 virus to proteotoxicity-opportunity for repurposed chemotherapy of COVID-19 infection. Front Pharmacol, 2020;11:585408. Doi: 10.3389/fphar.2020.585408

37. Chang SJ, Huang SH, Lin YJ, Tsou YY, Lin CW. Antiviral activity of *Rheum palmatum* methanol extract and chrysophanol against Japanese encephalitis virus. Arch Pharm Res, 2014;37(9):1117-1123. Doi: 10.1007/s12272-013-0325-x

38. Lin CW, Tsai FJ, Tsai CH, Lai CC, Wan L, Ho TY, et al. Anti-SARS coronavirus 3C-like protease effects of *isatis indigotica* root and plant-derived phenolic compounds. Antiviral Res, 2005;68(1):36-42. Doi: 10.1016/j.antiviral.2005.07.002

39. Khanal P, Patil BM, Chand J, Naaz Y. Anthraquinone derivatives as an immune booster and their therapeutic option against COVID-19. Nat Prod Bioprospect, 2020;10(5):325-335. Doi: 10.1007/s13659-020-00260-2

40. Bhoj VG, Chen ZJ. Ubiquitylation in innate and adaptive immunity. Nature, 2009;458(7237):430-437. Doi: 10.1038/nature07959

41. Lindner HA, Fotouhi-Ardakani N, Lytvyn V, Lachance P, Sulea T, Ménard R. The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. J Virol, 2005;79(24):15199-15208. Doi: 10.1128/JVI.79.24.15199-15208.2005

42. Kuhn JH, Li W, Choe H, Farzan M. Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus. Cell Mol Life Sci, 2004;61(21):2738-2743. Doi: 10.1007/s00018-004-4242-5

43. Delcanale P, Uriati E, Mariangeli M, Mussini A, Moreno A, Lelli D, et al. The interaction of hypericin with SARS-CoV-2 reveals a multimodal antiviral activity. ACS Appl Mater Interfaces, 2022;14(12):14025-14032. Doi: 10.1021/acsami.1c22439

44. Barot KP, Jain SV, Kremer L, Singh S, Ghate MD. Recent advances and therapeutic journey of coumarins: current status and perspectives. Med Chem Res, 2015;24(7):2771-2798. Doi: 10.1007/s00044-015-1350-8

45. Stefanachi A, Leonetti F, Pisani L, Catto M, Carotti A. Coumarin: a natural, privileged and versatile scaffold for bioactive compounds. Molecules, 2018;23(2):250. Doi: 10.3390/molecules23020250

46. Calcio Gaudino E, Tagliapietra S, Martina K, Palmisano G, Cravotto G. Recent advances and perspectives in the synthesis of bioactive coumarins. RSC Adv, 2016;6(52):46394-46405. Doi: 10.1039/c6ra07071j

47. Rambabu D, Mulakayala N, Ismail, Kumar KR, Kumar GP, Mulakayala C, et al. Synthesis and pharmacological evaluation of N-substituted 2-(2-oxo-2H-chromen-4-yloxy) propanamide as cyclooxygenase inhibitors. Bioorg Med Chem Lett, 2012;22(21):6745-6749. Doi: 10.1016/j.bmcl.2012.08.082

48. Guan Z, Ding M, Sun Y, Yu S, Zhang A, Xia S, et al. The synthesis of two long-chain N-hydroxy amino coumarin compounds and their applications in the analysis of aldehydes. RSC Adv, 2017;7(32): 19707-19716. Doi: 10.1039/c7ra02177a

49. Venugopala KN, Rashmi V, Odhav B. Review on natural coumarin lead compounds for their pharmacological activity. Biomed Res Int, 2013;2013:963248. Doi: 10.1155/2013/963248

50. Murray RD. The naturally occurring coumarins. Fortschr Chem Org Naturst, 2002;83:1-619. Doi: 10.1007/978-3-7091-6172-2_1

51. Singh S, Sk MF, Sonawane A, Kar P, Sadhukhan S. Plant-derived natural polyphenols as potential antiviral drugs against SARS-CoV-2 via RNA-dependent RNA polymerase (RdRp) inhibition: an *in-silico* analysis. J Biomol Struct Dyn, 2021;39(16):6249-6264. Doi: 10.1080/07391102.2020.1796810

52. Mishra S, Pandey A, Manvati S. Coumarin: an emerging antiviral agent. Heliyon, 2020;6(1):e03217. Doi: 10.1016/j.heliyon.2020.e03217

53. Maurya AK, Mishra N. *In silico* validation of coumarin derivatives as potential inhibitors against main Protease, NSP10/NSP16-Methyltransferase, Phosphatase and Endoribonuclease of SARS CoV-2. J Biomol Struct Dyn, 2021;39(18):7306-7321. Doi: 10.1080/07391102.2020.1808075

54. Abdizadeh R, Hadizadeh F, Abdizadeh T. *In silico* analysis and identification of antiviral coumarin derivatives against 3-chymotrypsin-like main protease of the novel coronavirus SARS-CoV-2. Mol Divers, 2022;26(2):1053-1076. Doi: 10.1007/s11030-021-10230-6

55. Abdelmohsen UR, Albohy A, Abdulrazik BS, Bayoumi SAL, Malak LG, Khallaf ISA, et al. Natural coumarins as potential anti-SARS-CoV-2 agents supported by docking analysis. RSC Adv, 2021;11(28):16970-16979. Doi: 10.1039/d1ra01989a

56. Özdemir M, Köksoy B, Ceyhan D, Sayın K, Erçağ E, Bulut M, et al. Design and *in silico* study of the novel coumarin derivatives against SARS-CoV-2 main enzymes. J Biomol Struct Dyn, 2022;40(11):4905-4920. Doi: 10.1080/07391102.2020.1863263

57. Mun CS, Hui LY, Sing LC, Karunakaran R, Ravichandran V. Multi-targeted molecular docking, pharmacokinetics, and drug-likeness evaluation of coumarin-based compounds targeting proteins involved in the development of COVID-19. Saudi J Biol Sci, 2022;29(12):103458. Doi: 10.1016/j.sjbs.2022.103458

58. Suleimen YM, Jose RA, Suleimen RN, Ishmuratova MY, Toppet S, Dehaen W, et al. Isolation and *in silico* SARS-CoV-2 main protease inhibition potential of jusan coumarin, a new dicoumarin from *Artemisia glauca*. Molecules, 2022;27(7):2281. Doi: 10.3390/molecules27072281

59. Malik HN, Jabeen A, Ashraf S, Ul Haq Z, Salar U, Arshia UZ, et al. Benzophenone and coumarin derivatives as 3-CLPro inhibitors: targeting cytokine storm through *in silico* and *in vitro* approaches. J Mol Struct, 2022;1265:133478. Doi: 10.1016/j.molstruc.2022.133478

60. Patil SM, Martiz RM, Ramu R, Shirahatti PS, Prakash A, Chandra S J, et al. *In silico* identification of novel benzophenone-coumarin derivatives as SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) inhibitors. J Biomol Struct Dyn, 2022;40(23):13032-13048. Doi: 10.1080/07391102.2021.1978322

61. Singh R, Kumar A, Rane JS, Khan R, Tripathi G, Ajay AK, et al. Arylcoumarin perturbs SARS-CoV-2 pathogenesis by targeting the S-protein/ACE2 interaction. Sci Rep, 2022;12(1):17038. Doi: 10.1038/s41598-022-20759-7

62. Milenković DA, Dimić DS, Avdović EH, Marković ZS. Several coumarin derivatives and their Pd(II) complexes as potential inhibitors of the main protease of SARS-CoV-2, an *in silico* approach. RSC Adv, 2020;10(58):35099-35108. Doi: 10.1039/d0ra07062a

63. Omar RA, Koparir P, Sarac K, Koparir M, Safin DA. A novel coumarin-triazole-thiophene hybrid: synthesis, characterization, ADMET prediction, molecular docking and molecular dynamics studies with a series of SARS-CoV-2 proteins. J Chem Sci (Bangalore), 2023;135(1):6. Doi: 10.1007/s12039-022-02127-0

ORIGINAL ARTICLES

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Determining the effect of benzo[a]pyrene exposures toward innate and adaptive immunity profiles in post-measles vaccination mouse model

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ABSTRACT

Measles is an infectious disease caused by a virus of the genus Morbillivirus and is considered as global health problem. Several causes have been postulated that affect the success rate of vaccination, including benzo[a]pyrene exposure which widely distributed in air pollution. This study aims to evaluate the effect of BaP exposure to mouse immunity after measles vaccination. Several immunological parameters observed in this study were the expression of $CD4^+TNF\text{-}\alpha^+$, $CD4^+IFN\text{-}\gamma^+$, $CD4^+IFN\text{-}\alpha^+$, $CD8^+IFN\text{-}\alpha^+$, $CD11b^+IL17^+$, $B220^+CD25^+$, and the ratio of $CD4^+$:$B220^+$ from benzo[a]pyrene-exposured BALB/c mouse post-measles vaccination. Each sample was analyzed using a flow cytometry analysis. According to our findings, we found that benzo[a] pyrene interferes several parameters of immune system in measles-vaccinated mice. These findings suggested that the pollutant compounds especially benzo[a]pyrene can suppress the success rate of vaccination.

Keywords: adaptive immunity, benzo[a]pyrene, innate immunity, measles, vaccination

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INTRODUCTION

Measles is an infectious disease caused by a virus of the genus Morbillivirus. Initial infection with measles affects the respiratory and lymphatic systems. The measles virus replicates and enters the bloodstream[1]. Furthermore, the measles virus will then replicate and flow into the bloodstream. In the circulation, viruses infect erythrocytes and leukocytes. Infected leukocytes diminish lymphocytes and exacerbate immunosuppression[2]. Measles also cause secondary infections such as pneumonia, respiratory tract infections, and inflammation of the brain. A report showed that secondary infection has high possibility causing deaths[3]. According to Widagdo, about 90% of measles patients have a history of contact with other patients. Transmission of this disease can be through large droplets from the respiratory tract, but also through small droplets such as through inhaled air[4].

A prominent strategy to reduce the incidence of measles is expanding the vaccination program among society. Vaccination is an activity to induce specific antigens in the body to trigger a specific immune response[5]. Generally, the measles vaccination can be divided into a single or a combination vaccine administration. Measles vaccine can induce body protection for a long time and the very common measles vaccine that widely used is a live attenuated vaccine[2].

The success rate of vaccination can be influenced by internal and external factors. The success rate of the measles vaccination depends on the presence of maternal inhibitory antibodies, the maturity of the immune system of the vaccine recipient, and the dose of vaccine administered to the patient[6]. In addition, external factors such as pollution are also thought to affect the success rate of vaccination by reducing antibody responses to the vaccine[7]. One of the pollutant compounds contained in vehicle exhaust gas, cigarette smoke, and the residue of organic materials combustion such as benzo[a]pyrene (BaP). Research has shown that BaP can affect the immune system[8]. As widely known, the immune system components such as lymphocytes, macrophages, or pro-inflammatory cytokines have an important role in the immune response after vaccination. For an instance, the TNF-α activity post-vaccination is critical for regulating the cascade of pro-inflammatory cytokine production and recruitment of immunocompetent cells. Therefore, from above explanation we aim to evaluate the effect of BaP exposure to mouse innate and adaptive immunity after measles vaccination.

METHODOLOGY

Sample preparation and treatment groups

This study was occupied about 24 pathogen-free female BALB/c mice aged 2-week-old which obtained from LPPM Gadjah Mada University. In this study, the treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB). The measles vaccine used in this present study is a live attenuated type of measles vaccine. Each dose of vaccine contains dry measles virus, kanamycin sulfate and erythromycin (Bio Farma, Indonesia). The dose of measles vaccine injected was 20 mg/kg BW. Similarly, the dose of BaP injected was 20 mg/kg BW. This study has been evaluated by Research Ethics Commission of Brawijaya University with ethical clearance no. 930-KEP-UB.

Treatment procedures

The experimental treatment of mice was carried out after a week acclimatization process. The injection of BaP was carried out every 3 days for 5 weeks (9 times injection), except for the vehicle treatment and the measles vaccine. Measles vaccine injection was carried out once at the beginning of the treatment through subcutaneous approach. On the other hand, BaP injection was performed intraperitoneally after finishing vaccination procedures (Figure 1).

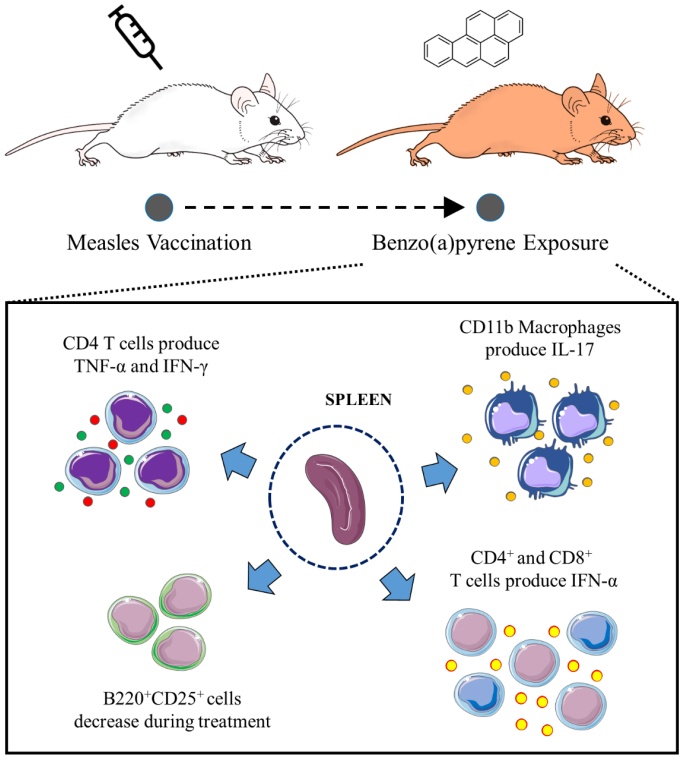

Figure 1. Schematic picture showed how benzo[a]pyrene interferes the measles vaccination effects of the immune system, including the innate and adaptive immunity and the production of cytokines on BALB/c mice.

Isolation of splenocytes and antibody staining

The protocol used for splenocytes and antibody staining was based on our previous studies[9-12]. Spleen organs from each treatment were isolated and washed using phosphate buffer saline to remove fat and debris. The homogenate of sample was put in a propylene tube and stored in an ice box. All homogenates were centrifuged at 2500 rpm for five minutes at 4°C to separate the pellet and supernatant. The pellet was resuspended using 1 ml of PBS. Each sample was put in a microtube for next antibody staining procedures. The sample was then centrifuged at 2500 rpm for five minutes at 4°C. The supernatant portion was discarded, while the pellet portion was added with 50 µl of extracellular antibody. The added antibodies were anti-CD4, anti-CD8, anti-CD11b, and anti-B220 (Biolegend, San Diego). Samples were incubated for 20 minutes in an ice box. Samples containing extracellular antibodies were added with 50 µl of cytofix. The samples were then incubated for 20 minutes in an ice box. Then

500 µl of washperm was added to the sample. The sample was centrifuged again at 2500 rpm for five minutes at 4°C. The supernatant was discarded while the pellet was added with 50 µl of intracellular antibody. The intracellular antibodies used were anti-TNF-α, anti-IFN-α, anti-IFNγ, and anti-IL-17 (Biolegend, San Diego). Samples were incubated for 20 minutes in an ice box.

Flow cytometry, chemical interaction prediction, and data analysis

Stained samples were added with 300 µl of PBS. Each sample was put into a cuvette for analysis using a flow cytometer. Each sample was analyzed using the BD Bioscience FACSCalibur™ flow cytometer (BD Biosciences). Data analysis of flow cytometry results was carried out using the BD CellQuest Pro™ program (BD Biosciences). Furthermore, STITCH database (http://stitch.embl.de/) was used to evaluate the interaction between chemical (benzo[a]pyrene) with certain protein[13,14]. Statistical test was performed by one way ANOVA analysis. The analysis was carried out with SPSS Ver. 20 software. The further tests used were the Tukey-HSD and Games Howell tests with $p<0.05$.

RESULTS and DISCUSSION

Effect of BaP exposures on $CD4^+TNF\text{-}\alpha^+$ expression

The relative number of $CD4^+TNF\text{-}\alpha^+$ molecules in the BaP group did not have a significant difference with the vehicle group (Figure 2). Oppositely, the research conducted by Lu et al. showed that BaP exposure increases TNF-α production and SIRT1[15]. SIRT1 is a regulatory protein that plays a role in the control of inflammation. SIRT1 protects cells from chronic inflammation by regulating NF-κB activity[16]. Activation of Nf-κB could enhance the production of pro-inflammatory cytokines including IL-1β and TNF-α[17].

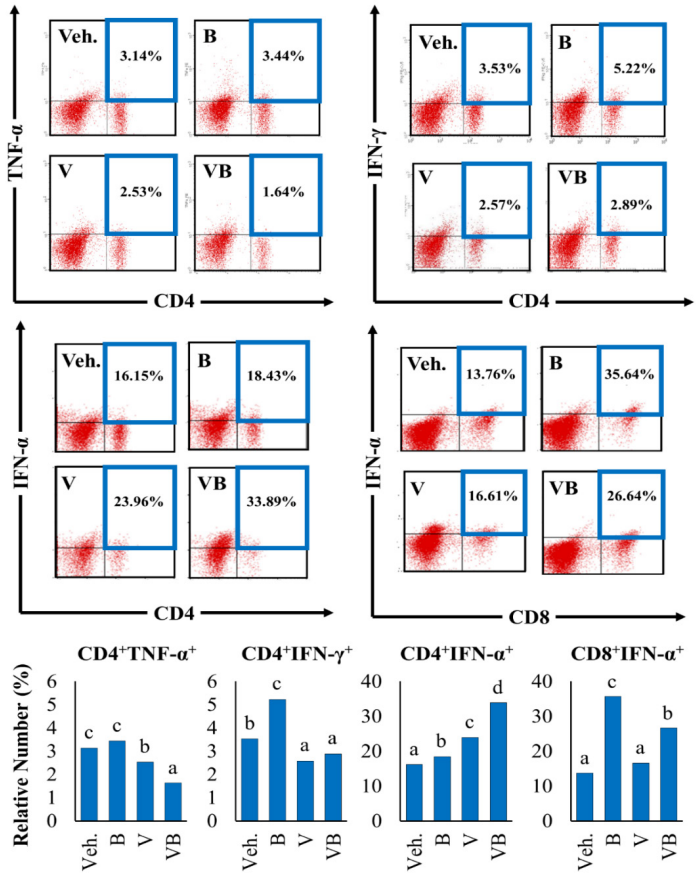

Figure 2. Relative number of $CD4^+TNF\text{-}\alpha^+$, $CD4^+IFN\text{-}\gamma^+$, $CD4^+IFN\text{-}\alpha$+, $CD8^+IFN\text{-}\alpha^+$ on experimental mice. Different number on graph showed the significant difference among experimental group ($p<0.05$).

The treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB).

Furthermore, vaccine group showed that the relative number of $CD4^+TNF\text{-}\alpha^+$ was significantly lower than the vehicle group. Research conducted by Ovsyannikova et al. stated that the administration of the vaccine increases in the amount of TNF-α[18]. The increase in TNF-α is one of the stages of the body's response when an antigen in the form of vaccine is inserted until it finally succeeds in forming specific antibodies. The decrease in $CD4^+TNF\text{-}\alpha^+$ production in the vaccine treatment when compared to the control could be caused by several possibilities. In general, the TNF-α cytokine would experience a significant increase after the second dose of measles vaccine was given.

BaP administration to vaccinated-mice caused a decrease in the relative number of $CD4^+TNF\text{-}\alpha^+$ molecules. BaP is a chemical compound that can cause cell damage and can be converted into metabolites which have immunosuppressant activity. BaP metabolites are immunosuppressant that leading in DNA mutations and DNA damage. In addition, decreased TNF-α production might occurs due to inhibition of CD4 T cell proliferation[19]. Similarly, the decrease in $CD4^+TNF\text{-}\alpha^+$ in VB treatment group could also be caused by an increase in the number of regulatory T cells. Regulatory T cells can produce TGF-β which can suppress the production of pro-inflammatory cytokines[20].

The administration of antigen in the form of a vaccine caused the activation of CD4 T cells. Activated CD4 T cells had a higher number of AhRs protein when compared to inactivated CD4 T cells[21]. AhR protein is a BaP receptor for initiating the production of CYP1B1 or CYP1A1 enzymes. These enzymes are involved in the metabolism of BaP to produce metabolites that are immunosuppressant. This fact is in line with our *in silico* finding (Figure 3) about the possible interaction between BaP and other type of target protein such as CYP1A2, CYP1A1, AHR, CYP1B1, TREX1, CYP4B1, CYP4X1, CYP2C8, CYP2J2, and CYP2D6. According to the predicted functional partner score, there are three proteins with the highest value including CYP1A2, CYP1A1, and AHR. The above prediction might confirm that BaP might have the closest influence with these proteins. Furthermore, the predicted proteins were involved in several pathways related to the BaP induction, including metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis, serotonergic synapse, and others (Table 1). To the greater extend, the prediction also showed BaP included in several biological activity including small molecule metabolic process, oxidation-reduction process, cellular catabolic process, cellular lipid metabolic process, monocarboxylic acid metabolic process, and others (Figure 4).

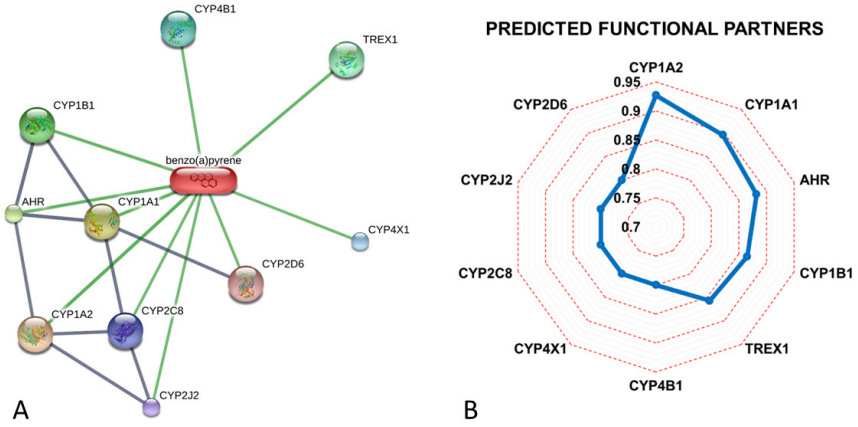

Figure 3. (A) The interaction among benzo[a]pyrene and possible targeted protein that include or affected by benzo[a]pyrene. (B) The predicted functional partners involved within the interaction with benzo[a]pyrene.

Table 1. The list of KEGG enrichment related to the target proteins which involved in the interaction with benzo[a]pyrene

No	Pathway Description	False Discovery Rate	Genes Involved
1	Metabolism of xenobiotics by cytochrome P450	6.19E-06	CYP1A1,CYP1A2,CYP1B1,CYP2D6
2	Chemical carcinogenesis	6.19E-06	CYP1A1,CYP1A2,CYP1B1,CYP2C8
3	Serotonergic synapse	2.34E-05	CYP2C8,CYP2D6,CYP2J2,CYP4X1
4	Linoleic acid metabolism	2.70E-05	CYP1A2,CYP2C8,CYP2J2
5	Tryptophan metabolism	6.00E-05	CYP1A1,CYP1A2,CYP1B1
6	Ovarian steroidogenesis	0.000107	CYP1A1,CYP1B1,CYP2J2
7	Steroid hormone biosynthesis	0.000109	CYP1A1,CYP1A2,CYP1B1
8	Retinol metabolism	0.000126	CYP1A1,CYP1A2,CYP2C8
9	Drug metabolism - cytochrome P450	0.000144	CYP1A2,CYP2C8,CYP2D6
10	Arachidonic acid metabolism	0.0136	CYP2C8,CYP2J2

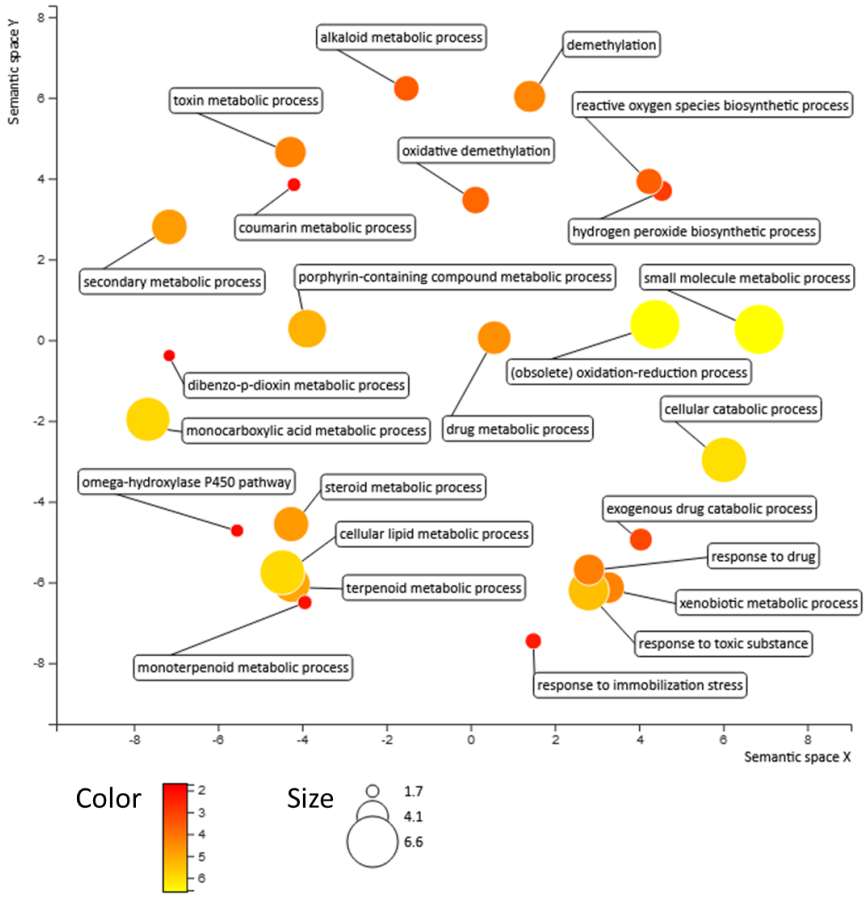

Figure 4. Possible biological process related to the target proteins which involved in the interaction with benzo[a]pyrene

Effect of BaP exposures on $CD4^+IFN\text{-}\gamma^+$ expression

In this present study, the BaP increase the relative number of $CD4^+IFN\text{-}\gamma^+$ (Figure 2). This is probably because CD4 T cells are still able to carry out repair mechanisms against DNA damage caused by BaP metabolites. On the other hand, the production of IFN-γ was lower in the vaccine treatment compared to the vehicle group.

Vaccination will stimulate the immune system to increase immunity against incoming pathogens. Research conducted by Ovsyannikova et al. stated that administering measles vaccine to experimental animals caused an increase in the production of pro-inflammatory cytokines, including IFN-γ[22]. The immune

system has a time limit of activation against the injected measles virus antigen. Antibody production, which indicates the last stage of the immune response to measles virus antigen, occurred on day 35 after vaccination[23]. Meanwhile, the maximum production of IFN-γ as a pro-inflammatory cytokine occurred on day 12 post-vaccination[24].

Furthermore, the results showed that the relative number of $CD4^+IFN\text{-}\gamma^+$ expression did not have a significant difference between the vaccine and BaP-vaccine treatments. In general, BaP administration led to an increase in post-vaccination IFN-γ production. Research conducted by Hur et al. stated that BaP can trigger FasL gene transcription in macrophages. FasL is one of the proteins involved in cell apoptosis[25].

As research conducted by Wu et al. showed that BaP is also a combustion derived particulate matter group that has the potential to damage macrophage cells located in mucosal areas[26]. The IFN-γ is one of the pro-inflammatory cytokines that play a role in macrophage activation[27]. Therefore, increasing the amount of IFN-γ is one of the body's defense mechanisms to balance the number of macrophages in the body.

IFN-γ and TNF-α are pro-inflammatory cytokines that work together in macrophage activation[28-30]. When the production of TNF-α by CD4 T cells decreases, then CD4 T cells will produce more IFN-γ to compensate for the decreased production of TNF-α. In addition, one other function of IFN-γ is to increase the amount of TNF-α[31,32]. Increasing the amount of IFN-γ becomes important to meet the body's need for TNF-α which has decreased due to BaP. Continuous exposure to BaP causes the increasing the expression of $CD4^+IFN\text{-}\gamma^+$. The continuous production of pro-inflammatory cytokines will activate immunocompetent cells. Activated immune cells will cause chronic inflammatory reaction.

Effect of BaP exposures on IFN-α production by $CD4^+$ and $CD8^+$ T cells

Interestingly, we found the significant differences between all treatment groups. There is an increasing trend in the percentage of IFN-α expression in $CD4^+$ T cells. Meanwhile, the percentage of IFN-α expression by $CD8^+$ T cells in Veh. and V treatment groups did not show any significant difference. However, a significant increase occurred in the treatment groups B and VB (Figure 2).

The increase in IFN-α expression on $CD4^+$ and $CD8^+$ occurred in BaP treatment, either without or in combination with measles vaccine. It is possible that the increase in IFN-α is a response to cell damage caused by BaP injection. The

higher IFN-α production compared to Veh. and V treatment groups aimed to increase the cytotoxicity of cytotoxic T cells. The important reason behind the increase cytotoxic T cells aim to kill damaged cells that have been affected by exposure to BaP. This refers to Li et al. who stated that one of the functions of IFN-α is to increase the cytotoxicity of cytotoxic T cells[33]. Meanwhile, the high $CD8^+IFN\text{-}\alpha^+$ T cells in BaP group were thought to be a form of compensation for the low IFN-α expression in $CD4^+$ T cells.

Meanwhile, when compared with vehicle group, the percentage of $CD4^+IFN\text{-}\alpha^+$ T cells in the vaccine treatment was higher. Similarly, Gibbert et al. stated that IFN-α is one of the signaling proteins produced by the body due to exposure to antigens such as viruses. Meanwhile, the injected vaccine is an attenuated measles virus that has the potential to increase the production of IFN-α in cells[34].

Effect of BaP exposures on $CD11b^+IL\text{-}17^+$ expression

BaP administration to mice significantly increased the production of $CD11b^+IL\text{-}17^+$ (Figure 5). Research conducted by Mohinta et al. stated that BaP can cause an increase in IL-17 gene expression[35]. Measles vaccine administration in experimental animals caused an increase in the relative $CD11b^+IL\text{-}17^+$ count but there was no significant difference with the vehicle group. Research conducted by Nelson et al., stated that IL-17 was produced on day 10, 35, and optimally on day 52 after the entry of measles antigen[36]. The experimental animal surgery in this study was carried out on the 35th day after the measles vaccine injection. Therefore, it is possible that the body has started to produce IL-17 but has not reached the optimal amount.

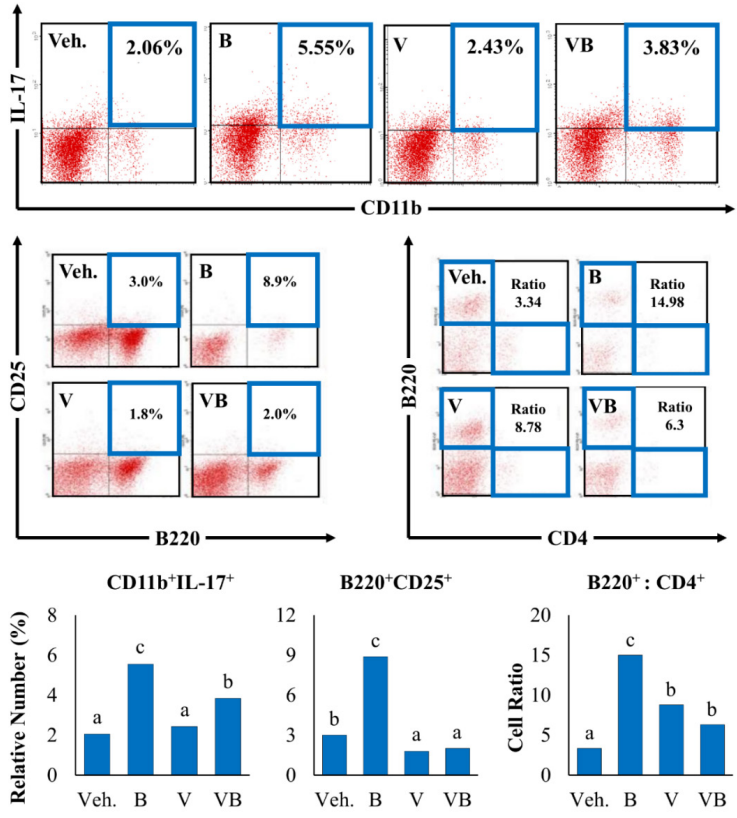

Figure 5. Relative number of CD11b+IL17+, B220+CD25+, and the ratio of CD4+:B220+ on experimental mice. Different number on graph showed the significant difference among experimental group ($p<0.05$). The treatment groups were vehicle (Veh.), mice injected with BaP (B), mice injected with vaccine (V), and mice injected with both vaccine and BaP (VB).

Administration of BaP to vaccinated mice significantly increased the relative number of $CD11b^+IL\text{-}17^+$. Research conducted by Mohinta et al. stated that BaP can cause an increase in the expression of the gene encoding IL-17[35]. The relative number of $CD11b^+IL\text{-}17^+$ in VB treatment was lower than in BaP group. This was due to the presence of regulatory T cells. The increase in IL-17 is always accompanied by an increase in regulatory T cells. The relative number of $CD11b^+IL\text{-}17^+$ had a relationship with the number of regulatory T cells and TGF-β production. TGF-β is a cytokine that widely produced by regulatory T cells and plays a role in controlling the number of pro-inflammatory cytokines[20]. The continuous increase in the number of IL-17 has an unfavorable impact because IL-17 is a cytokine that plays a role in controlling autoimmune mechanisms. Autoimmunity is a disorder of control of immune cells and the

production of auto-antibodies that result in damage to the body's own tissues[37]. Excessive IL-17 expression will cause chronic inflammation. The amount of IL-17 in normal measles virus antigen response is always opposite to the amount of IFN-γ. This is because the inducer of IFN-γ, namely IL-12, also acts as an inhibitor of IL-17 production[38].

Effect of BaP exposures on $B220^+CD25^+$ expression

A significant increase of memory B cell was found in BaP group (Figure 5). This is relatively far above the normal limit as reported by Amu et al. that the expression of memory $B220^+CD25^+$ cells in the spleen of healthy mice is about 2% of the total concentration of B cells[39]. The increase in the percentage of $B220^+CD25^+$ expression in BaP group could be due to BaP being able to increase $CD25^+$ expression in B cells through activation of the NF-κB pathway. This is supported by Ba et al. who stated that long-term accumulation of BaP can increase the activity of the NF-κB promoter[40]. Brisslert et al. showed NF-κB pathway blockade can inhibit the production of $CD25^+$ by B cells[41]. Meanwhile, the low $CD25^+$ expression in the VB group was thought to be due to the presence of the injected vaccine increasing the proliferation of B cells.

Effect of BaP exposures on $B220^+$:$CD4^+$ ratio

In this present study we found vehicle group has the lowest $B220^+$:$CD4^+$ ratio. Meanwhile, a significant increase occurred in BaP treatment group. Meanwhile, the VB treatment group experienced a significant decrease compared to BaP group, while vaccine group did not differ significantly from VB group (Figure 5).

The injection of BaP can increase the ratio by decreasing the number of $CD4^+$ T cells. The decrease in the number of $CD4^+$ T cells is related to the nature of BaP as an immunotoxic. Furthermore, BaP suppresses immunity through p53-dependent pathways which modulates signaling in lymphocytes and oxidative stress[42]. Meanwhile, the high ratio in the vaccine treatment was thought to be due to the fact that the vaccination was only carried out once, so that the accumulation of effector cells such as large numbers of $CD4^+$ T cells in the spleen did not occur. This can be explained that the injected antigen is only transient and is unable to induce the accumulation of effector cells in the spleen. According to Sarkander et al., when the antigen is transient, more immune cells will accumulate in the bone marrow[43].

Finally, based on the result, we found that BaP interferes several parameters of immune system in measles-vaccinated mice. These findings suggested that the chemicals compounds such as BaP can suppresses the success rate of vaccination.

STATEMENTS OF ETHICS

The protocols used in this study were following the guide for the care and use of experimental animals and the study passed the institutional ethical clearance by Research Ethics Commission of Brawijaya University with No. 930-KEP-UB.

CONFLICT OF INTEREST STATEMENT

All authors declare there is no conflict of interest.

AUTHOR CONTRIBUTIONS

W.E.P. was involved in data collection and analysis, methodology, and draft preparation. F.R.P.N. and M.I.F. were involved in data collection and analysis & review, and editing. M.R. was involved in study design, funding acquisition, supervision, review, and editing. All authors contributed to revision and approval of the final manuscript.

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REFERENCES

1. Fernandez R, Rammohan A, Awofeso N. Correlates of first dose of measles vaccination delivery and uptake in Indonesia. Asian Pac J Trop Med, 2011;4(2):140-145. Doi: 10.1016/s1995-7645(11)60055-2

2. Goodson JL, Seward JF. Measles 50 years after use of measles vaccine. Infect Dis Clin North Am, 2015;29(4):725-743. Doi: 10.1016/j.idc.2015.08.001

3. Perry RT, Halsey NA. The clinical significance of measles: a review. J Infect Dis, 2004;189(1):4-16. Doi: 10.1086/377712

4. Widagdo. Masalah dan tatalaksana penyakit anak dengan demam (Problems and management of children with fever). Jakarta: Sagung Seto; 2012.

5. Ovsyannikova IG, Larrabee BR, Schaid DJ, Poland GA. Immunoglobulin GM and KM genes and measles vaccine-induced humoral immunity. Vaccine, 2017:35(41):5444-5447. Doi: 10.1016/j.vaccine.2017.02.046

6. Lahariya C. Vaccine epidemiology: a review. Fam Med Prim Care Rev, 2016;5(1):7-15. Doi: 10.4103/2249-4863.184616

7. Jusko TA, De Roos AJ, Lee SY, Thevenet-Morrison K, Schwartz SM. A birth cohort study of maternal and infant serum PCB-153 and DDE concentrations and responses to infant tuberculosis vaccination. Environ Health Perspect, 2016;124(6):813-821. Doi: 10.1289/ehp.1510101

8. Hwang JA, Lee JA, Cheong SW, Youn HJ, Park JH. Benzo(a)pyrene inhibits growth and functional differentiation of mouse bone marrow-derived dendritic cells. Downregulation of RelB and eIF3 p170 by benzo(a)pyrene. Toxicol Lett, 2007;169(1):82-90. Doi: 10.1016/j.toxlet.2007.01.001

9. Putra WE, Soewondo A, Rifa'i M. Effect of dexamethasone administration toward hematopoietic stem cells and blood progenitor cells expression on BALB/c mice. JPACR, 2016;4(3):100-108. Doi: 10.21776/ub.jpacr.2015.004.03.221

10. Putra WE, Soewondo A, Rifa'i M. Expression of erythroid progenitor cells and erythrocytes on dexamethasone induced-mice. Biotropika, 2015;3(1):42-45.

11. Putra WE, Insani AF, Nurhayati D, Hidayatullah A, Rifa'i M. Assessing the immunomodulatory and hepatoprotective activities of aqueous tuber extract of *Typhonium flagelliforme* (Lood) Blume in BALB/c mouse. Jordan J Biol Sci, 2023;16(4):655-664. Doi: 10.54319/jjbs/160411

12. Putra WE, Maulana AR, Ramadhan ATK, Rifa'i M. T cells regulation modulated by *Sambucus javanica* extracts in DMBA-exposed mice. J Herbmed Pharmacol, 2020;9(4):408-411. Doi: 10.34172/jhp.2020.51

13. Putra WE, Waffareta E, Ardiana O, Januarisasi ID, Soewondo A, Rifa'i M. Dexamethasone-administrated BALB/c mouse promotes proinflammatory cytokine expression and reduces $CD4^+CD25^+$ regulatory T cells population. Biosci Res, 2017;14(2):201-213.

14. Putra WE, Agusinta AK, Ashar MSAA, Manullang VA, Rifa'i M. Immunomodulatory and ameliorative effect of *Citrus limon* extract on DMBA-induced breast cancer in mouse. Karbala Int J Mod Sci, 2023;9(2):1-14. Doi: 10.33640/2405-609X.3273

15. Lu J, Zhang M, Huang Z. SIRT1 in B[a]P-induced lung tumorigenesis. Oncotarget, 2015;6(29):27113-27129. Doi: 10.18632/oncotarget.4729

16. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol, 2009;1(6):1-11. Doi: 10.1101/cshperspect.a001651

17. Ji K, Xing C, Jiang F, Wang X, Guo H, Nan J, et al. Benzo[a]pyrene induces oxidative stress and endothelial progenitor cell dysfunction via the activation of the NF-κB pathway. Int J Mol Med, 2013;31(4):922-930. Doi: 10.3892/ijmm.2013.1288

18. Ovsyannikova IG, Reid KC, Jacobson RM, Oberg AL, Klee GG, Poland GA. Cytokine production patterns and antibody response to measles vaccine. Vaccine, 2003;21(25):3946-3953. Doi: 10.1016/s0264-410x(03)00272-x

19. Carlson EA, Li Y, Zelikoff JT. Benzo[a]pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*): Relationship between lymphoid CYP1A activity and humoral immune suppression. Toxicol Appl Pharmacol, 2004;201(1):40-52. Doi: 10.1016/j.taap.2004.04.018

20. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. Immunity, 2019;50(4):924-940. Doi: 10.1016/j.immuni.2019.03.024

21. Prigent L, Robineau M, Jouneau S, Morzadec C, Louarn L, Vernhet L, et al. The aryl hydrocarbon receptor is functionally upregulated early in the course of human T-cell activation. Eur J Immunol, 2014;44(5):1330-1340. Doi: 10.1002/eji.201343920

22. Ovsyannikova IG, Dhiman N, Jacobson RM, Vierkant RA, Pappouli R, Bagnoli F. Vaccine design: innovative approaches and novel strategies. United Kingdom:Caister Academic; 2011.

23. Kim D, Niewiesk S. Synergistic induction of interferon α through TLR-3 and TLR-9 agonists identifies CD21 as interferon α receptor for the B cell response. PLoS Pathogens, 2013;9(3):1-12. Doi: 10.1371/journal.ppat.1003233

24. Griffin DE. The immune response in measles: virus control, clearance and protective immunity. Viruses, 2016;8(10):1-8. Doi: 10.3390/v8100282

25. Hur D, Jeon JK, Hong S. Analysis of immune gene expression modulated by benzo[a] pyrene in head kidney of olive flounder (*Paralichthys olivaceus*). Comp Biochem Physiol B Biochem Mol Biol, 2013;165(1):49-57. Doi: 10.1016/j.cbpb.2013.03.001

26. Wu W, Jin Y, Carlsten C. Inflammatory health effects of indoor and outdoor particulate matter. J Allergy Clin Immunol, 2018;141(3):833-844. Doi: 10.1016/j.jaci.2017.12.981

27. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leuko Biol, 2004;75(2):163-189. Doi: 10.1189/jlb.0603252

28. Putra WE, Puspaningrum KR, Hidayatullah A, Rifa'i M. Immunomodulatory properties of *Citrus limon* extracts on BALB/c Mouse lymphoid and myeloid lineage cells. Jordan J Biol Sci, 2023;16(3):445-454. Doi: 10.54319/jjbs/160308

29. Salim T, Sershen CL, May EE. Investigating the role of TNF-α and IFN-γ activation on the dynamics of iNOS gene expression in LPS stimulated macrophages. PLoS One, 2016;11(6):1-35. Doi: 10.1371/journal.pone.0153289

30. Putra WE, Rifa'i M. Immunomodulatory activities of *Sambucus javanica* extracts in DMBA exposed BALB/c mouse. Adv Pharm Bull, 2019;9(4):619-623. Doi: 10.15171/apb.2019.071

31. Putra WE, Rifa'i M. Assessing the immunomodulatory activity of ethanol extract of *Sambucus javanica* berries and leaves in chloramphenicol-induced aplastic anemia mouse model. Trop Life Sci Res, 2020;31(2):175-185. Doi: 10.21315/tlsr2020.31.2.9

32. Mak TW, Saunders ME. The immune response basic and principle. Amsterdam: Academic Press; 2006.

33. Li SF, Gong MJ, Zhao FR, Shao JJ, Xie YL, Zhang YG, et al. Type I interferons: distinct biological activities and current applications for viral infection. Cell Physiol Biochem, 2018;51(5):2377-2396. Doi: 10.1159/000495897

34. Gibbert K, Schlaak JF, Yang D, Dittmer U. IFN-α subtypes: distinct biological activities in anti-viral therapy. Br J Pharmacol, 2013;168(5):1048-1058. Doi: 10.1111/bph.12010

35. Mohinta S, Kannan AK, Gowda K, Amin SG, Perdew GH, August A. Differential regulation of Th17 and T regulatory cell differentiation by aryl hydrocarbon receptor dependent xenobiotic response element dependent and independent pathways. Toxicol Sci, 2015;145(2):233-243. Doi: 10.1093/toxsci/kfv046

36. Nelson AN, Putnam N, Hauer D, Baxter VK, Adams RJ, Griffin DE. Evolution of T cell responses during measles virus infection and RNA clearance. Sci Rep, 2017;7(1):1-10. Doi: 10.1038/s41598-017-10965-z

37. Tabarkiewicz J, Pogoda K, Karczmarczyk A, Pozarowski P, Giannopoulos K. The role of IL-17 and Th17 lymphocytes in autoimmune diseases. Arch Immunol Ther Exp (Warsz), 2015;63(6):435-449. Doi: 10.1007/s00005-015-0344-z

38. Hoeve MA, Savage ND, de Boer T, Langenberg DM, de Waal MR., Ottenhoff TH, et al. Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. Eur J Immunol, 2006;36(3):661-670. Doi: 10.1002/eji.200535239

39. Amu S, Gjertsson I, Tarkowski A, Brisslert M. B-cell CD25 expression in murine primary and secondary lymphoid tissue. Scand J Immunol, 2006;64(5):482-492. Doi: 10.1111/j.1365-3083.2006.01832.x

40. Ba Q, Li J, Huang C, Qiu H, Li J, Chu R, et al. Effects of benzo[a]pyrene exposure on human hepatocellular carcinoma cell angiogenesis, metastasis, and NF-κB signaling. Environ Health Perspect, 2015;123(3):246-254. Doi: 10.1289/ehp.1408524

41. Brisslert M, Bokarewa M, Larsson P, Wing K, Collins LV, Tarkowski A. Phenotypic and functional characterization of human $CD25^+$ B cells. Immunology, 2006;117(4):548-557. Doi: 10.1111/j.1365-2567.2006.02331.x

42. Allmann S, Mayer L, Olma J, Kaina B, Hofmann TG, Tomicic MT, et al. Benzo[a]pyrene represses DNA repair through altered E2F1/E2F4 function marking an early event in DNA damage-induced cellular senescence. Nucleic Acids Res, 2020;48(21):12085-12101. Doi: 10.1093/nar/gkaa965

43. Sarkander J, Hojyo S, Tokoyoda K. Vaccination to gain humoral immune memory. Clin Transl Immunol, 2016;5(12):1-6. Doi: 10.1038/cti.2016.81

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Association between 25-hydroxy Vitamin D_3 levels and thyroiditis staged by using ultrasonography

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ABSTRACT

Vitamin D deficiency has been linked to a higher prevalence of thyroid gland impairment. The association between 25-hydroxy Vitamin D_3 concentrations and sonographic changes of thyroiditis was investigated. A total of 55 patients were divided into 3 groups according to the thyroid ultrasonography (US) findings: Group 1: control cases (n=17); Group 2: intermediate (early) cases (n=14); and Group 3: active and late-stage thyroiditis cases (n=24). Serum 25-hydroxy Vitamin D concentrations, thyroid hormones (free T3, free T4, TSH) and antibody levels (TPO Ab, Tg Ab) were assessed in different stages of thyroiditis. The Vitamin D concentrations of Group 1 (28.54 ± 20.43 ng/dL) were significantly higher than those of Group 2 (14.48 ± 5.87 ng/mL) and Group 3 (14.3 ± 9.2 ng/dL) ($p=0.025$ and $p=0.004$, respectively). Tg Ab and TPO Ab significantly increased in Group 3 compared to Groups 1 and 2 ($p<0.001$, $p<0.001$, respectively). 25-hydroxy Vitamin D_3 deficiency was associated with thyroid gland morphological changes detected by ultrasonography.

Keywords: 25-OH Vitamin D_3, autoimmune thyroiditis, anti-Tg, anti-TPO, sonography

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INTRODUCTION

Vitamin D is a well-known autoimmune system regulator. Vitamin D deficiency has been linked to a higher prevalence of thyroid autoimmunity in certain populations, including children, adolescents, and obese individuals. Moreover, Vitamin D supplementation has shown promise in reducing antithyroid antibody levels, improving thyroid function[1-3]. However, while some papers publish evidence that Vitamin D is an important factor in developing autoimmune thyroiditis[4-9], some papers report that their data do not provide any relationship[10,11].

Conventional method of diagnosing autoimmun thyroiditis (AT) is detecting anti-thyroglubulin (anti-Tg) and anti-thyroperoxidase (anti-TPO) high in serum[12]. During the development of autoimmune thyroiditis, structural changes occur in the gland. Hashimato Thyroiditis (HT) is characterized pathologically by lymphocytic infiltration of the interstitium, mainly lymphocytes with some plasma cells and macrophages[12,13]. The lymphoid tissue is distributed within and around the lobules and often exhibits large follicles with prominent germinal centers[12,14]. The inflammatory process also results in oxyphilic changes in follicular epithelial cells, parenchymal atrophy of thyroid tissue, and varying degrees of fibrosis, imparting a firm consistency to the thyroid[15]. These changes in the gland can be observed by ultrasonography. Thyroid ultrasonographic evaluation of patients with HT generally reveals diffuse enlargement of the gland, a heterogeneous background, and a general decrease in echogenicity. Other sonographic findings of HT often include hypervascularity and the presence of hypoechoic micronodules with an echogenic rim[16,17]. Some authors advocate the use of ultrasonography in cases of AT[18,19] because it can provide information about the level of inflammatory activity[20] and thyroiditis severity[21]. The histopathological findings and sonographic features in different stages of AT have been reported and used in clinical practice[22].

In this study, the association between thyroiditis and 25-hydroxy Vitamin D_3 levels was evaluated during different stages of thyroiditis based on morphological changes in the thyroid gland by using ultrasonography.

METHODOLOGY

The study groups

All patients (n=253) who were referred to our study radiologist for thyroid gland ultrasound (US) between June 2013 and February 2014 were included in the data. Among the patients, pregnant and breastfeeding women, pediatric patients, and patients who had undergone surgery and those with known malignancy, short neck, marked goiter, and thyroid nodules were excluded.

After exclusion, the study was conducted with 55 cases (15 male, 40 female). All patients underwent physical examination, thyroid hormones (free T_3, free T_4 and TSH) and 25-hydroxy Vitamin D_3 were analyzed, and thyroid sonography evaluation was performed. This study was approved by the Medipol University Ethics Committee.

Sonographic evaluation of thyroid gland

In this study, thyroiditis staging by ultrasonography was performed and reported as described by Ormeci et al.[22]. Briefly, normal thyroid parenchyma has a homogeneous medium-high level of echogenicity compared to periglandular muscles, diffuse tissue enlargement, parenchymal hypoechogenicity and coarsening. During US examination, the radiologist reported the thyroid gland sonographic findings as the echo texture (homogeneous or heterogeneous), echogenicity (hypo- or hyperechoic), contouring (regular or irregular), nodulation or pseudonodulation, and vascularization of the thyroid gland (decreased, normal, or increased) (Table 1)[22].

Table 1. Sonographic features of the groups[22]

Sonographic criteria	Group 1 Normal thyroid	Group 2 Early/indeterminate thyroiditis	Group 3 Chronic thyroiditis
Volume	Normal	Increased, normal	Decreased
Echotexture	Homogeneous	Homogeneous, slightly heterogenous	Heterogeneous
Echogenicity (compare to the muscles)	Hyperechoic	Hyperechoic, slightly hypoechoic	Hypoechoic
Contouring	Smooth	Smooth	Irregular
Nodulation/ pseudonodulation	No	No, slightly nodular	Pseudonodulation
Vascularization	Normal	Normal, increased	Increased, normal, decreased

Patients were grouped by thyroid gland sonographic features according to the properties described above, and the patients were divided into 3 groups according to sonography results as described in Table 1.

Group 1 (control cases; Figure 1-a) consisted of cases with normal thyroid gland ultrasonography, clinical, and laboratory findings. Group 2 (intermediate/earlystage cases; Figure 1-b) consisted of cases with minimal parenchymal hypoechogenicity and suspected heterogeneity but were still clinically suspected of autoimmune thyroiditis. Group 3 (active and advanced-late-stage thyroiditis patients; Figure 1-c and 1-d) consisted of patients in whom the parenchyma was heterogeneous/hypoechogenic in appearance and who had interlobular septa, irregular contours, pseudonodules, and glands of reduced size[22]. Ultrasound evaluation of thyroid gland was performed by a single radiologist who was blind to the thyroid hormones data. A Doppler device (LOGIQ P6 Pro, GE Healthcare GmBH, Germany) fitted with a linear 11 MHz probe was used.

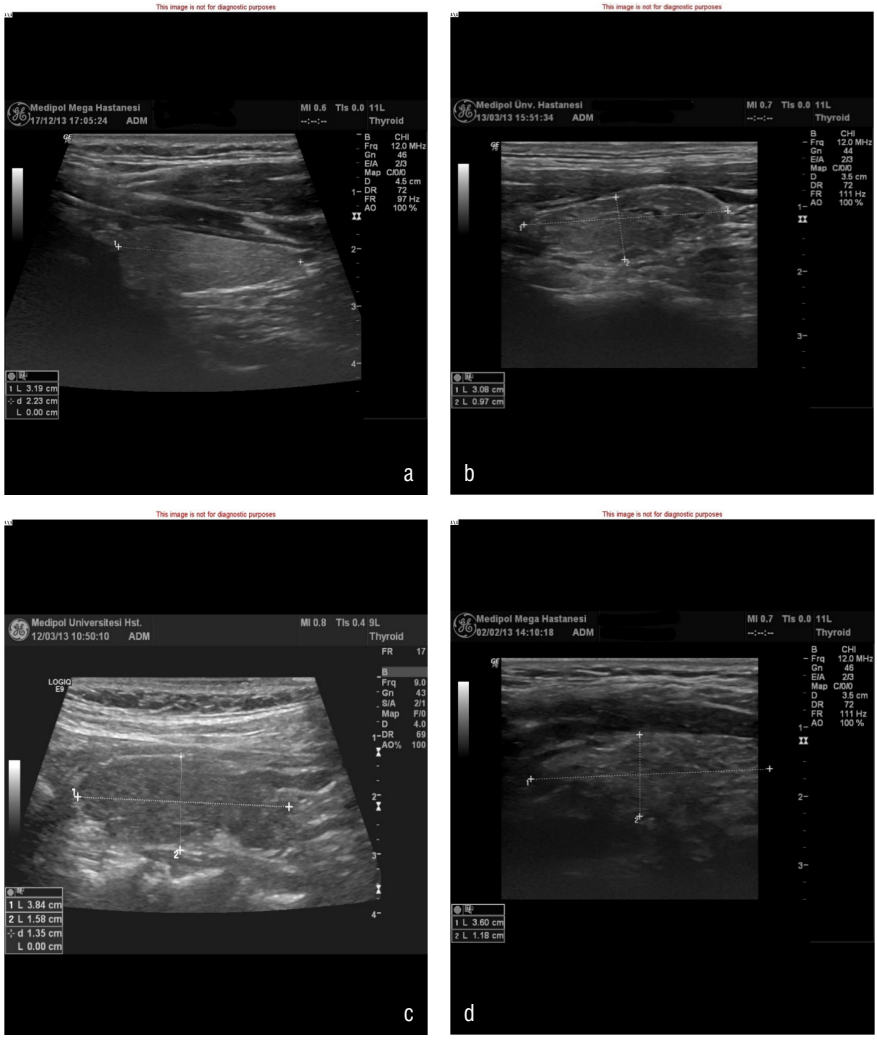

Figure 1. (a) Group 1 (control cases) normal thyroid gland ultrasonography, **(b)** Group 2 (intermediate/early-stage cases); minimal parenchymal hypoechogenicity and suspected heterogeneity, **(c)** Group 3 (active and advanced-late stage) parenchyma was heterogeneous/hypoechogenic, **(d)** Group 3 (chronic stage) with parenchyma was heterogeneous/hypoechogenic.

Hormone and 25-hydroxy Vitamin D_3 analysis

Free triiodothyronine (fT3), free thyroxine (fT4), thyroid-stimulating hormone (TSH), anti-thyroid antibody (TPO Ab), anti-thyroglobulin (Tg Ab) and 25-hydroxy Vitamin D tests were performed by using electrochemiluminescence kits and an automated analyzer (COBAS e 6000 ROCHE). Serum samples from all patients were kept at -20°C until the end of data collection.

We used a double-blind approach throughout the data collection period. Both the clinical biochemist and radiologist were blinded to the patients' clinical and study data throughout the data collection period.

Statistical analysis

SPSS for Windows was used for statistical analysis. The data are presented as the mean ± standard deviation (SD), median, frequency, ratio, and minimum to maximum. According to the Kolmogorov-Smirnov test, the distributions of free triiodothyronine (fT3), free thyroxine (fT4), thyroid-stimulating hormone (TSH), anti-thyroid antibody (TPO Ab), anti-thyroglobulin (Tg Ab) and 25-hydroxy Vitamin D_3 test data were nonuniform ($p<0.05$). The Kruskal-Wallis test was used to compare three or more groups, and the Mann-Whitney U test was used to identify the group responsible for any observed difference.

RESULTS and DISCUSSION

Characteristics of the study groups

A total of 55 patients, consisting of 40 (72.7%) female and 15 (27.3%) male patients, were included in the study after the exclusion criteria. The mean age of all patients was 37.49 ± 11.76 (range between 18-64) years (Table 2).

As in the current study, thyroiditis was staged according to sonographic evaluation as described by Ormeci et al. in Table 1, and patients were grouped into 3 stages[22]. Subjects with no glandular disruption in the thyroid gland were called the group-1 control group and consisted of 17 patients (7 Female/10 Male). Early- and intermediate-stage thyroiditis cases in group 2 consisted of 14 (10 Female/4 Male) individuals. In group 3, chronic and late-stage thyroiditis cases consisted of 24 (23 Female/1 Male) people. Among the groups, we observed that the number of women increased significantly as the thyroiditis stage progressed. There was no significant difference between the groups in terms of age distribution ($p=0.607$; Kruskal-Wallis test) (Table 2).

Table 2. Characteristics of the subjects and thyroid hormones, anti-thyroid antibodies and 25-hydroxy Vitamin D_3

Thyroiditis staging according to sonography evaluation	N	Age years Mean ± SD Median min-max	Sex	free T3 (pg/mL) Mean ± SD Median min-max	free T4 (ng/dL) Mean ± SD Median min-max	TSH (μIU/mL) Mean ± SD Median min-max	anti-Tg Ab (IU/mL) Mean ± SD Median min-max	anti-TPO Ab (IU/mL) Mean ± SD Median min-max	25-OH Vit D3 (ng/mL) Mean ± SD Median min-max
Group 1 Control Cases no confirmed thyroiditis	17	35,6 ± 11,5 36,5 18-64	7F 10M	2,99 ± 0,42 (3,00) 2,14-3,60	1,23 ± 0,25 (1,17) 0,92-1,93	2,11 ± 1,68 (1,73) 0,45-9,59	18,97 ± 10,87 (17,00) 10,00-58,00	10,80 ± 13,37 (5,87) 5,00-63,50	28,54 ± 20,43 (22,4) 6,39-70,00
Group 2 Early/ intermediate-stage thyroiditis cases	14	38,4 ± 10,3 (36,0) 20-60	10F 4M	3,16 ± 0,36 (3,26) 2,51-3,89	1,18 ± 0,42 (1,24) 0,1-2,19	4,31 ± 11,64 (1,90) 0,016-65,0	21,82 ± 9,63 (20,00) 10,00-42,28	24,92 ± 55,97 (7,07) 5,00-238	14,48 ± 5,87# (15,00) 4,91-23,62
Group 3 Active and late-stage thyroiditis cases	24	37,9 ± 12,5 (34,0) 19-63	23F 1M	3,71 ± 2,95 (2,83) 2,15-18,66	1,43 ± 1,69 (1,08) 0,30-13,0	8,68 ± 19,04** (3,41) 0,005-100	275 ± 300** (174,5) 14-1262	306 ± 423** (266) 5-2550	14,3 ± 9,2* (12,66) 3,16-34,79
p-value Kruskal-Wallis Test		0,607	-	0,474	0,214	0,006	0,001	0,001	0,028

*p<0,05 Group 2 compared to Control group by using Mann-Whitney U Test.
** p<0,01 Group 3 compared to Control group by using Mann-Whitney U Test.
# p<0,05 Group 2 compared to Control group by using Mann-Whitney U Test.
F stands for female
M stands for male

Relationship between thyroid hormones, anti-thyroid antibodies and 25-hydroxy Vitamin D_3

There was no significant difference between free T_3 and free T_4 hormones among the 3 groups. However, TSH was significantly elevated in group 3, which included patients with chronic active thyroiditis (p<0.01; Mann-Whitney U Test). This shows us that the TSH level changes significantly and noticeably only in the active and chronic stages (group 3), and the thyroid gland has to work harder. When we examined the anti-Tg Ab and anti-TPO Ab levels that we used in the diagnosis of autoimmune thyroiditis, we observed that they increased significantly in the chronic and active thyroiditis stages (group 3) compared to group 1 and group 2 (p<0.01, Mann-Whitney U test).

When we analyzed 25-hydroxy Vitamin D_3 levels, they were 28.54 ± 20.43 ng/dL in people with normal thyroiditis (group 1), 14.48 ± 5.87 ng/mL in group 2 and 14.3 ± 9.2 ng/mL in group 3. It was determined to be 14.3 ± 9.2 ng/mL. The vitamin D results of patients in both group 2 and group 3 were found to be significantly lower than those in group 1 ($p<0.05$, Mann-Whitney U test). Even before anti-thyroid antibodies are produced in the body and provide evidence about the nature of thyroiditis disease, patients have already developed Vitamin D deficiency.

In the current study, the association between autoimmune thyroiditis and 25-hydroxy Vitamin D_3 levels was associated during different stages of thyroiditis based on morphological changes in the thyroid gland by using ultrasonography. According to the results, 25-hydroxy Vitamin D_3 levels were found significantly lower in patients who develop early signs of morphological changes in thyroid gland which were evaluated by ultrasound examination. When anti-thyroid antibodies (anti-Tg and anti-TPO) were positive in the serum, thyroid gland showed severe destructions and patients had already developed 25-hydroxy Vitamin D_3 deficiency.

In the literature, there were conflicting results regarding whether 25-hydroxy Vitamin D_3 levels play an important role in the development of autoimmune thyroiditis. While some papers gave evidence that 25-hydroxy Vitamin D_3 was an important factor in autoimmune thyroiditis[23, 24] and some papers reported their data do not provide any relationship[25].

In our opinion, the design of grouping patients in each study was based on different criteria, which is one of the main reasons for conflicting results in the literature. The diagnosis of AT is conventionally based on clinical findings and laboratory tests, such as elevated thyroglobulin antibody (Tg Ab) and thyroid peroxidase antibody (TPO Ab). However, antibodies can be negative in cases of thyroiditis, as evidenced by a histological examination[11]. Some authors advocate the use of ultrasonography in cases of AT[15,16], where it was found to be useless by other authors[17]. The combination of US with clinical and serological assessments significantly improves sensitivity and specificity for diagnosing AT[18,19].

It has been demonstrated that 25-hydroxy Vitamin D_3 plays an important role in the immune system. The possible effects of low 25-hydroxy Vitamin D in the early stages of thyroiditis D'Aurizio et al. have discussed in depth[26]. Calapkulu et al. also found decreased levels of Vitamin D among subacute thyroiditis patients[27].

Orbach et al. found lower 25-hydroxy Vitamin D_3 levels in patients with AT than in healthy volunteers[28], and Kivity et al. found a significantly 25-hydroxy Vitamin D_3 deficiency in patients with HT compared to age-matched healthy individuals[29].

The sonographic characteristics of AT are well defined and used during US examinations. The histopathological findings and sonographic features in different stages of AT have been reported and used in clinical practice[22].

In the current study design, patients were staged according to the ultrasonographical morphological changes in the thyroid gland which occur even before anti-Tg Ab and TPO Ab levels were high enough in serum to diagnose AT.

According to Gierach and Junik , a lower level of Vitamin D was connected with a higher level of TSH, and they also found a strong, negative correlation between TSH and Vitamin D levels[30]. Moreover, there was a weak, negative correlation between antithyroid peroxidase antibody (anti-TPO) and anti-thyroglobulin antibody (anti-TG) and Vitamin D levels in females with HT regardless of Vitamin D status: < 20 ng/mL, 20-30 ng/mL, and > 30 ng/mL[30].

The main limitation of this study was the relatively small number of cases in the series.

In the study, the association between autoimmune thyroiditis and 25-hydroxy Vitamin D_3 levels was evaluated during different morphological changes in the thyroid gland by using ultrasonography. According to our results, 25-hydroxy Vitamin D_3 deficiency was detected even at early stage of thyroid gland destruction.

The association between significantly decreased 25 OH Vitamin D_3 concentrations and early morphological changes in the thyroid gland suggests that Vitamin D deficiency might play a role in the early development of thyroiditis.

STATEMENT OF ETHICS

This study was approved by the Medipol University Ethics Committee (10840098-604.01.01-E.28917).

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

MC was responsible for training LK, contributed to designing the research study, writing and editing the manuscript; LK was responsible for specimen handling, generating experimental data; TO was responsible for ultrasound examination of patients.

REFERENCES

1. Dipasquale V, Lo Presti G, Milani GP, Corsello A, Agostoni C, Romano C. Vitamin D in prevention of autoimmune diseases. Front Biosci (Landmark Ed), 2022;27(10):288. Doi: 10.31083/j.fbl2710288

2. Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. Am J Clin Nutr, 2004;80(6):1717S-1720S. Doi: 10.1093/ajcn/80.6.1717S

3. Lebiedziński F, Lisowska KA. Impact of vitamin D on immunopathology of hashimoto's thyroiditis: from theory to practice. Nutrients, 2023;15(14):3174. Doi: 10.3390/nu15143174

4. Sönmezgöz E, Ozer S, Yilmaz R, Önder Y, Bütün I, Bilge S. Hypovitaminosis D in children with hashimoto's thyroiditis. Rev Med Chil, 2016;144(5):611-616. Doi: 10.4067/S0034-98872016000500009

5 Kim D. Low vitamin D status is associated with hypothyroid hashimoto's thyroiditis. Hormones (Athens), 2016;15(3):385-393. Doi: 10.14310/horm.2002.1681

6. Metwalley KA, Farghaly HS, Sherief T, Hussein A. Vitamin D status in children and adolescents with autoimmune thyroiditis. J Endocrinol Invest, 2016;39(7):793-797. Doi: 10.1007/s40618-016-0432-x

7. Wang X, Lu K, Shen J, Xu S, Wang Q, Gong Y, Xia Y, Wang X, Chen L, Yan S, Tang Z, Li C. Correlation between meteorological factors and vitamin D status under different season. Sci Rep, 2023;13(1):4762. Doi: 10.1038/s41598-023-31698-2

8. Vondra K, Stárka L, Hampl R. Vitamin D and thyroid diseases. Physiol Res, 2015;64(Suppl 2):95-100. Doi: 10.33549/physiolres.933083

9. Ma J, Wu D, Li C, Fan C, Chao N, Liu J, Li Y, Wang R, Miao W, Guan H, Shan Z, Teng W. Lower Serum 25-Hydroxyvitamin D level is associated with 3 types of autoimmune thyroid diseases. Medicine (Baltimore), 2015;94(39):e1639. Doi: 10.1097/MD.0000000000001639

10. Yasmeh J, Farpour F, Rizzo V, Kheradnam S, Sachmechi I. Hashimoto thyroiditis not associated with vitamin D deficiency. Endocr Pract, 2016;22(7):809-813. Doi: 10.4158/EP15934.OR

11. Zhou P, Cai J, Markowitz M. Absence of a relationship between thyroid hormones and vitamin D levels. J Pediatr Endocrinol Metab, 2016;29(6):703-707. Doi: 10.1515/jpem-2015-0210

12. Caturegli P, De Remigis A, Rose NR. Hashimoto thyroiditis: clinical and diagnostic criteria. Autoimmun Rev, 2014;13(4-5):391-397. Doi: 10.1016/j.autrev.2014.01.007

13. Pearce EN, Farwell AP, Braverman LE. Thyroiditis. N Engl J Med, 2003;348(26):2646-2655. Doi: 10.1056/NEJMra021194

14. Ahmed AM, Ahmed NH. History of disorders of thyroid dysfunction. East Mediterr Health J, 2005;11(3):459-469.

15. Amani HK. Histopathologic and immunohistochemical features of Hashimoto thyroiditis. Indian J Pathol Microbiol, 2011;54(3):464-471. Doi: 10.4103/0377-4929.85076

16. Butch RJ, Simeone JF, Mueller PR. Thyroid and parathyroid ultrasonography. Radiol Clin North Am, 1985;23(1):57-71.

17. Anderson L, Middleton WD, Teefey SA, Reading CC, Langer JE, Desser T, Szabunio MM, Hildebolt CF, Mandel SJ, Cronan JJ. Hashimoto thyroiditis: part 1, sonographic analysis of the nodular form of hashimoto thyroiditis. AJR Am J Roentgenol, 2010;195(1):208-215. Doi: 10.2214/AJR.09.2459

18. Raber W, Gessl A, Nowotny P, Vierhapper H. Thyroid ultrasound versus antithyroid peroxidase antibody determination: a cohort study of four hundred fifty-one subjects. Thyroid, 2002;12(8):725-731. Doi: 10.1089/105072502760258712

19. Pedersen OM, Aardal NP, Larssen TB, Varhaug JE, Myking O, Vik-Mo H. The value of ultrasonography in predicting autoimmune thyroid disease. Thyroid, 2000;10(3):251-259. Doi: 10.1089/thy.2000.10.251

20. Willms A, Bieler D, Wieler H, Willms D, Kaiser KP, Schwab R. Correlation between sonography and antibody activity in patients with hashimoto thyroiditis. J Ultrasound Med, 2013;32(11):1979-1986. Doi: 10.7863/ultra.32.11.1979

21. Serres-Créixams X, Castells-Fusté I, Pruna-Comella X, Yetano-Laguna V, Garriga-Farriol V, Gallardo-Agromayor E. Paratracheal lymph nodes: a new sonographic finding in autoimmune thyroiditis. J Clin Ultrasound, 2008;36(7):418-421. Doi: 10.1002/jcu.20504

22. Ormeci T, Çolakoğulları M, Orhan İ. Importance of delphian lymph node evaluation in autoimmune thyroiditis: fact or fiction? Pol J Radiol, 2016;81:72-79. Doi: 10.12659/PJR.895761

23. Tamer G, Arik S, Tamer I, Coksert D. Relative vitamin D insufficiency in hashimoto's thyroiditis. Thyroid, 2011;21(8):891-896. Doi: 10.1089/thy.2009.0200

24. Mansournia N, Mansournia MA, Saeedi S, Dehghan J. The association between serum 25OHD levels and hypothyroid hashimoto's thyroiditis. J Endocrinol Invest, 2014;37(5):473-476. Doi: 10.1007/s40618-014-0064-y

25. Effraimidis G, Badenhoop K, Tijssen JG, Wiersinga WM. Vitamin D deficiency is not associated with early stages of thyroid autoimmunity. Eur J Endocrinol, 2012;167(1):43-48. Doi: 10.1530/EJE-12-0048

26. D'Aurizio F, Villalta D, Metus P, Doretto P, Tozzoli R. Is vitamin D a player or not in the pathophysiology of autoimmune thyroid diseases? Autoimmun Rev, 2015;14(5):363-369. Doi: 10.1016/j.autrev.2014.10.008

27. Calapkulu M, Sencar ME, Sakiz D, Unsal IO, Ozbek M, Cakal E. The importance of vitamin D level in subacute thyroiditis disease and the effect of vitamin D on disease prognosis. Endocr Pract, 2020;26(10):1062-1069. Doi: 10.4158/EP-2020-0046

28. Orbach H, Zandman-Goddard G, Amital H, Barak V, Szekanecz Z, Szucs G, et al. Novel biomarkers in autoimmune diseases: prolactin, ferritin, vitamin D, and TPA levels in autoimmune diseases. Ann N Y Acad Sci, 2007;1109:385-400. Doi: 10.1196/annals.1398.044

29. Kivity S, Agmon-Levin N, Zisappl M, Shapira Y, Nagy EV, Dankó K, et al. Vitamin D and autoimmune thyroid diseases. Cell Mol Immunol, 2011;8(3):243-247. Doi: 10.1038/cmi.2010.73

30. Gierach M, Junik R. The role of vitamin D in women with hashimoto's thyroiditis. Endokrynol Pol, 2023;74(2):176-180. Doi: 10.5603/EP.a2022.0095

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Formulation and optimization: Liquisolid of domperidone for solubility enhancement

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ABSTRACT

In the present study, liquisolid formulations of domperidone were prepared using Tween 80, microcrystalline cellulose, Aerosil 200 as solvent, carrier, and coating material, respectively. 2-factors, 3-level central composite experimental design was employed to examine the effect of independent variables (excipient ratio and load factor) on dependent variables (solubility and drug content). Differential scanning calorimetry (DSC), Fourier transform infrared (FTIR), X-ray powder diffraction (XRD) and scanning electron microscopy (SEM) studies were utilized to characterize the optimized formulation. The results of solubility studies of different batches of liquisolid formulations revealed an improvement in solubility ranging from 17.02-58.10 µg/mL as compared to pure drug domperidone (7.47 µg/mL). The *in-vitro* dissolution profile of optimized batch of liquisolid formulation depicted higher rate of drug release (93.63%) when compared with conventional marketed tablets (Dompy®, 81.98%) following non fickian diffusion ($n<0.5$) as mechanism of drug release from the matrix.

Keywords: liquisolid, domperidone, solubility enhancement, optimization

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INTRODUCTION

Domperidone (Domperidonum), 5-chloro-1-[1-[3-(2,3-dihydro-2-oxo-1H-ben zimidazol-1-yl) propyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazole-2-one, having molecular formula of $C_{22}H_{24}ClN_5O_2$ belongs to biopharmaceutical classification system (BCS) class II drug. It is a selective D_2 and D_3 dopamine receptor antagonist with a peripheral effect. It reduces the nausea by blocking dopamine receptor. It has poor bioavailability (13-17%) because of extensive presystemic metabolism (83-87%) with short biological half-life. Moreover, domperidone is insoluble in water, resulting in poor absorption from the Gastrointestinal tract (GIT)[1]. The poor bioavailability of domperidone has influenced a number of researchers to develop novel techniques like solid dispersion of domperidone[2-4], nanoparticles[5], nanocrystals[6], nanostructured lipid carrier[7], solid lipid nanoparticles[8], microemulsion nasal spray[9], emulgel alginate based beads[10,11], microcrystals[12] and also liquisolid compacts of domperidone[13-15] indicating a considerable increase in solubility and dissolution rate of domperidone. The above approaches have some limitations such as instability during storage, agglomeration, sticky product and need innovative expensive tools. Out of these, liquisolid strategy is particularly interesting and attractive because of manufacturing process simplicity, low production costs and ease of scale-up to industrial tablet production.

Spireas and Sadu first presented the liquisolid formulation in 1998 which incorporated water-insoluble drugs into rapid-release solid dosage forms[16]. The liquisolid system (LS) refers to "formulations comprised of water-insoluble drugs (liquid drugs, drug suspensions or drug solution) dissolved or dispersed in a suitable water-miscible nonvolatile solvent system later transformed into dry, non-adherent, free-flowing and compressible mixtures by blending the suspension or solution with selected carrier and coating materials". Carrier materials are compression-enhancing, relatively massive, ideally porous particles with adequate absorption properties that aid in liquid absorption examples includes starch lactose, eudragit RL and RS, sorbitol, microcrystalline cellulose, Neusilin® and Fujicalin®. Coating materials are flow enhancing, very tiny (10 nm-5,000 nm in diameter), strongly adsorptive coating particles (e.g. Aerosil 200, Cab-O-Sil M5, Syloid 244FP, etc.) that support to cover the wet carrier particles and exhibiting a dry looking powder by adsorbing extra liquid[17]. Non-volatile solvents are "Organic solvent systems that are inert, have a high boiling point, are usually water-miscible, and are not viscous". Several non-volatile solvents are employed in the formulation of liquisolid systems[18]. Examples includes propylene glycol, polysorbate 20 and 80, polyethylene gly-

col 200 and 400, Cremphor® EL, capryol 90, glycerine and transcutol HP. The non-volatile solvent functions as wetting agent in the liquisolid system thereby helps to enhance the dissolution rate by lowering the interfacial tension between the dissolution medium and the surface of particles. Besides formulating liquisolid, the solubility of poorly soluble drugs is increased because the high porous surface area improves molecular dispersion and wetting properties, ultimately boosting its dissolution and solubility. Pre-compression tests[18] of the Liquisolid powder systems were recommended by Lu et al.[18]. In the literature, liquisolid has effectively exhibited enhancement in the *in-vitro* release of poorly soluble drugs like risperidone[19], iloperidone[20], progesterone[21], simvastatin[22,23], carvacrol[24,25], propranolol hydrochloride[26,27], tadalafil[28-30], naproxen[31,32], felodipine[33,34], furosemide[35-37], silymarin[38], efavirenz[39] hydrochlorothiazide[40] and chlorpromazine[41] etc. The primary goal of the current study is to use an optimization technique for systematic medicinal product development in order to achieve good performance, greater efficiency and high quality. The Design Expert® is regarded as a vital tool that gives maximum knowledge with least experimental work. In the current research work, liquisolid of domperidone were performed by using tween-80 as solvent medium, microcrystalline cellulose (MCC) as a carrier material and aerosil-200 as a coating material employing Design Expert® Software (11.0) and followed by characterization using DSC, FTIR, XRD and SEM examinations. In 1999, Spireas and Bolton suggested a mathematical model for the successful fabrication of a free-flowing and compressible liquisolid formulation, which was further used to calculate the optimum quantities of carrier and coating ingredients. The liquid load factor (Lf) is the weight ratio of the liquid drug (W) and carrier material (Q). The excipient ratio is the weight proportion of the carrier (Q) and coating material (q)[42].

METHODOLOGY

Materials

Domperidone, Microcrystalline cellulose (MCC), glycerin, sodium starch glycolate (SSG) were supplied by Hi-media Laboratories Pvt. Ltd., Mumbai, India. Aerosil-200 was supplied by Central Drug House Pvt. Ltd., New Delhi, India. Cremophor EL (Sigma-Aldrich, USA). PEG-600, propylene glycol (PG), and empty hard gelatin capsule shells (Patco Pharmaceuticals, India) were supplied by Loba Chemie Pvt. Ltd. All other chemicals and reagents used in the study were of analytical grade and used as received without further processing.

Selection of non-volatile solvent

Domperidone solubility was determined in distilled water and five other non-volatile solvents like PEG 400, tween 80, glycerin, cremophor-EL and propylene glycol. Saturated solution was made by mixing excess quantity of domperidone in 10 mL solvent vehicles. Above samples were set on a shaker incubator for 48 hours at room temperature. The samples were then filtered through a 0.45 µm whatman filter paper, diluted with methanol and analyzed by UV spectrophotometer at λ_{max} 284 nm for its drug content (n=3). Solvent showing highest solubility of domperidone was selected[43].

Preparation of liquisolid formulation

Liquisolid formulations were made by dispersing an appropriate quantity of drug (domperidone) and non-volatile solvent (Tween 80) by solvent evaporation method (heated at 60-80°C with continuous stirring) and the solution was sonicated for 15 minutes. The liquid drug solution was blended with the binary mixture of carrier (MCC) and coating (Aerosil-200) material into mortar and pestle uniformly followed by addition of sodium starch glycolate to the above mixture with continuous stirring for about 10 to 15 min. The resultant mixture was finally filled into empty capsule shell size 1 for further studies[44]. The final composition of different batches was shown in the results section.

Experimental design

Liquisolid formulation was optimized using 2-factor, 3-level central composite design (CCD). The excipient ratio (X_1) was varied from 5 to 15 and liquid load factor (X_2) 1.1 to 1.5 designed as independent variables whereas the solubility (Y_1) and drug content (Y_2) were selected as dependent variables (3). Each independent variable was taken at three levels (-1, 0 and +1) as given in Table 1.

Table 1. Optimization of domperidone LS via CCD at different variables and their respective levels

Independent variables	Level		
	-1	0	+1
X_1 (liquid load factor)	1.1	1.3	1.5
X_2 (Excipient ratio)	5	10	15
Dependent variables	Goal		
Y_1 (Solubility)	Maximum		
Y_2 (Drug content)	Maximum		

Solubility studies of liquisolid formulation

Liquisolid formulations (F_1 to F_{13}) containing domperidone equivalent to 10 mg were dispersed in 10 mL distilled water, separately and were kept on continuous shaking for 48 h at room temperature to determine the solubility of drug. The obtained solution was filtered by 0.45 μm whatman filter paper and the domperidone content was observed by taking absorbance at 284nm using UV-VIS spectrophotometer (n=3). The amount of drug was calculated using calibration curve in distilled water[45].

Determination of drug content

Liquisolid formulations (F_1 to F_{13}) containing domperidone equivalent to 10 mg were dissolved in 20 mL of phosphate buffer (pH 6.8), separately. All the samples were sonicated separately for 15 minutes and then filtered through 0.45 μm whatman filter paper[46]. The samples were diluted accurately and analyzed taking absorbance at 284 nm using UV-VIS spectrophotometer (n=3).

FTIR spectroscopy

The drug, MCC, Aerosil-200, and optimized batch of liquisolid formulation were exposed to FT-IR spectroscopy (Perkin Elmer Spectrum, BX II spectrophotometer) and the spectrum was documented in the wavelength region of 4000cm^{-1} to 400 cm^{-1} using KBr pellet method. The method involves dispersing of sample in potassium bromide (KBr) and compressing into disc by applying a pressure of 50 kg/cm^2 in hydraulic press[47].

Differential scanning calorimetry (DSC)

Thermal behavior of domperidone, MCC, Aerosil-200 and optimized batch of domperidone liquisolid formulation was studied using DSC (Q-10, TA instruments waters) by heating the samples within the temperature range of 10–400°C with a scanning rate of 10°C/min in aluminum pans under nitrogen flow at a rate of 50 mL/min[48].

X-ray powder diffraction

The domperidone, MCC, Aerosil-200 and optimized batch of domperidone liquisolid formulation powder samples were examined using an X-ray diffractometer (Miniflex 2, Rigaku, Japan) with Cu-Kα from 10° to 80° diffraction angle (2θ)[49].

Scanning electron microscopy (SEM)

The surface morphology of optimized batch of domperidone liquisolid formulation was examined using SEM (JSM-6100 scanning microscopy, Japan). The sample (optimized batch) after coated with gold was mounted on aluminum stub containing double-adhesive carbon tape. The photographs were observed at acceleration voltage of 10kV[50].

Flow properties of liquisolid formulation

The flow properties of powder were determined by angle of repose (θ) using fixed funnel method. Powders that had previously been sieved were allowed to pass through the funnel until the tip of the conical pile of powder just touched the tip of the funnel. The mean radius (R) and height (H) of powder base were measured, and the tangent of the angle of repose was calculated by $\tan\theta = H/R$. Powder compressibility was determined by calculating the bulk density, tapped density, Carr's index and Hausner ratio as per the method given in United State Pharmacopeia (USP). Carr's index (% CI) [% CI = 100(1-bulk density/tapped density)] and Hausner ratio (HR) [HR = tapped density/bulk density] values reflect the flow properties of a powder; the higher the value of the Hausner ratio, the worse the flow of the powder[25,52].

Weight variation

The weight variation test was performed by the method given in Indian Pharmacopeia (IP)[53].

In-vitro drug release study

In-vitro dissolution studies of pure domperidone, optimized batch of drug loaded LS and marketed domperidone tablets (Dompy® that was crushed and filled in empty capsule shells) equivalent to 20 mg, was carried out in USP type-II apparatus. The dissolution study was conducted in 900 mL phosphate buffer (pH 6.8) at a temperature 37 ± 0.5 °C and the paddle speed was set at 50 rpm. The samples of around 5 mL were taken at fixed time periods of 5, 15, 30, 45, 60, 90 and 120 min. and replaced with an equivalent volume of fresh dissolution medium to maintain the sink condition[23]. The samples were filtered using 0.45 μm Millipore filters and were examined using a uv-vis spectrophotometer at λ_{max} 284nm after appropriate dilution (n=3). To find out the mechanism of drug release, the release data were put in various models like Zero-order, First-order, Higuchi and Korsmeyer-Peppas.

Stability studies

The stability studies were carried out on optimized batch of liquisolid for 3 months in a stability chamber maintaining the temperature of 40 ± 2°C at 75% relative humidity. The samples were stored in hermetically sealed vials containing rubber plugs and aluminum bung. Following a three-month interval, samples were withdrawn and tested for drug content, solubility, and *in-vitro* drug release studies and also for any physical changes[54].

RESULTS and DISCUSSION

Selection of non-volatile solvent

The domperidone exhibited varying proportions of solubility in a variety of non-volatile solvents like Tween-80 (57.13 ± 0.60 µg/mL), PEG 600 (32.52 ± 0.42 µg/mL), Cremophor EL (23.11 ± 0.51 µg/mL), glycerine (3.11 ± 0.73 µg/mL) and Propylene glycol (2.08 ± 0.92 µg/mL). The solubility of domperidone was found to be highest in Tween-80 and hence, Tween-80 was selected as solvent vehicle.

Optimization studies

Domperidone liquisolid formulation was produced and optimization was performed using two-factor, three-level CCD and evaluated for designated responses that are given as below.

Solubility studies of liquisolid formulation

Table 2 shows the solubility (Y_1) and drug content (Y_2) of the domperidone liquisolid produced according to the Design Expert Software, version 11.0. The generated responses were fitted into several polynomial models. The response, solubility (Y_1) was fitted best into the linear response surface model with no data processing. Domperidone liquisolid solubility values range from 17.02 - 59.10 µg/mL in various batches whereas pure domperidone displayed a solubility of 7.478µg/mL. The liner regression fitted for solubility (Y_1) has a good correlation (R^2) of 0.921 is shown in equation (1).

$$Y_1 = 36.21 + 14.87\ X_1 + 5.16\ X_2 \quad (1)$$

Table 2. Formulation parameters and responses for experimental design

St. No.	Conc. of drug (mg)	Tween-80 (mg)	Liquid load factor (X_1)	Excipient ratio (X_2)	MCC (mg)	Aerosil-200 (mg)	SSG	Total (mg)	Solubility in Distilled water (Y_1) (µg/mL)	Drug content (Y_2) (%)	Weight Variation (mg)
F_1	25	100	1.1	15	90	6	19	240	28.01 ± 0.0043	93.82 ± 0.63	205.21 ± 0.45
F_2	25	100	1.1	5	90	18	7	240	17.02 ± 0.0054	90.65 ± 0.23	227.41 ± 0.52
F_3	25	100	1.1	10	90	9	16	240	22.01 ± 0.0036	91.06 ± 0.49	215.38 ± 0.38
F_4	25	100	1.5	10	66	6.6	43	240	51.06 ± 0.0055	98.98 ± 0.96	267.26 ± 0.59
F_5	25	100	1.3	10	75	7.5	33	240	34.02 ± 0.0056	95.18 ± 0.36	257.61 ± 0.65
F_6	25	100	1.3	10	75	7.5	33	240	34.11 ± 0.0045	95.81 ± 0.63	255.92 ± 0.70
F_7	25	100	1.3	10	75	7.5	33	240	35.08 ± 0.0049	96.21 ± 0.23	251.75 ± 0.62
F_8	25	100	1.3	15	75	5	35	240	39.03 ± 0.0051	97.05 ± 0.36	240.85 ± 0.82
F_9	25	100	1.3	5	75	15	25	240	32.04 ± 0.0054	93.26 ± 0.27	311.72 ± 0.72
F_{10}	25	100	1.3	10	90	18	7	240	37.08 ± 0.0045	96.21 ± 0.99	230.11 ± 0.48
F_{11}	25	100	1.3	10	75	7.5	33	240	36.03 ± 0.0044	96.62 ± 0.61	260.54 ± 0.57
F_{12}	25	100	1.5	15	66	4.4	45	240	59.10 ± 0.0058	98.99 ± 0.21	243.08 ± 0.48
F_{13}	25	100	1.5	5	66	13	36	240	46.11 ± 0.0037	97.58 ± 0.31	320.31 ± 0.77
Drug									7.478 ± 0.0032		

R: Excipient ratio (carrier: coating material), Q: Carrier material (microcrystalline cellulose), q: Coating material (Aerosil 200). All values are expressed as mean ± S.D., n=3

The results of the ANOVA test on the solubility and drug content response surface model, revealing that the model was deemed significant with lack of fit as non-significant. Adequate precision of solubility was found to be 48.66 and indicated a requisite signal. It is preferable to have an adequate precision measurement signal to noise ratio (higher than 4). The combined effect of liquid load factor (L_f) and excipients ratio (R) on solubility and drug content was shown in Figure 1. The plot indicates that independent and dependent variables have a curvilinear correspondence. The plot also shows that with increasing value of liquid load factor and excipient ratio the solubility also increases.

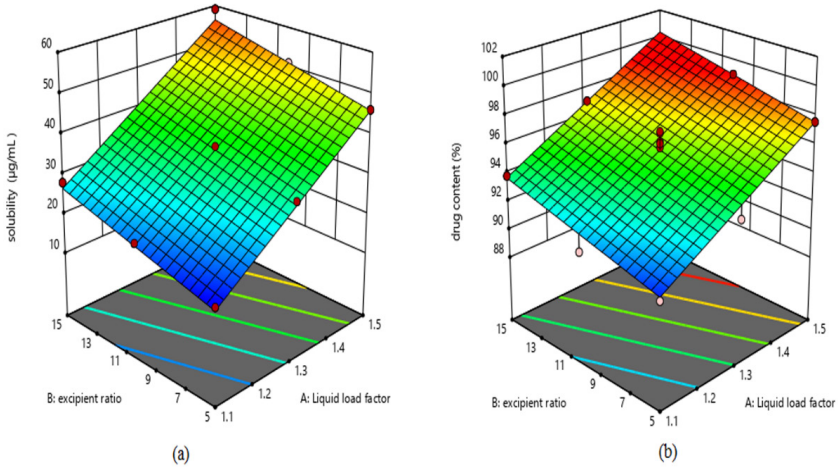


Figure 1. Response surface plots displaying effect of Load factor and excipients ratio on solubility (a) and drug content (b)

Desirability function for the selection of optimized batch

The different set of solutions was provided by the optimization tool in the software. The software combines all the response factors so that the final optimized batch has the optimal balance of all the required attributes. A desirability value of 0 indicates an inappropriate value for the responses while a value of 1 indicates the most desirable. The software assessed desirability ability to each response and calculated a composite desirability value for each batch through considering the geometric mean of all the responses desirability. The desirability index value was estimated to be 1 as shown in Figure 2.

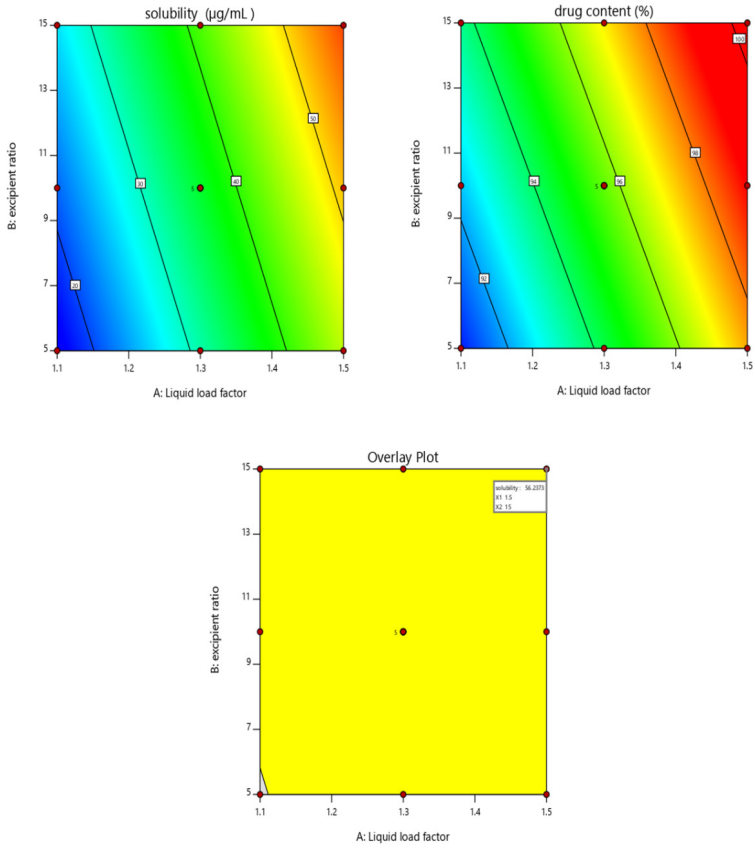


Figure 2. Desirability index of optimized formulation (F_{12})

Drug content

The response, drug content (Y_2) was fitted best into the linear response surface model with no data processing as shown in equation (2). Drug content values range from 90.65 to 98.99% in various batches. The linear regression fitted for drug content (Y_2) has a good correlation (R^2) of 0.9069.

$$Y_2 = 95.63 + 3.34\,X_1 + 1.39\,X_2$$

Adequate precision of drug content was found to be 21.94 (higher than 4) indicated a requisite signal.

The optimization Eqs. (1) and (2), relating the responses and independent factors, were acquired based on a quadratic and linear model. To the responses i.e. solubility and drug content the desirability function was applied with con-

straints to obtain the higher magnitude of both the factors. In this manner, the formulation having liquid load factor (1.5) and excipient ratio (15) established the maximum desirability, was organized, and evaluated. The optimization of independent variables was done with constraints of maximum solubility and maximum drug content. The parameters suggested by the design were liquid load factor (1.5) & ratio of excipients (15) that provided liquisolid with solubility of 56.00 μg/mL (predicted value 56.24 μg/mL) and drug content 98.77% (predicted value 100.0%). The closer concordance between observed and predicted values discerned high predictive ability of the model. Based on solubility and drug content data, batch F_{12} (optimized batch suggested by design expert) containing Lf (1.5) and R (15) was selected for further examination[23,55].

Characterization

Fourier Transform Infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy (FT-IR characterization) was performed to examine the possible interactions between drug and excipients, the FTIR absorption spectra for the drug, MCC, Aerosil-200 and LS formulation (F_{12}) represented in Fig. 3a. The FTIR spectra of domperidone showed characteristic absorption band at 3354 cm^{-1} to 3706 cm^{-1} (-N-H stretching) and the peak appearing at 1728 cm^{-1} (-C=O stretching), 3028 cm^{-1} to 3139 cm^{-1} (=C-H stretching), 2865 cm^{-1} to 3028 cm^{-1} (Sp_3–C-H stretching) and 1493 cm^{-1} to 1605 cm^{-1} (-C=C stretching). The FTIR spectra of MCC showed the characteristic absorption band at 1406 cm^{-1} (-N-H stretching), 1648 cm^{-1} (-C=O stretching), 1236 cm^{-1} (-C=C stretching), 1430 cm^{-1} to 1374 cm^{-1} (–CH_2 stretching), 2902 cm^{-1} (-C-H stretching) and 3352 cm^{-1} to 3802 cm^{-1} (-OH stretching). The FTIR spectra of Aerosil-200 showed characteristic absorption band at 1648 cm^{-1} (-OH stretching), 1670 cm^{-1} (-C=O stretching), 1108 cm^{-1} (Si-O-Si stretching) and 2896 cm^{-1} to 2958 cm^{-1} (-C-H stretching). The FTIR spectra of LS formulation showed peak at 1112 cm^{-1} to 1374 cm^{-1} (-OH stretching), 1488 cm^{-1} (-C=C stretching), 1694 cm^{-1} (-C=O stretching), 3014 cm^{-1} (-N-H stretching) and 3885 cm^{-1} (-C-H stretching). The intensity of the absorption bands of domperidone was found to be diminished in the LS formulation, which could be explained to the hydrogen bonding interaction between the carboxylic group of domperidone[56].

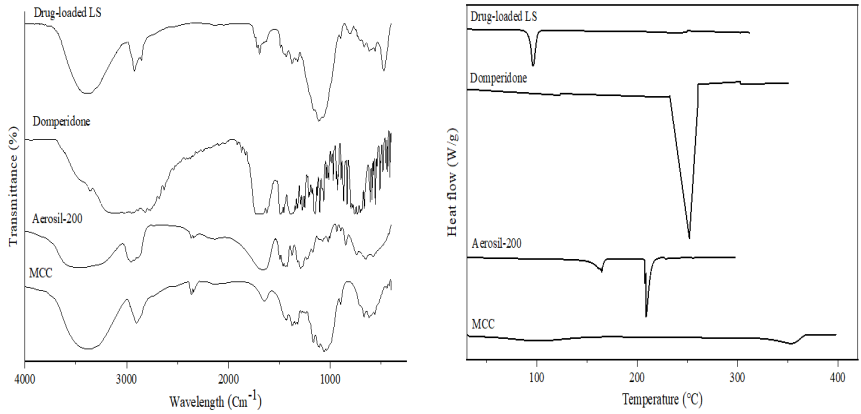


Figure 3. (a) FTIR and (b) DSC Spectra of MCC, Aerosil-200, Domperidone and LS formulation (F_{12})

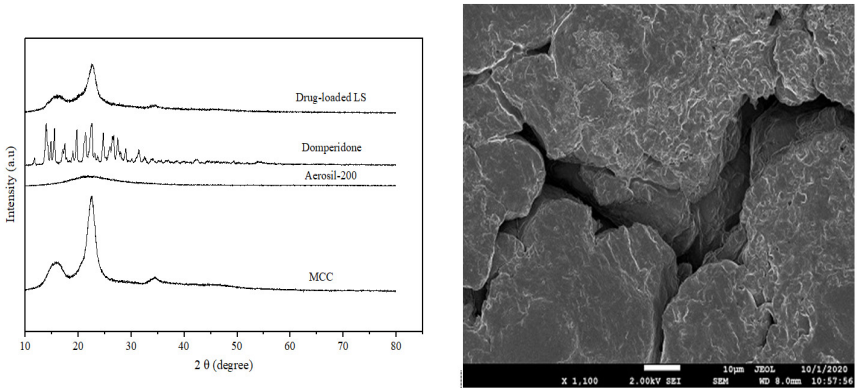
Differential scanning calorimetry (DSC)

The DSC thermograms for the drug, MCC, Aerosil-200 and LS formulation (F_{12}) are shown in Fig. 3b. Domperidone exhibited a sharp peak at 251.8°C that corresponds to its melting point with a fusion enthalpy (ΔH) of -14.08W/g. MCC and Aerosil-200 exhibited a sharp peak at 103.029°C and 208.77°C corresponds to the melting point of MCC and Aerosil-200. However, LS formulation (F_{12}) exhibited a strong peak at 96.15°C. The thermogram of the liquisolid system showed the disappearance of the domperidone endothermic peak, which corresponds with the establishment of drug solution in the LS formulation (i.e. the drug was molecularly disseminated inside the liquisolid matrix). Such disappearance of the drug peaks during formation of the liquisolid system was in accordance with Brittain and Mccauley[13]. The thermogram of LS also indicated that the constituents' crystalline habit was disrupted, which might be explained to physical interactions between the constitutional components during processing.

Powder x-ray diffraction analysis (P-XRD)

Fig. 4a shows the x-ray diffraction spectra of drug, MCC, Aerosil-200 and LS formulation (F_{12}). The x-ray diffraction spectra of domperidone exhibited different characteristic peaks at 11.77°, 13.96°, 15.54°, 17.5°, 19.76°, 21.42°, 24.8°, 26.46°, 27.48°, 29° and 31.46° that indicate the crystalline nature of domperidone. The diffraction spectra of MCC exhibited characteristic peaks at 14.6°, 22.02°, 34.62° indicates the crystalline nature of MCC. On other hand aero-

sil-200 has no characteristic peak because it is amorphous in nature. The diffraction spectra of LS formulation (F_{12}) exhibited characteristic peak at 13.24°, 16°, 18.66° and 25.32°. The reduction in the intensity and the disappearance of many peaks of domperidone in the LS formulation suggest that the molecule was dissolved molecularly in the LS formulation. The drug may become solubilized in a liquid vehicle, which could explain this behavior. This also implies that domperidone was in an amorphous form in the LS formulation[57].



(a) **(b)**

Figure 4. (a) XRD and (b) SEM images of MCC, Aerosil-200, Domperidone and LS formulation (F_{12}) flow properties and weight variation of Liquisolid formulation

Scanning Electron Microscope (SEM)

The SEM image of optimized domperidone liquisolid (F_{12}) was shown in Fig. 4b. Liquisolid had an uneven form and a dimension of around 10 μm. The disappearance of domperidone crystals in the liquisolid system was reported to be associated with drug solvation in non-volatile solvent. The surface of liquisolid seems to be uneven and porous and this porosity, makes it more soluble[58].

The angle of repose, bulk densities, Carr's index, tapped densities and Hausner's ratio were determined to evaluate the flow parameters of the prepared LS powder. Powders with an angle of repose value of less than 45° are deemed to have acceptable flow characteristics, according to the USP. The liquisolid optimized batch has bulk density (0.34), tapped density (0.38), Carr's index (10.5), Hausner's ratio (1.11) and angle of repose (34.06°) shows the good flow. The average weight of liquisolid capsules ranged between 205.21 mg and 320.31 mg was found to be in acceptable limits.

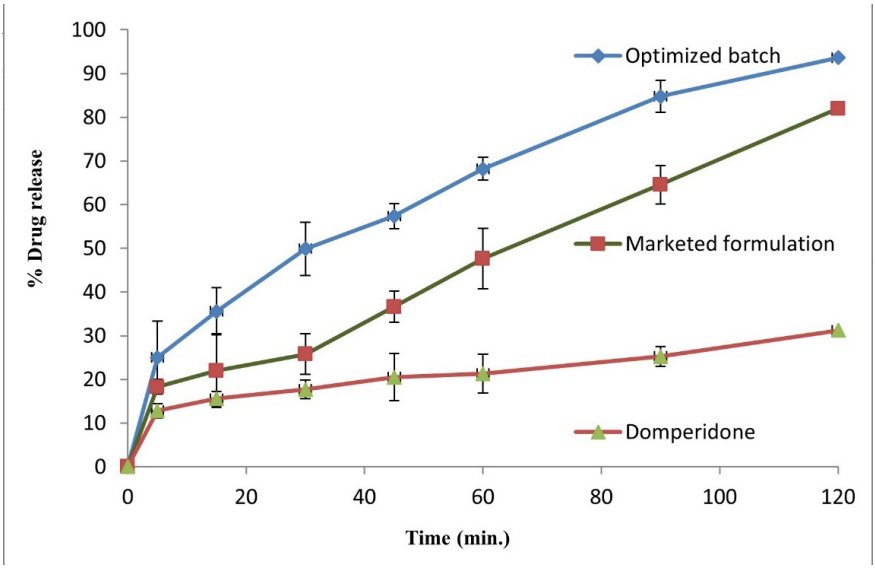


Figure 5. Graph showing comparison between drug release kinetics of pure domperidone, liquisolid optimized batch (F_{12}) and marketed formulation (Dompy ®)

Stability studies

After the stability period of 3 months, there was no discernible change in the physiochemical characteristics of optimized LS formulation (F_{12}). The solubility and drug content differed slightly, but all were confirmed to be within acceptable limits[61,62]. There was no discernible influence on the dissolution profile also as shown in Table 3.

Table 3. Stability studies of optimized LS formulation

Parameter	Before	After 3 months
Solubility (µg/mL)	58.10 ± 0.0058	56.04 ± 0.0047
Drug content (%)	98.77 ± 0.21	96.61 ± 0.68
% drug release at 120 min.	93.63 ± 3.68	90.93 ± 2.81

Domperidone has a high permeability across biological membranes, but its absorption is limited following oral administration due to its low dissolution rate and poorly water solubility. The liquisolid-capsule strategy could be a good way to speed up the dissolution of water-insoluble drugs like domperidone. In the liquisolid formulation, the liquid vehicle aids in increasing the dissolution characteristics of an aqueous insoluble drug. To improve the domperidone liquisolid formulation, a 3^2 Central Composite Design was used. Aerosil-200,

MCC, Tween-80 and sodium starch glycolate are selected as coating material, carrier material, solvent and disintegrant respectively, in the liquisoild formulation. The results of solubility studies of different batches of liquisolid formulations revealed an improvement in solubility ranging from 17.02-58.10 μg/mL as compared to pure drug domperidone (7.47 μg/mL). There was no interaction between the drug and the additives, as per FTIR and DSC investigations and XRD revealed the absence of the drug characteristic peaks in the optimized liquisolid formulation, implying that the pure medication had been partially transformed into an amorphous or solubilized form. The disapperance of crystallinity of drug in liquisolid formulation, which is then, adsorbed onto the coating and carrier materials. Pure drugs that have been amorphous or soluble may have a fast rate of dissolution. Further the SEM study conform the drug is in amorphous form and solubilized into the liquisolid formulation. The data on drug release at 120 min. was found to be 93.63% further it is mathematically analyzed and fitted into zero-order release kinetics. A stability analysis of optimized batch for solubility, drug content and drug release were done which revealed that there was no significant change seen after three months of storage. This novel approach to the formulation may be helpful in improving solubility of poorly soluble drug domperidone.

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

Meenakshi Bhatia- Conceptualization, Supervision; Geeta Rani- Writing – Original Draft Preparation; Sunita Devi - Review & Editing, Software; Kavita Bahmani- Review & Editing, Data Curation.

REFERENCES

1. Reddymasu SC, Soykan I, Mc Callum RW. Domperidone: review of pharmacology and clinical applications in gastroenterology. ACG, 2007;102(9):2036-2045. Doi: 10.1111/j.1572-0241.2007.01255

2. X Patel DM, Patel SP, Patel CN. Formulation and evaluation of fast dissolving tablet containing domperidone ternary solid dispersion. Int J Pharm Investig, 2014;4(4):174. Doi: 10.4103%2F2230-973X.143116

3. Swami G, Koshy MK, Pandey M, Saraf SA. Preparation and characterization of Domperidone-ß-cyclodextrin complexes prepared by kneading method. Int J Adv Pharm Sci, 2010;1(1):68-74. Doi: 10.5138/ijaps.2010.0976.1055.01008

4. Nagpal M, Kaur L, Chander J, Sharma P. Dissolution enhancement of domperidone fast disintegrating tablet using modified locust bean gum by solid dispersion technique. JPTRM, 2016;4(1):1-11 Doi: 10.15415/jptrm.2016.41001

5. Al-lami MS, Oudah MH, Rahi FA. Preparation and characterization of domperidone nanoparticles for dissolution improvement. IJPS, 2018;27(1):39-52. Doi: 10.31351/vol27iss1pp39-52

6. Ndlovu ST, Ullah N, Khan S, Ramharack P, Soliman M, de Matas M, et al. Domperidone nanocrystals with boosted oral bioavailability: fabrication, evaluation, and molecular insight into the polymer-domperidone nanocrystal interaction. Drug Deliv Transl Res, 2019;9(1):284-297. Doi: 10.1007/s13346-018-00596-w

7. Tetyczka C, Griesbacher M, Absenger-Novak M, Fröhlich E, Roblegg E. Development of nanostructured lipid carriers for intraoral delivery of Domperidone. Int J Pharm, 2017;526(1-2):188-198. Doi: 10.1016/j.ijpharm.2017.04.076

8. Shazly GA, Alshehri S, Ibrahim MA, Tawfeek HM, Razik JA, Hassan YA, et al. Development of domperidone solid lipid nanoparticles: *in vitro* and *in vivo* characterization. AAPS Pharmscitech, 2018;19(4):1712-1719. Doi: 10.1208/s12249-018-0987-2

9. Rathod M, Suthar D, Patel H, Shelat P, Parejiya P. Microemulsion based nasal spray: a systemic approach for non-CNS drug, its optimization, characterization, and statistical modelling using QbD principles. J Drug Deliv Sci Technol, 2019;49:286-300. Doi: 10.1016/j.jddst.2018.11.017

10. Daihom BA, Bendas ER, Mohamed MI, Badawi AA. Development and *in vitro* evaluation of domperidone/Dowex resinate embedded gastro-floatable emulgel and effervescent alginate beads. J Drug Deliv Sci Technol, 2020;59:101941. Doi: 10.1016/j.jddst.2020.101941

11. Ismail A, Kerdpol K, Rungrotmongkol T, Tananuwong K, Ueno T, Ekasit S, et al. Solubility enhancement of poorly water soluble domperidone by complexation with the large ring cyclodextrin. Int J Pharm, 2021;606:120909. Doi: 10.1016/j.ijpharm.2021.120909

12. Enteshari S, Varshosaz J. Solubility enhancement of domperidone by solvent change in situ micronization technique. Adv Biomed Res, 2018;7. Doi: 10.4103%2Fabr.abr_219_17

13. Ibrahim EH, El-Faham TH, Mohammed FA, El-Eraky NS. Enhancement of solubility and dissolution rate of domperidone by utilizing different techniques. Bull Pharm Sci, 2011;34(2):105-120. Doi: 10.21608/bfsa.2011.63250

14. Aparna TN, Rao AS. Liquisolid compacts: an approach to enhance the dissolution rate of domperidone. World J Pharm Pharm Sci, 2017;6(7):1219-1232. Doi: 10.20959/wjpps20177-9529

15. Kudamala S, Murthy KVR. Applicability of a novel carrier, Neusilin UFL2, for the preparation of domperidone liquisolid tablets. World J Pharm Pharm Sci, 2017:6:1662-1671 Doi: 10.20959/wjpps20171-8524

16. Javadzadeh Y, Musaalrezaei L, Nokhodchi A. Liquisolid technique as a new approach to sustain propranolol hydrochloride release from tablet matrices. Int J Pharm, 2008;362(1-2):102-108. Doi: 10.1016/j.ijpharm.2008.06.022

17. Chella N, Shastri N, Tadikonda RR. Use of the liquisolid compact technique for improvement of the dissolution rate of valsartan. ACTA Pharm Sci, 2012:2(5):502-508. Doi: 10.1016/j.apsb.2012.07.005

18. Lu M, Xing H, Jiang J, Chen X, Yang T, Wang D, et al. Liquisolid technique and its applications in pharmaceutics. AJPS, 2017;12(2):115-123. Doi: 10.1016/j.ajps.2016.09.007

19. Khames A. Investigation of the effect of solubility increase at the main absorption site on bioavailability of BCS class II drug (risperidone) using liquisolid technique. Drug Deliv, 2017;24(1):328-338. Doi: 10.1080/10717544.2016.1250140

20. Suram D, Narala A, Veerabrahma K. Development, characterization, comparative pharmacokinetic and pharmacodynamic studies of iloperidone solid SMEDDS and liquisolid compact. Drug Dev Ind Pharm, 2020;46(4):587-596. Doi: 10.1080/03639045.2020.1742142

21. Jadhav NR, Irny PV, Patil US. Solid state behavior of progesterone and its release from Neusilin US2 based liquisolid compacts. J Drug Deliv Sci Technol, 2017;38:97-106. Doi: 10.1016/j.jddst.2017.01.009

22. Elkadi S, Elsamaligy S, Al-Suwayeh S, Mahmoud H. The development of self-nanoemulsifying liquisolid tablets to improve the dissolution of simvastatin. AAPS PharmSciTech, 2017;18:2586-2597. Doi: 10.1208/s12249-017-0743-z

23. El-Say KM, Ahmed TA, Ahmed OA, Elimam H. Enhancing the hypolipidemic effect of simvastatin in poloxamer-induced hyperlipidemic rats via liquisolid approach: pharmacokinetic and pharmacodynamic evaluation. AAPS PharmSciTech, 2020;21:1-14. Doi: 10.1208/s12249-020-01754-5

24. Taghizadeh Z, Rakhshani S, Jahani V, Rajabi O, Haghighi HM, Abbaspour M. Preparation and *in vitro* characterization of carvacrol pellets by combination of liquisolid technique and extrusion-spheronization. J Drug Deliv Sci Technol, 2021;61:102232. Doi: 10.1016/j.jddst.2020.102232

25. Baranauskaite J, Kopustinskiene DM, Masteikova R, Gajdziok J, Baranauskas A, Bernatoniene J. Effect of liquid vehicles on the enhancement of rosmarinic acid and carvacrol release from oregano extract liquisolid compacts. Colloids Surf A: Physicochem Eng Asp, 2018;539:280-290. Doi: 10.1016/j.colsurfa.2017.12.034

26. Javadzadeh Y, Musaalrezaei L, Nokhodchi A. Liquisolid technique as a new approach to sustain propranolol hydrochloride release from tablet matrices. Int J Pharm, 2008;362(1-2):102-108. Doi: 10.1016/j.ijpharm.2008.06.022

27. Ward A, Walton K, Stoycheva S, Wallis M, Adebisi A, Nep E, Asare-Addo K. The use of visible and UV dissolution imaging for the assessment of propranolol hydrochloride in liquisolid compacts of *Sesamum radiatum* gum. J Drug Deliv Sci Technol, 2020;56:101511. Doi: 10.1016/j.jddst.2020.101511

28. Lu M, Xing H, Yang T, Yu J, Yang Z, Sun, Y, et al. Dissolution enhancement of tadalafil by liquisolid technique. Pharm Dev Technol, 2017:22(1):77-89. Doi: 10.1080/10837450.2016.1189563

29. Alotaibi FO, Alhakamy NA, Omar AM, El-Say KM. Clinical pharmacokinetic evaluation of optimized liquisolid tablets as a potential therapy for male sexual dysfunction. Pharmaceutics, 2020;12(12):1187. Doi: 10.3390/pharmaceutics12121187

30. Badr-Eldin SM, Elkheshen SA, Ghorab MM. Improving tadalafil dissolution via surfactant-enriched tablets approach: statistical optimization, characterization, and pharmacokinetic assessment. J Drug Deliv Sci Technol, 2017;41:197-205. Doi: 10.1016/j.jddst.2017.07.014.

31. Tiong N, Elkordy AA. Effects of liquisolid formulations on dissolution of naproxen. Eur J Pharm Biopharm, 2009;73(3):373-384. Doi: 10.1016/j.ejpb.2009.08.002

32. Lam M, Ghafourian T, Nokhodchi A. Optimising the release rate of naproxen liqui-pellet: a new technology for emerging novel oral dosage form. Drug Deliv Transl Res, 2020;10:43-58. Doi: 10.1007/s13346-019-00659-6

33. Pezzini BR, Beringhs AO, Ferraz HG, Silva MAS, Stulzer HK, Sonaglio D. Liquisolid technology applied to pellets: evaluation of the feasibility and dissolution performance using felodipine as a model drug. Chem Eng Res Des, 2016;110:62-69. Doi: 10.1016/j.cherd.2016.01.037

34. Basalious EB, El-Sebaie W, El-Gazayerly O. Rapidly absorbed orodispersible tablet containing molecularly dispersed felodipine for management of hypertensive crisis: development, optimization and *in vitro/in vivo* studies. Pharm Dev Technol, 2013;18(2):407-416. http://doi.org/10.3109/10837450.2012.659258

35. Akinlade B, Elkordy AA, Essa EA, Elhagar S. Liquisolid systems to improve the dissolution of furosemide. Sci Pharma, 2010;78(2):325-344. Doi: 10.3797/scipharm.0912-23

36. Dalal L, Allaf AW, El-Zein H. Formulation and *in vitro* evaluation of self-nanoemulsifying liquisolid tablets of furosemide. Sci Rep, 2021;11(1):1315. Doi: 10.1038/s41598-020-79940-5

37. Ludhiani S, Maheshwari R. Novel application of mixed solvency concept to develop and formulate liquisolid system of a poorly water-soluble drug, furosemide and their evaluations. IJPRT, 2022;12(1):28-57. Doi: 10.31838/ijprt/12.01.05

38. Sheta NM, Elfeky YA, Boshra SA. Cardioprotective efficacy of silymarin liquisolid in isoproterenol prompted myocardial infarction in rats. AAPS PharmSciTech, 2020;21(81):1-16. Doi: 10.1208/s12249-019-1609-3

39. Jaydip B, Dhaval M, Soniwala MM, Chavda J. Formulation and optimization of liquisolid compact for enhancing dissolution properties of efavirenz by using DoE approach. SPJ, 2020;28(6):737-745. Doi: 10.1016/j.jsps.2020.04.016

40. Dholakiya A, Dudhat K, Patel J, Mori D. An integrated QbD based approach of SMEDDS and liquisolid compacts to simultaneously improve the solubility and processability of hydrochlorthiazide. J Drug Deliv Sci Technol, 2021;61. Doi: 10.1016/j.jddst.2020.102162

41. Patel H, Gupta N, Pandey S, Ranch K. Development of liquisolid tablets of chlorpromazine using 3^2 full factorial design. Indian J Pharm Sci, 2019;81(6);1107-1114. Doi: 10.36468/pharmaceutical-sciences.609

42. Chandel P, Kumari R, Kapoor A. Liquisolid technique: an approach for enhancement of solubility. JDDT, 2013;3(4):131-137. Doi: 10.22270/jddt.v3i4.556

43. Devi S, Kumar S, Verma V, Kaushik D, Verma R, Bhatia M. Enhancement of ketoprofen dissolution rate by the liquisolid technique: optimization and *in vitro* and *in vivo* investigations. Drug Deliv Transl Res, 2022;12(11):2693-2707. Doi: 10.1007/s13346-022-01120-x

44. Spireas S, Sadu S. Enhancement of prednisolone dissolution properties using liquisolid compacts. Int J Pharm, 1998;166(2):177-188. Doi: 10.1016/S0378-5173(98)00046-5

45. Kamble PR, Shaikh KS, Chaudhari PD. Application of liquisolid technology for enhancing solubility and dissolution of rosuvastatin. Adv Pharm Bull, 2014;4(2):197-204. Doi: 10.5681%2Fapb.2014.029

46. Jhaveri M, Nair AB, Shah J, Jacob S, Patel V, Mehta T. Improvement of oral bioavailability of carvedilol by liquisolid compact: optimization and pharmacokinetic study. Drug Deliv Transl Res, 2020;10(4):975-985. Doi: 10.1007/s13346-020-00734-3

47. Devi S, Kumar A, Kapoor A, Verma V, Yadav S, Bhatia M. Ketoprofen–FA Co-Crystal: *In vitro* and *in vivo* investigation for the solubility enhancement of drug by design of expert. AAPS PharmSciTech, 2022;23(101). Doi: 10.1208/s12249-022-02253-5

48. Bhatia M, Kumar A, Verma V, Devi S. Development of ketoprofen-*p*-aminobenzoic acid co-crystal: formulation, characterization, optimization, and evaluation. Med Chem Res, 2021;30(11):2090-2102. Doi: 10.1007/s00044-021-02794-7

49. Bhatia M, Devi S. Development, characterisation and evaluation of PVP K-30/PEG solid dispersion containing ketoprofen. ACTA Pharm Sci, 2020;58(1):83-99. Doi: 10.23893/1307-2080.APS.05806

50. Bonthagarala B, Dasari V, Kotra V, Swain S, Beg S. Quality-by-Design based development and characterization of pioglitazone loaded liquisolid compact tablets with improved biopharmaceutical attributes. J Drug Deliv Sci Technol, 2019;51:345-355. Doi: 10.1016/j.jddst.2019.03.033

51. Vraníková B, Svačinová P, Marushka J, Brokešová J, Holas O, Tebbens JD, et al. The importance of the coating material type and amount in the preparation of liquisolid systems based on magnesium aluminometasilicate carrier. Eur J Pharm Sci, 2021;165. Doi: 10.1016/j.ejps.2021.105952

52. Government of India, Ministry of Health & Family Welfare, 2007. Pharmacopoeia of India (Indian Pharmacopoeia volume 1, page no. 182). Ghaziabad: Sector-23, Rajnagar.

53. Jacob S, Shirwaikar A, Nair A. Preparation and evaluation of fast-disintegrating effervescent tablets of glibenclamide. Drug Dev Ind Pharm, 2009;35(3):321-328. Doi: 10.1080/03639040802337021

54. Sayyad FJ, Tulsankar SL, Kolap UB. Design and development of liquisolid compact of candesartan cilexetil to enhance dissolution. J Pharm Res, 2013;7(5):381-388. Doi: 10.1016/j.jopr.2013.05.012

55. Vittal GV, Deveswaran R, Bharath S, Basavaraj BV, Madhavan V. Formulation and characterization of ketoprofen liquisolid compacts by Box-Behnken design. Int J Pharm Investig, 2012;2(3):150-156. Doi: 10.4103%2F2230-973X.104398

56. Kapure VJ, Pande VV, Deshmukh PK. Dissolution enhancement of rosuvastatin calcium by liquisolid compact technique. Journal of Pharmaceutics, 2013;2013:315902.Doi: 10.1155/2013/315902

57. Shazly GA, Alshehri S, Ibrahim MA, Tawfeek HM, Razik JA, Hassan YA, et al. Development of domperidone solid lipid nanoparticles: *in vitro* and *in vivo* characterization. AAPS PharmSciTech, 2018;19:1712-1719. Doi: 10.1208/s12249-018-0987-2

58. Tayel SA, Soliman II, Louis D. Improvement of dissolution properties of carbamazepine through application of the liquisolid tablet technique. Eur J Biopharm, 2008;69(1):342-347. Doi: 10.1016/j.ejpb.2007.09.003

59. Bhatia M, Srivastav M, Devi S, Sharma SK, Kakkar V, Saini K. Optimization and evaluation of ketoconazole loaded nanostructured lipid carriers employing microwave-assisted technique. Indian J Pharm Sci, 2022;84(1):162-172. Doi: 10.36468/pharmaceutical-sciences.907

60. Devi S, Sharma K. Preparation and evaluation of ibuprofen loaded sodium alginate/ sodium CMC mucoadhesive drug delivery system for sustained release. ACTA Pharm Sci, 2022;60(3):217-234. Doi: 10.23893/1307-2080.APS.6015

61. Devi S, Bhatia M, Grewal S. Polymers for mucoadhesive drug delivery systems. ACTA Pharm Sci, 2021;59(2):343-362. Doi: 10.23893/1307-2080.APS.05919

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The assessment of the antimicrobial effect of gemfibrozil alone or in combination with ceftriaxone or gentamycin on several types of bacteria

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ABSTRACT

The present work aimed to investigate the potential antimicrobial action of gemfibrozil alone and in combination with ceftriaxone or gentamycin against specific bacterial strains isolates (*S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli*) with an evaluation of minimum inhibitory concentration (MIC) values, which revealed that gemfibrozil demonstrated the lowest MIC values against all studied bacterial isolates and the combination of gemfibrozil with either ceftriaxone or gentamycin results in an improvement in the MIC values to levels lower than those obtained with ceftriaxone or gentamycin alone, which revealed that there is a synergistic effect of the gemfibrozil combination with ceftriaxone or gentamycin on antibacterial activity against the studied pathogens, which appear more pronouncedly in the effect of combined antibacterial effect upon *Staphylococcus aureus, Staphylococcus epidermis,* and *Streptococcus spp*. In conclusion, the current study demonstrated a synergistic effect between gemfibrozil and both ceftriaxone and gentamycin, indicating that the combination of these compounds is a potential therapeutic option for treating resistant bacterial strains.

Keywords: ceftriaxone, gemfibrozil, gentamycin, synergism

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INTRODUCTION

The continual demand for novel antibacterial agents for the treatment of bacterial infections that should be effective against multidrug-resistant bacteria has prompted researchers to evaluate a variety of antimicrobial development strategies[1]. The extensive and frequent use of antibacterial agents favors the generation of resistant bacteria, which may cause serious infections[2]. In spite of the availability of antibacterial agents, they may also be associated with a limited number of factors such as side effects, adverse effects, and the emergence of new strains of resistant bacteria[3].

Moreover, resistance to antibacterial agents has led to the generation of worldwide infections, causing an increase in morbidity and mortality, with about thirteen million deaths per year within the past century[4].

Antibiotic-resistant pathogens such as *Staphylococcus* species are the most prevalent multidrug-resistant pathogens in both community and hospital infections, and they are the most prevalent in hospitals[5]. Therefore, it is mandatory to investigate new strategies in the treatment of bacterial infections.

Gemfibrozil is peroxisome proliferator-activated receptor-alpha (PPAR-alpha)[6]. It acts by inducing changes in the biometabolism of fats, subsequently lowering triglyceride (TG) levels and also increasing the level of high-density lipoprotein (HDL)[7]. PPAR-alpha causes activation of the lipoprotein lipase (LPL) in both adipose tissues and muscles, leading to a downregulation of the TG concentration. In addition, gemfibrozil also decreases lipolysis and the removal of free fatty acids via the liver, leading to decreased TG production. Gemfibrozil also inhibits the synthesis and stimulates the catabolism of very-low-density lipoprotein (VLDL)[8]. The reduction of the levels of VLDL can cause a drop in serum TG levels to be decreased by about 30–60 percent. On the other hand, gemfibrozil can also upregulate HDL by different mechanisms[9].

Studies showed that gemfibrozil reduces the export of different organic anions, such as penicillin and quinolone antibiotics, in murine macrophages, so it can increase the intracellular levels of these agents and improve their ability to inhibit the intracellular growth of certain bacterial pathogens, such as Listeria monocytogenes[10]. In addition, gemfibrozil can play an essential role in the potentiation of host immune defense activity against different pathogenic bacteria[11,12]. Especially, it has a significant inhibitory effect on the replication of *Pseudomonas aeruginosa*[13] Furthermore, studies showed that gemfibrozil has a significant effect on the treatment of sepsis associated with acute kidney injury [14]. So, by exploring this system, we can investigate the promoting ef-

fect of gemfibrozil on the antimicrobial action of ceftriaxone and gentamycin antibiotics on several types of bacteria. Gentamicin is an antibacterial agent, considered the prototype of the aminoglycoside group, used in the eradication of various bacterial infections; it acts through binding to the 30s subunit of susceptible bacterial ribosomes, leading to inhibition of protein synthesis[15].

Ceftriaxone sodium, a third-generation cephalosporin[16], has been used to treat different bacterial events; however, it is associated with increased resistance that may contribute to treatment failure[17].

The present study was to investigate the possible direct or adjuvant antibacterial effect of gemfibrozil alone or in combination with Ceftriaxone and Gentamycin in an *in vitro* study to identify its spectrum of action in the treatment of infections with major pathogens such as *Staphylococcus aureus, Staph epidermidis, Streptococcus spp., Pseudomonas aeruginosa,* and *E. coli*.

METHODOLOGY

Microorganisms

Bacterial species, including *Staphylococcus aureus* and *epidermidis, Streptococcus sp., Pseudomonas aeruginosa,* and *Escherichia coli*, were carefully isolated from patients. The samples were collected, then carefully enriched in selective media and examined by microscopic and biochemical identification.

Procedure

The antibacterial activity of gemfibrozil alone, gemfibrozil with ceftriaxone, and gemfibrozil with gentamycin was tested against bacterial isolates (*S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli*). The agar-well diffusion method was used. and compare the result with the antimicrobial activity of ceftriaxone and gentamycin alone.

1. Prepare a bacterial plate by using Mueller-Hinton agar to measure the inhibition zone.

2. Sterile swabs were used to spread the bacterial inoculum ($1.5x10^8$ CFU/ml McFarland standard) onto Mueller-Hinton agar in three directions.

a. A sterile stainless-steel borer was used to punch out wells (6 mm in diameter) in the plates. There were three wells each containing 0.10 µl of gemfibrozil solution with concentrations of 100, 50, and 25 µg/ml.

The plates were kept at 37^oC for a period of 24 hours, and a ruler was used to measure the inhibitory zone width in mm.

b. Repeat the step (a.) with another prepared bacterial plate for *S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli*, and put 0.10 µl of gemfibrozil solution with concentrations of 100, 50, and 25 µg/ml separately with a ceftriaxone disc (30 µg) in each well. The plates were also kept at 37°C for a period of 24 hours. A ruler was used to measure the inhibitory zone width in mm.

c. Repeat the step (a.) with another prepared bacterial plate for *S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli* and put 0.10 l of gemfibrozil solution with concentrations of 100, 50, and 25 µg/ml separately with gentamycin disc (10 µg) in each well; the plates were then kept at 37°C for a period of 24 hours. A ruler was used to measure the inhibitory zone width in mm.

d. With another prepared bacterial plate for *S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli*, a sterile stainless-steel borer was used to punch out one well (6 mm in diameter) in the plates and put a ceftriaxone disc (30 µg) alone in this well. The plates were kept at 37°C for a period of 24 hours. A ruler was used to measure the inhibitory zone width in mm.

e. with another prepared bacterial plate (*S. aureus, S. epidermidis, Streptococcus spp.,* and *E. coli*). A sterile stainless-steel borer was used to punch out one well (6 mm in diameter) in the plates. Put the gentamycin disc (10 µg) alone in this well. The plates were kept at 37°C for a period of 24 hours. A ruler was used to measure the inhibitory zone width in mm.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined accurately by serial dilutions according to the National Institute of Clinical Laboratory Standards[18]. Briefly, the investigated drugs were diluted serially and then added to well plates containing molten Muller-Hinton Gold II agar. Then, the plates were set aside to cool, then dried thoroughly. Finally, bacterial species were distributed among the wells of a plate using a steer replicator, with each drop containing $5x10^4$ colony units. At the end of an 18-hour incubation at 37°C, well plates are then accurately read. MIC of an antibacterial agent is defined as the lowest concentration of the agent, in milligrams per liter (µg/mL), that completely inhibits the growth of the designated bacterial strain under *in vitro* conditions. Well plates were read in duplicate, and then the highest MIC value was determined. The reference number is indicated in the tables of the National Committee of Laboratories, which were used to decide whether it was susceptibility or resistance.

Chemicals

All drugs used in this study were purchased from Sigma-Aldrich, and prior to MIC testing, they were diluted to a concentration of 1 mg/ml in dimethyl sulfoxide (DMSO). All substances were used in their purest forms. As a surfactant, dimethyl sulfoxide helped the evaluated drugs dissolve better. However, DMSO has zero antibacterial activity and serves as a negative control because of this fact.

Statistics

GraphPad Prism was used to conduct the statistical analysis (version 4.0, GraphPad Software, CA). To test for statistical significance, we used both one-way ANOVA and Tukey's post-hock test. Significant results were defined as p-values ≤ 0.05[19].

RESULTS and DISCUSSION

Results obtained in the current study regarding the antibacterial effect of either Gemfibrozil alone or in combination with ceftriaxone or gentamycin on standard bacterial strains: *Staphylococcus aureus, Staphylococcus epidermis, Streptococcus spp., Pseudomonas aeruginosa,* and *E. coli* revealed that gemfibrozil alone or in combination with either ceftriaxone or gentamycin induced variable degrees of antibacterial action, with ceftriaxone plus gemfibrozil and gentamycin plus gemfibrozil being more potent than either ceftriaxone or gentamycin alone.

Results illustrated in Table 1 and Figure 1 showed that gemfibrozil demonstrated the lowest MIC values against all studied bacterial isolates. The results also showed that the combination of gemfibrozil with either ceftriaxone or gentamycin results in an improvement in the MIC values to levels lower than those obtained with ceftriaxone or gentamycin alone, which revealed that there is a synergistic effect of the gemfibrozil combination with ceftriaxone or gentamycin on antibacterial activity against the studied pathogens, which appear more pronouncedly in the effect of combined antibacterial effect upon *Staphylococcus aureus, Staphylococcus epidermis,* and *Streptococcus spp.*

The antibacterial activity of gemfibrozil was evaluated against five important bacterial strains by measuring MIC values. In Table 1, results showed that various responses of antibacterial activity were induced by bacterial statins, where ceftriaxone plus gemfibrozil and gentamycin plus gemfibrozil were the most potent combinations compared to either gentamycin or ceftriaxone alone ($p \leq 0.05$). Nevertheless, *Staphylococcus aureus, Staphylococcus epidermis,*

and Streptococcus spp. were more sensitive to ceftriaxone plus gemfibrozil compared to gentamycin alone or in combination with gemfibrozil (P≤0.05). In contrary, *Pseudomonas aeruginosa* and *E. coli* showed to be more sensitive to gentamycin plus gemfibrozil compared to ceftriaxone alone or in combination with gemfibrozil (p≤0.05).

Table 1. Minimum inhibitory concentrations (MIC; µg/mL) of different bacterial strains

Bacterial spp	Gemfibrozil	Ceftriaxone	Gentamycin	Ceftriaxone + Gemfibrozil	Gentamycin + Gemfibrozil
Staphylococcus aureus	63.3 ± 16	350 ± 24	143 ± 12	233.6 ± 14	113.5 ± 12
Streptococcus spp	69.9 ± 12	321 ±12	143 ± 16	250 ± 19	80.5 ± 15
Pseudomonas aeruginosa	83.8 ± 22	159 ± 14	243 ± 15	140 ± 12	223.5 ± 5.8
Staphylococcus epidermis	70 ± 10	388 ± 12	113 ± 17	310 ± 19	70.9 ± 14
Escherichia coli	130 ± 13	190 ±15	290 ± 16	180 ± 11	270.4 ± 24

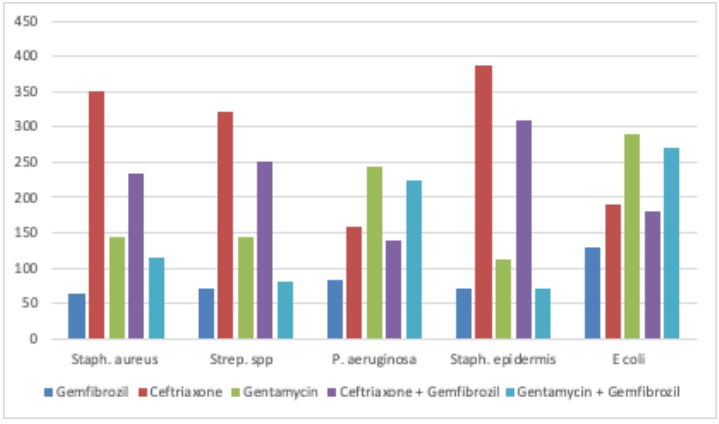

Figure 1. Minimum inhibitory concentrations (MIC; µg/mL) of different bacterial strains

The emergence of drug resistance, antibacterial adverse effects, and poor patient compliance indicates a potential need for a therapy regimen with similar or higher antibacterial beneficial activity yet with fewer side effects. Studies involving combinations for synergism have been prescribed as mandatory for multidrug-resistant bacteria species[20]. Therefore, there is an urgent need to investigate new antibacterial modalities. Results of this study showed potential antibacterial effects for gemfibrozil and indicated the superior antibac-

terial effect of adding gemfibrozil to both ceftriaxone and gentamycin compared with both of them alone. Gemfibrozil is a member of the fibrate group of medications, which were used to lower cholesterol and plasma triglycerides 40 years ago[20]. It is an agonist of the peroxisome proliferator-activated receptors (PPARs)[22]. This agent has recently been demonstrated to have an additional effect through reducing inflammation, decreasing serum interferon and tumor necrosis factor (TNF)[23], as well as interleukin-6 (IL-6)[24].

Gemfibrozil also produces a variety of anti-inflammatory cytokines, such as IL-4, and protects against autoimmune encephalitis[25]. Furthermore, gemfibrozil inhibits TNF, IL-1, IL-6, and nitric oxide[26]. In addition to its antihyperlipidemic effects, gemfibrozil was shown to have other, related effects, including an anticoagulant property, antioxidative activity, and immunomodulatory effect[27,28]. The current study indicated the antimicrobial effect of gemfibrozil against *Staphylococcus aureus, Staphylococcus epidermis, Streptococcus spp., Pseudomonas aeruginosa,* and *E. coli.* Results of the present study also showed that the use of gemfibrozil could potentially improve the antibacterial activity of both ceftriaxone and gentamycin. The antibacterial action of the investigated drugs was validated through the measurement of MIC values against the bacterial species involved in this study. As mentioned earlier, MIC of an antibacterial agent is defined as the lowest concentration of the agent, in milligrams per liter (μg/mL), that completely inhibits the growth of the designated bacterial strain under *in vitro* conditions[29].

Other studies showed that gemfibrozil noncompetitively abolished the growth of Legionella pneumophila and Mycoplasma tuberculosis by inhibiting their enoyl reductases[10]. It is well known that bacterial fatty acid synthesis occurs within membrane phospholipid synthesis, so substances that inhibit fatty acid synthesis will inhibit bacterial growth[30]. Moreover, gemfibrozil could aid the action of ceftriaxone and gentamycin through their reported pleiotropic actions[31]. Additionally, our study showed that gemfibrozil plus ceftriaxone or gentamycin was superior to both drugs alone. These distinct actions could be related to the antimicrobial effect of gemfibrozil, which is unrelated to its lipid-lowering action[32]. The MIC value for gemfibrozil plus ceftriaxone was lower for *E. coli* than pseudomonas strains as compared with gemfibrozil plus gentamycin, while the first combination was better against *Staphylococcus aureus, Staphylococcus epidermis,* and *Streptococcus spp*.

In conclusion, current study demonstrated synergistic activity between gemfibrozil and both ceftriaxone and gentamycin. The results depicted that the combination of these compounds is a potential therapeutic option for treatment resistant bacterial strains. This combination has essentially to be studied in pharmaceutical industry and clinical studies.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to the conception, drafting, and critical revision of the manuscript, and approve of the final version to be published.

REFERENCES

1. Mahdi MH, Dawood AH, Shaheed DQ. Synthesis and study of antimicrobial activity of some tetrahydrocarbazole derivatives substituted with NSAID. AJPS, 2022;22(2):44-54. Doi: 10.32947/ajps.v22i2.857

2. O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. Rev Antimicrob Resist, 2016;1-80.

3. Ali ZA, Jasim TM, Alani WMK. Antibacterial activity of chloroform extract from *Tagetes erecta* L. flowers. AJPS, 2019;19(4):7-15. Doi: 10.32947/ajps.v19i4.627

4. Taha DE, Raauf AMR, Ali KF. Design, synthesis, characterization, biological activity and ADME study of new 5-arylidene-4-thiazolidinones derivatives having. AJPS, 2019;19(4):77-88. Doi: 10.32947/ajps.v19i4.637

5. Bodnar GC, Martins HM, De Oliveira CF, Morey AT, Tavares ER, Cardosa JD, et al. Comparison of HRM analysis and three REP-PCR genomic fingerprint methods for rapid typing of MRSA at a Brazilian hospital. J Infect Dev Ctries, 2016;10(12):1306-1317. Doi: 10.3855/jidc.7887

6. Rasheed HA, Hussien NR, Al-Naimi MS, Al-Kuraishy HM, Al-Gareeb AI. Fenofibrate and *Crataegus oxyacantha* is an effectual combo for mixed dyslipidemia. Biomed Biotechnol Res J, 2020;4:259-265. Doi: 10.4103/bbrj.bbrj_26_20

7. Ito MK. Long-chain omega-3 fatty acids, fibrates and niacin as therapeutic options in the treatment of hypertriglyceridemia: a review of the literature. Atherosclerosis, 2015;242(2):647-656. Doi: 10.1016/j.atherosclerosis.2015.06.012

8. Bussière-Côté S, Omlin T, de Càssia Pinheiro E, Weber JM. Gemfibrozil disrupts the metabolism of circulating lipids in bobwhite quails. Comp Biochem Physiol C Toxicol Pharmacol, 2016;179:137-143. Doi: 10.1016/j.cbpc.2015.09.011

9. Kimoto E, Li R, Scialis RJ, Lai Y, Varma MV. Hepatic disposition of gemfibrozil and its major metabolite gemfibrozil 1-o-β-glucuronide. Mol Pharm, 2015;2;12(11):3943-3952. Doi: 10.1021/acs.molpharmaceut.5b00411

10. Reich-Slotky R, Kabbash CA, Della-Latta P, Blanchard JS, Feinmark SJ, Freeman S, et al. Gemfibrozil inhibits *Legionella pneumophila* and *Mycobacterium tuberculosis* enoyl coenzyme a reductases and blocks intracellular growth of these bacteria in macrophages. J Bacteriol, 2009;191(16):5262-5271. Doi: 10.1128/JB.00175-09

11. Gopal R, Mendy A, Marinelli MA, Richwalls LJ, Seger PJ, Patel S, et al. Peroxisome proliferator-activated receptor gamma (PPAR) suppresses inflammation and bacterial clearance during influenza-bacterial super-infection. Viruses, 2019;11:505. Doi: 10.3390/v11060505

12. Leopold Wager CM, Arnett E, Schlesinger LS. Macrophage nuclear receptors: emerging key players in infectious diseases. PLoS Pathog, 2019;15:e1007585. Doi: 10.1371/journal.ppat.1007585

13. Gugliandolo E, Fusco R, Ginestra G, D'Amico R, Bisignano C, Mandalari G, et al. Involvement of TLR4 and PPAR-alpha receptors in host response and NLRP3 inflammasome activation, against pulmonary infection with *Pseudomonas aeruginosa*. Shock, 2019;51,221-227. Doi: 10.1097/SHK.0000000000001137

14. Iwaki T, Bennion BG, Stenson EK, Lynn JC, Otinga C, Djukovic D, et al. PPAR-alpha contributes to protection against metabolic and inflammatory derangements associated with acute kidney injury in experimental sepsis. Physiol Rep, 2019;7(10):e14078. Doi: 10.14814/phy2.14078

15. Al-kuraishy HM, Al-Gareeb AI, Al-nami MS. Irbesartan attenuates gentamicin-induced nephrotoxicity in rats through modulation of oxidative stress and endogenous antioxidant capacity. Int J Prev Med, 2020;11(16):1-8. Doi: 10.4103/ijpvm.IJPVM_567_18

16. Salman AT. RP- HPLC estimation of ceftriaxone sodium in pharmaceuticals. Egypt J Chem, 2021;64(9):4901-4906. Doi: 10.21608/EJCHEM.2021.68537.3504

17. Muhsin EA, Al-Juboriv SS, Said, LA. Prevalence of efflux pump and porin-related antimicrobial resistance in clinical *Klebsiella pneumoniae* in Baghdad, Iraq. Archives of Razi Institute, 2022;77(2):785-798.

18. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. Pathogens, 2021;10(2):165. Doi: 10.3390/pathogens10020165

19. Norman G. Likert scales, levels of measurement and the "laws" of statistics. Adv Health Sci Educ Theory Pract, 2010;15:625-632. Doi: 10.1007/s10459-010-9222-y

20. Xiao G, Li J, Sun Z. The combination of antibiotic and non-antibiotic compounds improves antibiotic efficacy against multidrug-resistant bacteria. Int J Mol Sci, 2023;24(20):15493. Doi: 10.3390/ijms242015493

21. Nikkila EA, Ylikahri R, Huttunen JK. Gemfibrozil: effect on serum lipids, lipoproteins, postheparin plasma lipase activities and glucose tolerance in primary hypertriglyceridaemia. Proc R Soc Med, 1976;2:58-63.

22. Berger J, Moller DE. The mechanisms of action of PPARs. Annu Rev Med, 2002;53:409-435. Doi: 10.1146/annurev.med.53.082901.104018

23. Madej A, Okopien B, Kowalski J, Zielinski M, Wysocki J, Szygula B, et al. Effects of fenofibrate on plasma cytokine concentrations in patients with atherosclerosis and hyperlipoproteinemia IIb. Int J Clin Pharmacol Ther, 1998;36:345-349.

24. Staels B, Koenig W, Habib A, Merval R, Lebret M, Torra IP, et al. Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by PPARγ activators. Nature, 1998;393:790-793. Doi: 10.1038/31701

25. Lovette-Racke AE, Hussain RZ, Northrop S, Choy J, Rocchini A, Matthes L, et al. Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease. J Immunol, 2004;172:5790-5798. Doi: 10.4049/jimmunol.172.9.5790

26. Xu J, Chavis JA, Racke MK, Drew PD. Peroxisome proliferator-activated receptor-alpha and retinoid X receptor agonists inhibit inflammatory responses of astrocytes. J Neuroimmunol, 2006;176:95-105. Doi: 10.1016/j.jneuroim.2006.04.019

27. Mohanalakshmi S, Bhatt S, Ashok Kumar CK. Enhanced antihyperlipidemic potential of gemfibrozil under co-administration with piperine. Curr Res Pharmacol Drug Discov, 2021;2:2571-2590. Doi: 10.1016/j.crphar.2021.100021

28. Roy A, Pahan K. Gemfibrozil, stretching arms beyond lipid lowering. Immunopharmacol Immunotoxicol, 2009;31(3):339-351. Doi: 10.1080/08923970902785253

29. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. Pathogens, 2021;10(2):165. Doi: 10.3390/pathogens10020165

30. Zhang YM, Rock CO. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol, 2008;6,222-233. Doi: 10.1038/nrmicro1839

31. Doncheva NI , Nikolov KV, Vassileva DP. Lipid-modifying and pleiotropic effects of gemfibrozil, simvastatin and pravastatin in patients with dyslipidemia. Folia Med, 2006;48(34):56-61.

32. Seral C, Carryn S, Tulkens PM, Van Bambeke F. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by *Listeria monocytogenes* or *Staphylococcus aureus*. J Antimicrob Chemother, 2003;51(5):1167-1173. Doi: 10.1093/jac/dkg223

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Research article
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Cytotoxic activity of the fruit extracts of *Heptaptera cilicica* (Boiss. & Balansa) Tutin

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ABSTRACT

The fruit extracts of *Heptaptera cilicica* (Boiss. & Balansa) Tutin were screened for cytotoxic activities on the colon (COLO205 and KM12) and kidney (UO31 and A498) cancer cell lines. The highest cytotoxic activities were observed in the dichloromethane extract of the fruits with IC_{50} values of 12.1 mg/mL and 12.2 mg/mL on the COLO205 and KM12 cell lines, respectively. In contrast, the ethyl acetate extract of the fruits exhibited moderate cytotoxic activity with IC_{50} values of 33.6 mg/mL and 37.2 mg/mL against the COLO205 and KM12 cell lines. Dichloromethane and ethyl acetate extracts of the fruits exhibited weak cytotoxic activity against the UO31 and A498 cancer cell lines with IC_{50} values greater than 50 mg/mL. Aqueous-methanol extract of the fruits showed cytotoxic activity against all cancer cell lines with IC_{50} value greater than 50 mg/mL.

Keywords: cytotoxic activity, *Heptaptera cilicica*, Apiaceae

INTRODUCTION

Cancer, a significant public health problem, is the second leading cause of death worldwide. Colorectal cancers are the third estimated new cancer cases and deaths among adult Americans in 2022, and kidney cancer (Renal cell can-

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cer-RCC) is the second most common cancer of the urinary system[1]. In 2023, there were approximately 81,800 new cases and 14,8900 deaths due to kidney and renal pelvis cancer, 153,020 new patients, and 52,550 deaths due to colon and rectum cancer[2]. Natural products are important sources for the discovery of new anticancer drugs[3]. This study was designed to evaluate the cytotoxic activities of *Heptaptera cilicica* fruits on colon and kidney cancer cells during our ongoing studies on the genus *Heptaptera* (Apiaceae). There are 11 species of *Heptaptera* Marg. & Reuter found in the world four of them: *H. anatolica* (Boiss.) Tutin, *H. anisoptera* (DC.) Tutin *H. cilicica* (Boiss. & Balansa) Tutin and *H. triquetra* (Vent.) Tutin are growing in Turkey[4,5]. *H. cilicica* is an endemic species distributed in the Mersin province of the Mediterranean region of Turkey[5]. *Heptaptera* species have been reported to contain sesquiterpene coumarin derivatives[6-12]. Coumarins are well-known plant secondary metabolites for their cytotoxic, anti-cancer, anti-inflammatory, antibacterial, antidiabetic, antileishmanial, antiviral, and anti-cholinesterase effects[11-18].

In this study, the fruit extracts of *Heptaptera cilicica* were investigated for their cytotoxic activities against the colon (COLO205 and KM12) and kidney (UO31 and A498) cancer cell lines.

METHODOLOGY

Plant material

The fruits of *Heptaptera cilicica* were collected in Mersin province between Tarsus and Karakütük village in June 2013 and identified by Prof. A. Duran. A voucher specimen (A. Duran 9591) was deposited in the Herbarium of Selçuk University, Faculty of Sciences, Department of Biology (KONYA).

Extraction

Pulverized fruits (50 g) of the plant were sequentially extracted by maceration with dichloromethane (CH_2Cl_2) and methanol. The extracts were individually concentrated in a rotary evaporator under reduced pressure to yield crude extracts. Dichloromethane and methanol extracts of the fruits were 0.72 g (1.44%) and 2.45 g (4.90%), respectively. The methanol extract was redissolved in a mixture of methanol/water (10:90) and then partitioned with ethyl acetate (EtOAc); the resulting extracts were separately concentrated in vacuo to dryness. The yields of ethyl acetate and aqueous-methanol extracts of the fruits were 0.92 g (1.84%) and 1.44 g (2.88%), respectively.

Cytotoxicity assay on cancer cells

The assay used for this study was a two-day, two-cell line XTT bioassay[19], an *in vitro* antitumor colorimetric assay developed by the MTL Assay Development and Screening Section. Cancer cell lines used were colon (COLO205 and KM12) and kidney (UO31 and A498). Sanguinarine was used as a positive control. The assay was performed as described previously[12].

RESULTS and DISCUSSION

In this study, the cytotoxic activity of the fruits of *H. cilicica* was reported for the first time. In our research, the dichloromethane extract of the fruits had a strong inhibitory activity on the colon cancer COLO205 and KM12 cell lines. The ethyl acetate extract of the fruits showed moderate inhibitory activity on the COLO205 and KM12 cell lines. Aqueous-methanol extract of the fruits showed cytotoxic activity against the COLO205 and KM12 cell lines with IC_{50} values greater than 50 μg/mL. All the extracts had cytotoxic activity on UO31 and A498 renal cell lines greater than 50 μg/mL concentrations. The cytotoxic activities detected in these extracts are shown in Table 1.

Table 1. Cytotoxic activities of the extracts

IC_{50} values (μg/mL)				
Extracts	**UO31**	**A498**	**COLO205**	**KM12**
1	> 50	> 50	12,1	12,2
2	> 50	> 50	33,6	37,2
3	> 50	> 50	> 50	> 50

1: CH_2Cl_2 extract; 2: EtOAc extract; 3: Aqueous-methanol extract

The highest cytotoxic activities were observed in the dichloromethane extract of the fruits with IC_{50} values of 12.1 mg/mL and 12.2 mg/mL on the COLO205 and KM12 cell lines, respectively. The ethyl acetate extract of the fruits exhibited moderate cytotoxic activity with IC_{50} values of 33.6 μg/mL and 37.2 μg/mL on the COLO205 and KM12 cell lines. Dichloromethane and ethyl acetate extracts of the fruits exhibited weak cytotoxic activity against the UO31 and A498 cancer cell lines with IC_{50} values greater than 50 mg/mL. Aqueous-methanol extract of the fruits showed cytotoxic activity against all cancer cell lines with IC_{50} value greater than 50 mg/mL. Previously, Tosun and Miski reported the

cytotoxic activities of the fruit extracts of *Heptaptera anisoptera*[20], *H. anatolica*[21], and *H. triquetra*[22] on the COLO205 and KM12 cell lines. The cytotoxic activities of the dichloromethane, ethyl acetate, and aqueous-methanol extract of the *H. cilicica* fruits on the COLO205 and KM12 cell lines were similar to those of the fruit dichloromethane, ethyl acetate, and aqueous-methanol extracts of *H. anatolica*. Among these four *Heptaptera* species, the dichloromethane and ethyl acetate extracts of the fruits of *H. anisoptera* showed the best cytotoxic activities. The ethyl acetate extracts of the *H. anatolica*, *H. triquetra,* and *H. cilicica* fruits showed moderate to weak cytotoxic activities. Aqueous-methanol extracts of the fruits of these four *Heptaptera* species showed cytotoxic activity against the COLO205 and KM12 cell lines with IC_{50} values greater than 50 mg/mL. Since some sesquiterpene coumarin derivatives were isolated from the chloroform extracts of the fruits of *Heptaptera anatolica*[9], *H. anisoptera*[9] and *H. cilicica*[23], and the cytotoxic activities of sesquiterpene coumarins were reported earlier[11-13], it can be concluded that the secondary metabolite(s) responsible for the cytotoxic activity of the fruits of *H. cilicica* may be sesquiterpene coumarin(s). The dichloromethane extract of the fruits of *H. cilicica* will be subjected to bioactivity-guided fractionation.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors made equal contributions to the study.

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REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics. CA Cancer J Clin, 2022;72:7-33. Doi: 10.3322/caac.21708

2. Siegel R, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin, 2023;73(1):17-48. Doi: 10.3322/caac.21763

3. Newman DJ, Cragg GM. Natural products as sources of new drugs over the nearly four decades from 01/ 1981 to 09/2019. J Nat Prod 2020;83:770-803. Doi: 10.1021/acs.jnatprod.9b01285

4. The WFO Plant List, A working list of all plant species, September 6, 2023. Available from: https://wfoplantlist.org/plant-list/taxon/wfo-4000017518-2023-06?page=1

5. Herrnstadt I, Heyn CC. Heptaptera Marg. & Reuter. In Flora of Turkey and the East Aegean Islands, Davis, P., Ed.; Edinburgh: Edinburgh University Press; 1972;Vol.4:388-390.

6. Ban'kovskii AI, Ermatov NE, Parel'son ME, Bubeva-Ivanova L, Pavlova NSt. Structure of the coumarins colladin and colladonin. Khim Prir Soedin, 1970;6(2):173-180.

7. Borisov VN, Ban'kovskii AI, Pavlova NS, Bubeva-Ilanova, Sheichenko VI, Kabanov VS. Khim Prir Soedin, 1975;247.

8. Appendino G, Özen HÇ, Tagliapietra S, Cisero M. Coumarins from *Heptaptera anisoptera*. Phytochemistry, 1992;31:3211-3213. Doi: 10.1016/0031-9422(92)83477-G

9. Appendino G, Özen HÇ, Nano GA, Cisero M. Sesquiterpene coumarin ethers from the genus *Heptaptera*. Phytochemistry, 1992;31:4223-4226. Doi: 10.1016/0031-9422(92)80447-M

10. Appendino G, Özen HÇ, Jakupovic J. A sesquiterpene coumarin ether and a coniferyl ester from *Heptaptera anisoptera*. Fitoterapia, 1993;64:505-506.

11. Tosun F, Beutler JA, Ransom TT, Miski M. Anatolicin, a highly potent and selective cytotoxic sesquiterpene coumarin from the root extract of *Heptaptera anatolica*. Molecules, 2019;24:1153-1160. Doi: 10.3390/molecules24061153

12. 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: 10.1002/ptr.3311

13. Nazari ZE, Iranshahi M. Biologically active sesquiterpene coumarins from *Ferula* species. Phytother Res, 2011;25:315-323. Doi: 10.1002/ptr.3311

14. Gliszczynska A, Brodelius PE. Sesquiterpene coumarins. Phytochem Rev, 2012;11:77-96. Doi: 10.1007/s11101-011-9220-6

15. Dastan D, Salehi P, Aliahmadi A, Gohari AR, Maroofi H, Ardalan A. New coumarin derivatives from *Ferula pseudalliacea* with antibacterial activity. Nat Prod Res, 2016;30(24):2747-2753. Doi: 10.1080/14786419.2016.1149705

16. Amin A, Tuenter E, Cos P, Maes L, Exarchou V, Apers S, Pieters L. Antiprotozoal and antiglycation activities of sesquiterpene coumarins from *Ferula narthex* exudate. Molecules, 2016;21:1287-1296. Doi: 10.3390/molecules21101287

17. Iranshahi M, Rezaee R, Najafi MN, Haghbin A, Kasaian J. Cytotoxic activity of the genus *Ferula* (Apiaceae) and its bioactive constituents. Avicenna J Phytomed, 2018;8(4):296-312.

18. Erdogan Orhan İ, Tosun F, Senol Deniz F S, Eren G, Mıhoğlugil F, Akalgan D, Miski M. Butyrylcholinesterase-inhibiting natural coumarin molecules as potential leads. Phytochem Lett, 2021;44:48-54. Doi: 10.1016/j.phytol.2021.05.001

19. 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

20. Tosun F, Miski M. Cytotoxic activity of the root and fruit extracts of *Heptaptera anisoptera* (DC.) Tutin. Acta Pharm Sci, 2020;58(1):21-25. Doi: 10.23893/1307-2080.APS.05801

21. Tosun F, Miski M. Cytotoxic activity of the fruit extracts of *Heptaptera anatolica* (Boiss.) Tutin. Acta Pharm Sci, 2022;60(2):125-129. Doi: 10.23893/1307-2080.APS.6009

22. Tosun F, Miski M. Potent colon cancer cell line toxicity of the fruit extracts of *Heptaptera triquetra* (Vent.) Tutin. Acta Pharm Sci, 2022;60(3):295-299. Doi: 10.23893/1307-2080.APS.6019

23. Güvenalp Z, Özbek H, Yılmaz G, Yerdelen KÖ, Kazaz C, Demirezer ÖL. Cholinesterase inhibition and molecular docking studies of sesquiterpene coumarin ethers from *Heptaptera cilicica*. Rec Nat Prod, 2017;11(5):462-467. Doi: 10.25135/rnp.58.17.03.051

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Essential oil composition of *Isatis floribunda* Boiss. ex. Bornm. and acetylcholinesterase inhibitory activity of its extract

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ABSTRACT

This study aims to investigate the essential oil composition of aerial parts of *Isatis floribunda*, and the acetylcholinesterase inhibitory activity of its extracts. Essential oil of the aerial parts of the plant material was obtained and the GC-MS analysis was performed. Then, extracts of the plant material were obtained, and *in vitro* acetylcholinesterase inhibitory assay was performed. GC-MS analysis demonstrated that the composition of *Isatis floribunda* is rich in fatty acid compounds, with the major compounds including dodecanoic acid, nanocosane, hexadecenoic acid, tetradecanoic acid, methyl octadecenoate, decanoic acid, and hexahydrofarnesyl acetone. Methanolic extract of the plant species has demonstrated strong acetylcholinesterase inhibitory activity with the IC_{50} value of 0.16 mg/mL. Essential oil composition of *Isatis floribunda* has been determined to be rich in fatty acid components, these compounds could demonstrate potent acetylcholinesterase inhibition. Therefore, a novel medication from *Isatis floribunda* extracts could be discovered against Alzheimer's disease.

Keywords: *Isatis floribunda*, acetylcholinesterase inhibition, Brassicaceae, GC-MS

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INTRODUCTION

Natural sources have been used for developing medicinal agents for centuries. Many modern medicines today have been developed from natural sources such as plants. Traditional medicine that suggests using plant extracts and essential oils of plants still provides treatment to most of the world's population[1]. Brassicaceae (Cruciferea) family is a major family which includes approximately 350 genera and 3000 species and distributes mainly in North Temperate Zone. The family species of Brassicaceae, also known as mustards, have been used for centuries for food and herbal remedy purposes[2]. Plants in the Brassicaceae family have been used for their antifungal, antibacterial, antidiabetic and anticancer properties[3]. Genus *Isatis* is one of the the most extensive genera in the Brassicaceae family, and they are known as biennial, herbaceous shrubs with yellow flowers. Leaves of the *Isatis* genera are known to have antiviral, antibacterial, anticancer, astringent, and febrifuge activities. *Isatis floribunda* is a member of the Brassicaceae family, and an endemic plant distributed in Türkiye and Iran. Mediterranean regions are the major distribution zones of *Isatis floribunda*, including Adana, Çankırı, Ankara, Eskişehir, Kayseri, Konya, and Nevşehir provinces[4].

In previous studies, extracts of *Isatis floribunda* have been analyzed for their antioxidative, antibacterial, and cytotoxic properties. Plant extracts have demonstrated rich phenolic and flavonoid content, which results in antioxidative activity. Additionally, extracts of *Isatis floribunda* demonstrated antibacterial activity against various Gram-positive and Gram-negative bacteria. Furthermore, HPLC analysis of the extracts of *Isatis floribunda* has shown to be rich in chlorogenic acid, quercetin, p-coumaric acid and caffeic acid[4,5]. Overall, *Isatis floribunda* extracts have been used for their various biological activities for decades. However, the possible biological activities of *Isatis floribunda* are still yet to be determined.

Alzheimer's disease is the most common type of dementia worldwide. It is characterized by dementia; however, the disease's pathogenesis is still could not be elucidated. The cholinergic hypothesis is believed to be the reason for the disease formation and progression. The cholinergic hypothesis is explained by insufficient endogenous acetylcholine in the cholinergic system[6]. Acetylcholine is a neurotransmitter that causes the signal transmission from one cholinergic neuron to another. Acetylcholine deficiency causes impaired memory, leading to dementia symptoms[7]. One reason for acetylcholine deficiency is the increased acetylcholinesterase (AChE) enzyme activity. AChE enzyme is responsible for the degradation of acetylcholine in the synaptic junction. Overactivation of the AChE enzyme can cause fast degradation of acetylcholine, therefore, deficiency

of acetylcholine, which leads to dementia symptoms[8]. Another hypothesis includes inhibition of the choline-acetyltransferase enzyme, which is the enzyme responsible for the synthesis of acetylcholine. Therefore, an insufficient amount of acetylcholine causes dementia symptoms. Depending on the first hypothesis, AChE inhibitors are used in the treatment of Alzheimer's disease, including Galantamine, Rivastigmine, Physostigmine, Tacrine, and Donepezil[9,10]. AChE inhibition is the essential treatment strategy for Alzheimer's disease. Therefore, emerging novel AChE inhibitors are essential treatment options[11].

In this study, the essential oil composition of *Isatis floribunda* was analyzed with GC-MS analysis, and the AChE inhibitory activity of the extracts of *Isatis floribunda* was tested to determine if the plant species is a promising compound in developing novel treatment options for Alzheimer's disease.

METHODOLOGY

Plant material

Isatis floribunda was collected from its natural habitat during the flowering season from Beypazarı, Ankara, Türkiye. The plant material was deposited to the Gaziosmanpaşa University Herbarium with the voucher specimen number GOPU 3028. Aerial parts of *Isatis floribunda* were used to obtain essential oil and extracts.

Isolation of essential oil

100 grams of dried aerial parts of the plant material were used to obtain the essential oil. Hydro-distillation method was used by Clevenger-type apparatus for 3 hours. At hour 3, the yield of the essential oil was 0.03%.

GC-MS analysis of essential oil

The essential oil analysis was conducted by Agilent 5977 MSD GC-MS system operating in EI mode. Temperatures of the MS transfer line and injector were adjusted at 250˚C, and spitless injection was used through the analysis. As a carrier gas, helium was used with a constant flow rate of 1 mL/min. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used, and the temperature was adjusted at 60˚C for 10 minutes and increased to 220°C at the rate of 4˚C/min. The temperature was kept stable at 220°C for 10 min and then increased to 240°C at a rate of 1˚C/min. Mass spectra were recorded at 70 eV with the mass range m/z 35 to 425. The relative percentage amounts of the separated compounds were calculated from the integration of the peaks in mass spectrum chromatograms. The results of the GC-MS analysis are provided in "Table 1".

Preparation of plant extracts

Air-dried plant material was ground to obtain a fine powder. Then, 10 grams of plant species were used to obtain dichloromethane and methanol extracts of the plant species by the Soxhlet extraction method for 3 hours, respectively. Finally, the excess solvent was evaporated with a rotary evaporator (Heidolph, Germany). The yields of the dichloromethane and methanol extracts were calculated as 5.89% and 34.73%, respectively.

In vitro acetylcholinesterase inhibition assay

The previously described method was used for the AChE inhibition assay[12]. The solution of the AChE inhibitory assay contained 240 µL, 1.25 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 192 µL acetylthiocholine iodide (AChI), 1200 µL, 100 mM Tris–HCl buffer pH 8.0 and 20, 40 and 60 µL extract solution. The inhibitory activity was tested for 10 mg/mL concentration stock solution in methanol. In addition, 3 different volumes were tested, including 20 µL, 40 µL, and 60 µL. The same buffer volume was added to the assay solution instead of the tested amounts in the blank solution. Galantamine hydrobromide was used as a positive control during the assay. To start the reactions, ≈ 0.03 U/mL of AChE (electric eel) was transferred into the reaction mixture. The reaction was monitored for 2 minutes using a spectrophotometer (Carry 60 single beam spectrophotometer, Agilent Technologies, USA) at 412 wavelengths. Following the activity obtained from the blank, as the percentage of the reaction rate, the enzymatic activity was calculated. The data obtained from the linear section of the initial 60 s were used to calculate the activities. The subtraction of the ratio of the sample activity versus blank activity from 100 calculated the AChE inhibitory activity. The results of the experiments were given as mean ± standard deviation of three parallel experiments. The analysis was performed only on methanolic extract due to the solubility and turbidity problem of the dichloromethane extract.

RESULTS and DISCUSSION

GC-MS analysis of essential oil

The essential oil composition of the plant species was detected to be rich in saturated fatty acids. The major compound of *Isatis floribunda* was determined as dodecanoic acid represents about 28.6%. Nanocosane, hexadecenoic acid, and tetradecanoic acid were also detected at about 11.0%, 10.0%, and 8.4%, respectively. In addition, methyl octadecenoate, decanoic acid, and hexahydrofarnesyl acetone were detected at the ratio of 4.8%, 4.6%, and 3.5%, respectively. Due to insufficient essential oil, a bioactivity assay could not be performed. Therefore, an extract of the plant material was obtained for further analysis. GC-MS chromatogram is presented in “Figure 1”.

Table 1. Compounds determined by GC-MS of *Isatis floribunda* essential oil, major compounds were presented in bold font type

No	[1]RT	[2]RRI	[3]RRI Lit	Compound	%	[4]Identification method
1	17,879	1229	1232	(E)-2 Hexanal	0.12	RI, MS
2	25,232	1403	1400	Nonanal	0.06	RI, MS
3	26,674	1443	1441	(E)-2-Octenal	0.09	RI, MS
4	32,831	1627	1624	cis-Dihydrocarvone	0.07	RI, MS
5	33,23	1640	1638	β-Cyclocitral	0.09	RI, MS
6	33,445	1647	1645	cis-Isodihydrocarvone	0.05	RI, MS
7	34,01	1666	1664	Phenylacetaldehyde	0.29	RI, MS
8	34,319	1676	1671	(Z)- β–Farnesene	0.08	RI, MS
9	35,397	1712	1709	α-Terpineol	0.15	RI, MS
10	37,019	1769	1765	Napthalene	0.46	RI, MS
11	38,236	1813	1815	Methyl dodecanoate	0.06	RI, MS
12	39,255	1850	1845	Anethole	0.08	RI, MS
13	39,325	1853	1853	Ethyl dodecanoate	0.13	RI, MS
14	39,798	1871	1868	(E)-Geranyl acetone	0.75	RI, MS
15	42,17	1962	1958	(E)-β-Ionone	0.59	RI, MS
16	42,544	1976	1956	2-Methyl-5-(1,1,5-trimethyl-5-hexenyl)furan	0.26	RI, MS
17	43,572	2017	1968	β-Ionol	0.09	RI, MS
18	43,778	2025		3,4-Dehydro-β-ionone	0.34	MS
19	**44,496**	**2055**	**2048**	**3,4-Dimethoxystyrene**	**1.15**	**RI, MS**
20	44,605	2059	2057	Ethyl tetradecanoate	0.07	RI, MS
21	44,92	2072	2084	Octanoic acid	0.11	RI, MS
22	45,619	2101	2096	(E)-Methyl cinnamate	0.06	RI, MS
23	45,717	2105	2100	Heneicosane	0.48	Ac, RI, MS
24	**46,5**	**2138**	**2131**	**Hexahydrofarnesyl acetone**	**3.50**	**RI, MS**
25	47,451	2179	2179	1-Tetradecanol	0.06	RI, MS
26	47,553	2183	2170	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	0.52	RI, MS
27	47,668	2188		Megastigmatrienone isomer*	0.07	MS
28	47,913	2199	2198	1-Docosene	0.09	RI, MS
29	48,506	2225	2218	p-Vinylguaicol	0.09	RI, MS
30	48,666	2232		Megastigmatrienone isomer*	0.46	MS

Table 1. Compounds determined by GC-MS of *Isatis floribunda* essential oil, major compounds were presented in bold font type (continued)

31	48,754	2236	2241	Heptadecanal	0.24	RI, MS
32	49,421	2266	2262	Ethyl hexadecanoate	0.11	RI, MS
33	**49,863**	**2286**	**2296**	**Decanoic acid**	**4.69**	**RI, MS**
34	50,314	2305	2300	Tricosane	0.50	Ac, RI, MS
35	50,996	2334		Megastigmatrienone isomer*	0.22	MS
36	51,567	2358	2353	Octadecanal	0.06	RI, MS
37	52,365	2391	2384	Farnesyl acetone	0.59	RI, MS
38	52,757	2407	2400	Tetracosane	0.34	Ac, RI, MS
39	**53,866**	**2446**	**2431**	**Methyl octadecanoate**	**4.84**	**RI, MS**
40	54,19	2458	2467	Ethyl octadecanoate	0.37	RI, MS
41	**55,392**	**2500**	**2503**	**Dodecanoic acid**	**28.62**	**RI, MS**
42	**55,601**	**2506**	**2500**	**Pentacosane**	**3.08**	**RI, MS**
43	57,808	2571	2560	Ethyl 3-hydroxytridecanoate	0.30	RI, MS
44	58,127	2580	2583	Methyl linolenate	0.07	RI, MS
45	59,14	2608	2600	Hexacosane	0.17	Ac, RI, MS
46	59,357	2613	2613	Ethyl linolenate	0.09	RI, MS
47	59,814	2624	2622	Phytol	0.43	RI, MS
48	**61,284**	**2659**	**2670**	**Ethyl eicosanoate**	**1.51**	**RI, MS**
49	61,521	2665	2670	Tetradecanoic acid	0.06	RI, MS
50	**63,429**	**2709**	**2700**	**Heptacosane**	**2.62**	**Ac, RI, MS**
51	**63,557**	**2712**	**2713**	**Tetradecanoic acid**	**8.54**	**RI, MS**
52	68,141	2809	2800	Octacosane	0.41	Ac, RI, MS
53	68,581	2818	2822	Pentadecanoic acid	0.13	Ac, RI, MS
54	70,485	2856	2857	Palmito-γ-lactone	0.25	RI, MS
55	**73,307**	**2911**	**2900**	**Nonacosane**	**11.03**	**Ac, RI, MS**
56	**74,049**	**2925**	**2931**	**Hexadecanoic acid**	**9.99**	**RI, MS**
57	78,749	3013	2290	Docosanol	0.52	RI, MS

RT: Retention Time, RRI: Relative Retention Index according to n-alkanes, RRI Lit: Relative Retention Index according to the literature. Identification Method; MS: According to mass similarity, RI: According to the similarity of RRI with the literature, Ac: Co-injection of the authentic compound

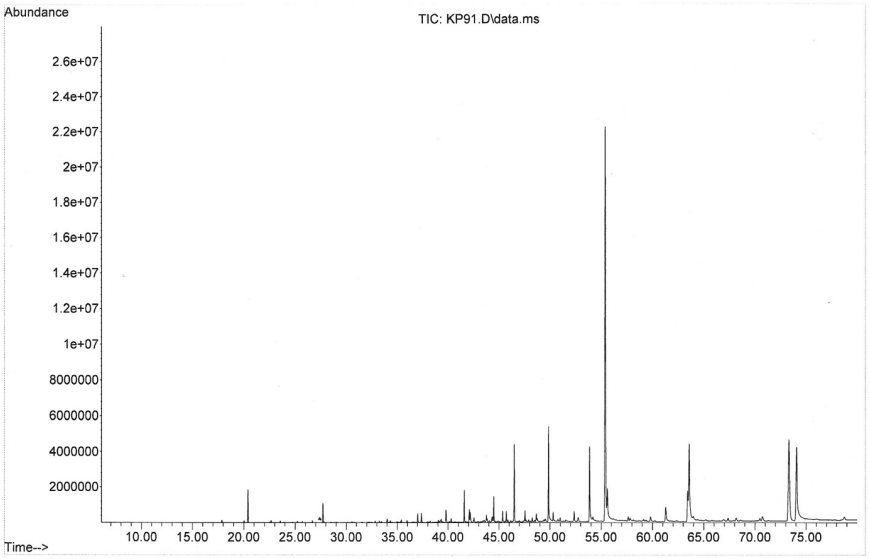

Figure 1. GC-MS chromatogram of *Isatis floribunda* essential oil

In vitro acetylcholinesterase inhibition assay

Due to the solubility and turbidity problem of the dichloromethane extract, an AChE inhibitory assay was performed only on the methanolic extract. All the tested volumes showed inhibitory activity. 20 µL, 40 µL, and 60 µL volumes demonstrated 35.17%, 50.44%, and 57.323% inhibition, respectively. The IC_{50} of positive control, galantamine hydrobromide, was calculated with the same equation which was 2.01 µL. The detailed results of the inhibitory assay are demonstrated in "Table 2".

Table 2. AChE inhibition results of the methanolic extracts of *Isatis floribunda*

Tested Volume, µL	[Inh], mg/mL	Inhibition, %	Standard Deviation ±
0	0	0	0
20	0.083	35.17	0.39
40	0.166	50.44	0.208
60	0.25	57.323	1.258

IC_{50} was calculated as 0.16 mg/mL according to the equation obtained from the AChE inhibitory curve. The AChE inhibition curve of *Isatis floribunda* is demonstrated in "Figure 2".

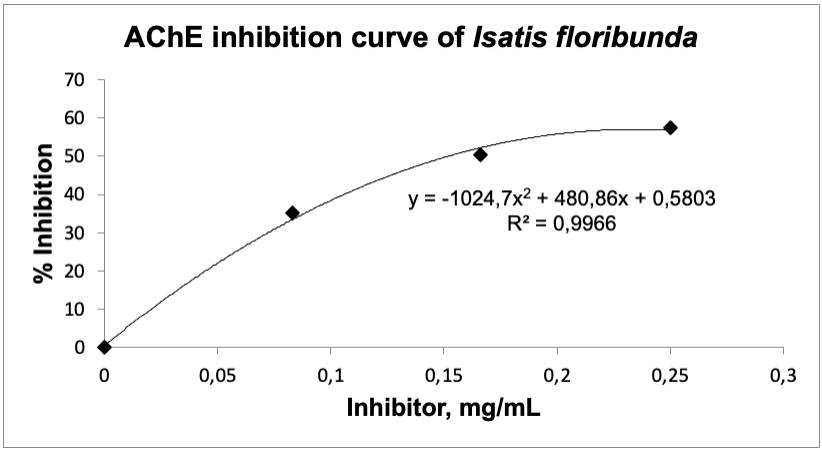

Figure 2. AChE inhibition curve of *Isatis floribunda* methanolic extract

Alzheimer's is the most common neurodegenerative disease and has become a major health issue worldwide. However, there is still no cure for the disease, which makes it one of the top research topics. AChE inhibition is one of the crucial treatment options in treating Alzheimer's disease. However, the use of the current AChE inhibitors is limited due to the side effects[13]. Physostigmine has a narrow therapeutic index and shoer half-life and has numerous side effects, including diarrhea, abdominal cramps, increased sweating, and increased saliva production. Due to these reasons, its user in treating Alzheimer's disease is not approved anymore[14]. Tacrine also has a short half-life and has side effects of nausea, vomiting, clumsiness, and diarrhea. Also, blood monitoring is required during Tacrine treatment because of its hepatotoxic effects. Therefore, its use in Alzheimer's disease is not continued anymore[15,16]. Another AChE inhibitor is Donepezil, which demonstrates side effects of insomnia, loss of appetite, muscle cramps, and muscle weakness. Additionally, it has been detected that those patients under Donepezil treatment have experienced low blood pressure, severe vomiting, breathing problems, and bradycardia. Therefore, its use in Alzheimer's disease is not approved in some countries. Rivastigmine also has similar side effects, including stomach pain, weight loss, diarrhea, loss of appetite, nausea, and vomiting[13]. Lastly, Galantamine is shown to be effective in treating cognitive symptoms of Alzheimer's disease. However, it also has side effects that limit medication use including convulsions, irregular breathing, stomach cramps, watery eyes, and confusion[17]. Due to all these reasons, novel AChE inhibitors are needed to treat Alzheimer's disease.

Natural sources such as plant species have been used to treat several diseases for centuries. As such, extracts, essential oils, and fractions of different plant species, including the families of Acanthaceae, Amaranthaceae, and Amaryllidaceae have been used for their AChE inhibitory effect[18]. As previously mentioned, Rivastigmine and Galantamine are also medications derived from natural sources[19]. In previous studies, *Isatis floribunda* extracts have been tested for its antioxidative, antibacterial, and cytotoxic properties[4]. However, the AChE inhibitory effect of the plant species is still a mystery. Therefore, *Isatis floribunda* extracts have been tested against their AChE inhibitory effect in this study. It has been found that the methanolic extracts have a promising AChE inhibitory effect with the IC50 value of 0.16 mg/mL according to the equation obtained from the AChE inhibitory curve. Previous studies have shown that the chemical composition of methanolic extracts of *Isatis floribunda* contains catechin, chlorogenic acid, caffeic acid, quercetin, and p-coumaric acid[4]. Therefore, it has been believed that the AChE inhibition of *Isatis floribunda* extract is due to these compounds[20-23]. Additionally, the essential oil composition of *Isatis floribunda* was demonstrated to be rich in fatty acids, which are also potent AChE inhibitors[24,25]. Therefore, sufficient *Isatis floribunda* essential oil could also demonstrate an AChE inhibitory effect.

This study determined the AChE inhibitory effect of *Isatis floribunda* extracts. Methanolic extracts of the plant species have demonstrated strong AChE inhibition with the IC_{50} of 0.16 mg/mL, which shows that it is a promising plant material for developing new AChE inhibitors. Additionally, it has been shown that the essential oil composition of *Isatis floribunda* is rich in compounds which are potentially effective AChE inhibitors. Therefore, even though the essential oil yield was insufficient, it is believed that the essential oil of the plant species could be a promising AChE inhibitor in the future. In conclusion, *Isatis floribunda* is a promising plant species in developing novel AChE inhibitors in the treatment of Alzheimer's disease.

STATEMENT OF ETHICS

Ethics approval is not required in this study, as no human and experimental animal samples are not involved.

CONFLICT OF INTEREST STATEMENT

Declared none.

AUTHORS CONTRIBUTIONS

Concept: E.G., Y.Y., K.P., Design: E.G., Y.Y., K.P., Data Collection and Processing: E.G., Y.Y., M.A., K.P., Analysis or Interpretation: E.G., Y.Y., K.P., Literature Search: E.G., Writing: E.G.

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REFERENCES

1. Nair RR, Kalariya T, Chanda S. Antibacterial activity of some selected Indian medicinal flora. Turk J Biol, 2005;29(1):41-47.

2. Dar MI, Khan FA, Rehman F, Masoodi A, Ansari AA, Varshney D, Naushin F, Naikoo MI. Roles of Brassicaceae in phytoremediation of metals and metalloids. Phytoremediation, 2015(1):201-215. Doi: 10.1007/978-3-319-10395-2_14

3. Soundararajan P, Kim J. Anti-carcinogenic glucosinolates in cruciferous vegetables and their antagonistic effects on prevention of cancers. Molecules, 2018;23(11)2983. Doi: 10.3390/molecules23112983

4. Karakoca K, Asan Ozusaglam M, Cakmak YS, Karaman Erkul S. Antioxidative, antimicrobial and cytotoxic properties of *Isatis floribunda* Boiss. ex Bornm. extracts. EXCLI J, 2013;(12):150-167.

5. Radwan HM, Shams KhA, Tawfik WA, Soliman AM. Investigation of the glucosinolates and lipids constituents of *Cakile maritima* (Scope) growing in Egypt and their biological activity. Res J Med Med Sci, 2008;3(2):182-187.

6. Cavdar H, Senturk M, Guney M, Durdagi S, Kayik G, Supuran CT, Ekinci D. Inhibition of acetylcholinesterase and butyrylcholinesterase with uracil derivatives: kinetic and computational studies. J Enzyme Inhib Med Chem, 2019;34(1):429-437. Doi: 10.1080/14756366.2018.1543288

7. Adewusi EA, Moodley N, Steenkamp V. Antioxidant and acetylcholinesterase inhibitory activity of selected Southern African medicinal plants. South Afr J Bot, 2011;77(3):638-644. Doi: 10.1016/j.sajb.2010.12.009.

8. Fu AL, Li Q, Dong ZH, Huang SJ, Wang YX, Sun MJ. Alternative therapy of Alzheimer's disease via supplementation with choline acetyltransferase. Neurosci Lett, 2004;368(3):258-262. Doi: 10.1016/j.neulet.2004.05.116

9. Dave KR, Syal AR, Katyare SS. Tissue Cholinesterases. A comparative study of their kinetic properties. Z Für Naturforschung C, 2000;55(1-2):100-108. Doi: 10.1515/znc-2000-1-219

10. Dïken ME, Yilmaz B. Inhibitory effect on acetylcholinesterase and toxicity analysis of some medicinal plants. Int J Second Metab, 2022;9(1):27-42. Doi: 10.21448/ijsm.1032863

11. Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol, 2013;11(3):315-335. Doi: 10.2174/1570159X11311030006

12. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, 1961;7(2):88-95. Doi: 10.1016/0006-2952(61)90145-9

13. Sharma K. Cholinesterase inhibitors as Alzheimer's therapeutics (Review). Mol Med Rep, 2019;20(2):1479-1487. Doi: 10.3892/mmr.2019.10374

14. Thal LJ, Fuld PA, Masur DM, Sharpless NS. Oral physostigmine and lecithin improve memory in Alzheimer disease. Ann Neurol, 1983;13(5):491-496. Doi: 10.1002/ana.410130504

15. Farlow M, Gracon SI, Hershey LA, Lewis KW, Sadowsky CH, Dolan-Ureno J. A controlled trial of tacrine in Alzheimer's disease. JAMA, 1992;268(18):2523-2529. Doi: 10.1001/jama.1992.03490180055026

16. Watkins PB. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. JAMA, 1994;271(13):992. Doi: 10.1001/jama.1994.03510370044030

17. Mehta M, Adem A, Sabbagh M. New acetylcholinesterase inhibitors for Alzheimer's disease. Int J Alzheimers Dis, 2012;2012:1-8. Doi: 10.1155/2012/728983

18. Murray A, Faraoni M, Castro M, Alza N, Cavallaro V. Natural AChE inhibitors from plants and their contribution to Alzheimer's disease therapy. Curr Neuropharmacol, 2013;11(4):388-413. Doi: 10.2174%2F1570159X11311040004

19. Devi P, Syad AN. Botanics: a potential source of new therapies for Alzheimer's disease?. Bot Targets Ther, 2014;2014(4):11-26. Doi: 10.2147/BTAT.S33554

20. Oboh G, Agunloye OM, Akinyemi AJ, Ademiluyi AO, Adefegha SA. Comparative study on the inhibitory effect of caffeic and chlorogenic acids on key enzymes linked to Alzheimer's disease and some pro-oxidant induced oxidative stress in rats' brain-*in vitro*. Neurochem Res, 2013;38(2):413-419. Doi: 10.1007/s11064-012-0935-6

21. Okello EJ, Mather J. Comparative kinetics of acetyl- and butyryl-cholinesterase inhibition by green tea catechins |relevance to the symptomatic treatment of Alzheimer's disease. Nutrients, 2020;12(4):1090. Doi: 10.3390/nu12041090

22. Orhan IE. Cholinesterase inhibitory potential of quercetin towards Alzheimer's disease - a promising natural molecule or fashion of the day? - a narrowed review. Curr Neuropharmacol, 2021;19(12):2205-2213. Doi: 10.2174%2F1570159X18666201119153807

23. Ouattara N, Tiero Meda RN, Hilou A, Guenne S, Konate K, Coulibaly AY, Kiendrebeogo M, Millogo JF, Nacoulma OG. Anti-acetylcholinesterase and antioxidant activities and HPLC-MS analysis of polyphenol from extracts of *Nelsonia canescens* (Lam.) Spreng. Asian Pac J Trop Di, 2013;3(5):382-388. Doi: 10.1016%2FS2222-1808(13)60088-2

24. Akay MB, Şener K, Sari S, Bodur E. Inhibitory action of omega-3 and omega-6 fatty acids alpha-linolenic, arachidonic and linoleic acid on human erythrocyte acetylcholinesterase. Protein J, 2023;42(2):96-103. Doi: 10.1007/s10930-022-10088-z

25. Itriago Yanes CV, Melo Filho AA, Ribeiro PRE, Reis Melo ACG, Takahashi JA, Ferraz VP, Mozombite DMS, Santos RC. Inhibition of acetylcholinesterase and fatty acid composition in *Theobroma grandiflorum* seeds. Orbital: Electron J Chem, 2017;9(3):127-130. Doi: 10.17807/orbital.v0i0.894

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Exploring the correlation between ibuprofen solubility and permeability in intestinal disease conditions

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ABSTRACT

In this study, physicochemical properties of Ibuprofen were evaluated using HPLC-DAD system to examine the effect of pH on the solubility and intestinal permeability of Ibuprofen. Ibuprofen is still one of the most used and safest non-selective NSAIDs among those approved. Ibuprofen solubility is dramatically affected by pH changes. There is not enough data to show if pH dependent water solubility of ibuprofen will affect its absorption/bioavailability. For this purpose, lipophilicity parameters (logkw) of ibuprofen were determined at various pH ranges using HPLC-DAD system and the results were compared with in-vivo intestinal permeability results obtained where a perfusion medium having different pH values used (pH 3.9, 4.9 and pH 7.4). The results confirmed that the acidic pH of the perfusion medium increased the permeability of ibuprofen,

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but in basic pH values (pH 7.4) the permeability was relatively lower. The permeability results were in correlation with determined lipophilicity.

Keywords: Ibuprofen, intestinal permeability, HPLC diffusion coefficient, acid dissociation constant, drug absorption

INTRODUCTION

Nonsteroidal anti-inflammatory drugs [NSAIDs] are a drug class FDA-approved for use as antipyretic, anti-inflammatory, and analgesic agents. These effects make NSAIDs useful for the treatment of muscle pain, dysmenorrhea, arthritic conditions, pyrexia, gout, migraines, and used as opioid-sparing agents in certain acute trauma cases[1,2]. Ibuprofen (IBU) is still one of the most commonly used and safest non-selective NSAIDs among others, and its use for various diseases beyond its primary purpose has been reported[3-6]. These are necrosis, bronchopulmonary dysplasia, cardiovascular diseases, neurodegenerative diseases and cancer prevention[7-11]. The other interesting thing about IBU is its common usage as an OTC medicine[9,12]. However, some studies show that IBU is not safe when it is used over-dose or in regular daily usage[9,13-16]. A basic search on Rxlist [www.rxlist.com] shows that there are more than fifty different formulations of IBU marketed in the USA and it is easy to reach as an OTC for a wide population. The chemical structure of IBU is shown in Figure 1.

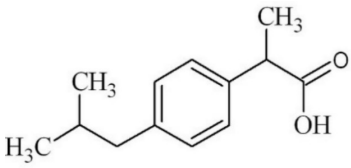

Figure 1. Chemical structure of Ibuprofen

A study published in 2005 by Savolainen et al. about the brain delivery of IBU presents that IBU concentration in both plasma and brain reached a steady state within 6 h. This study proves that limited brain penetration prevents the possible usage of IBU in treating or preventing neurodegenerative disorders such as Alzheimer's disease[17]. Mainly, these studies concentrated on the clinical aspects of NSAID use and its impact on gastrointestinal complications.

The main focus of our current study is to evaluate the use of an NSAID in cases involving certain gastrointestinal complications. These complications include conditions such as inflammatory bowel diseases. This evaluation is essential because the pH level in the bowels undergoes an acidic shift under such cir-

cumstances. This shift in pH can potentially result in unexpected pharmacokinetic profiles for IBU. These unexpected profiles may occur due to changes in the permeability of this weakly acidic drug. Essentially, the intraluminal pH of the gastrointestinal tract is variable. In healthy subjects, it is from highly acidic pH in the stomach (pH 1.5 – 3.5) to about pH 6.0 in the duodenum. The pH gradually increases in the small intestine from pH 6.0 to about pH 7.4 in the terminal ileum. Although the pH drops to 5.7 in the caecum, it gradually increases, reaching a pH 6.7 in the rectum[18]. However, as mentioned, intraluminal pH may vary in a disease state. For example, very low intraluminal colonic pH was reported for ulcerative colitis (pH 3.0 – 4.5) which is a form of inflammatory bowel disease[19]. The pKa value for IBU is 4.9 and in the disease conditions where the pH of the column is dramatically lower in comparison to the normal conditions, the solubility of the IBU is affected. When the solubility is affected, we assume that the permeability, and by that way, the pharmacokinetic profile will also be changed.

As a group working on pharmacokinetics and analytical method development, we primarily focused on the changes in the intestinal permeability of IBU in a case of a gastrointestinal complication. In this paper, an intestinal permeability study was performed in two different conditions and one of them refers to the healthy intestine [pH: 7.4 and 5.9] and the other [pH 3.9] refers the inflammatory bowel disease. pH 5.9 and pH 3.9 also refer the pKa ± 1 for IBU (pKa 4.9). To prove the dramatic solubility changes of IBU [a weakly acidic drug] in various pH, an *in vitro* experimental approach was designed and Log kw [20] for IBU was determined for pH 4.5, 5.5, 6.5 and 7.5. The results obtained from *in vitro* Log kw studies for the solubility of IBU depending on pH of the medium were compared with the results of the intestinal permeability of IBU while simulating a disease condition changing the pH of the colon.

METHODOLOGY

Chemicals

IBU, Metoprolol tartrate (MET), Phenol red (PR) was supplied from Sigma Aldrich (St. Louis, MO, USA). Sodium phosphatedibasic (NaH_2PO_4) and NaOH were from Merck (Darmstadt, Germany), disodium monohydrogenphosphate (Na_2HPO_4), potassium chloride (KCl), Sodium hydroxide (NaCl), Sodium Sulfate (Na_2SO_4), Sodium bi carbonate ($NaHCO_3$), Mannitol, Acetonitrile (ACN), Methanol (MeOH), ortho phosphoric acid (purity > 99%). 1-octanol was obtained from Sigma Aldrich. HPLC grade water was purchased from Carlo Erba (France) and it was used for the preparation of standard solutions and buffers. All solutions were of analytical grade.

Instrumentation

The chromatographic separation of all samples was carried out using an HPLC system (Shimadzu, Nexera–*i*, LC–2040C 3D Model, JAPAN) that was coupled to a Shimadzu Nexera–*i* 2040C 3D Model (Model, JAPAN) UV/DAD detector. Chromatographic separations were carried out on a C18 (150 mm 4.6 mm, 2.7 μm particle size) column (Restek Raptor ™) for *logkw* studies and C18 (250 mm x 460 mm, 5 μm particle size) column (Supelco) with a pH range of 3-6.50 and isocratic separation with a mobile phase consisting of 20 mM phosphate buffer solution (PBS): ACN (55:45, v/v) at a flow rate of 1 mL min^{-1}. This pH range is also used for the assessment of IBU's pKa value and permeability studies. The wavelength of the detector was set to 220 nm and 254 nm, and the retention times were determined automatically by an online computer running Shimadzu LabSolution software. The injection volume was 10 μL. The delivery of the perfusion medium to the jejunum was accomplished using a peristaltic pump. The perfusion medium was administered to the jejunum with the help of a Gilson Minipuls 3 peristaltic pump (USA).

Preparation of the standard stock solutions, buffer solutions, mobile phases and perfusion medium

Standard stock solution of Ibuprofen, metoprolol tartrate and phenol red (1000 μg mL^{-1} in MeOH)

Standard stock solution was prepared by dissolving 25 mg of IBU, 25 mg of PR and 25 mg MET in 20 mL volumetric flasks. All solutions were stored at 4°C in the fridge during experiments. Each standard was prepared in 75% (v/v) MeOH for IBU, MeOH for PR and Milli Q water for MET, respectively.

20 mM Phosphate buffer solution (PBS)

PBS was prepared by dissolving 2.84 g of Na_2HPO_4 in approximately 800 mL water and then increasing the volume to 1000 mL with water once the disodium Na_2HPO_4 solution was completely dissolved.

20 mM PBS:MeOH (from 60:40 v/v to 30:70 v/v) solutions (pH 4.50 –7.50) for log kw determination using HPLC

PBS and MeOH in a range between 60:40 v/v and 30:70 v/v were mixed to reach 500 mL final volume. The pH of the mobile phases (60:40 v/v and 30:70 v/v) was arranged to 4.5, 5.5, 6.5 and 7.5.

Perfusion medium

The perfusion medium contains 25 mM NaCl, 10 mM KCl, 40 mM Na_2SO_4, 20 mM $NaHCO_3$ and 80 mM mannitol. O-phosphoric acid was utilized to modify the pH of the buffer depending on the process. Perfusion medium was made fresh and filtered through a 0.22 μm membrane filter before being used in the experiment.

Procedures

Method optimization

MET which is used as reference standard and PR (zero permeability marker) are commonly used compounds in Single Pass Intestinal Perfusion (SPIP) technique. For this purpose, IBU, MT and PR mixture solution were used for optimization studies. After some preliminary studies, the mobile phase decided to be as (pH 6.61 10 mM Na2HPO4:ACN)/ (45/55,v/v). Flow rate was set at 0.8 ml min^{-1} and wavelength at 220 nm. Column temperature was adjusted 25°C and injection volume was 10 μL. under these conditions, the obtained chromatogram of IBU, MET and PR is shown in Figure 2.

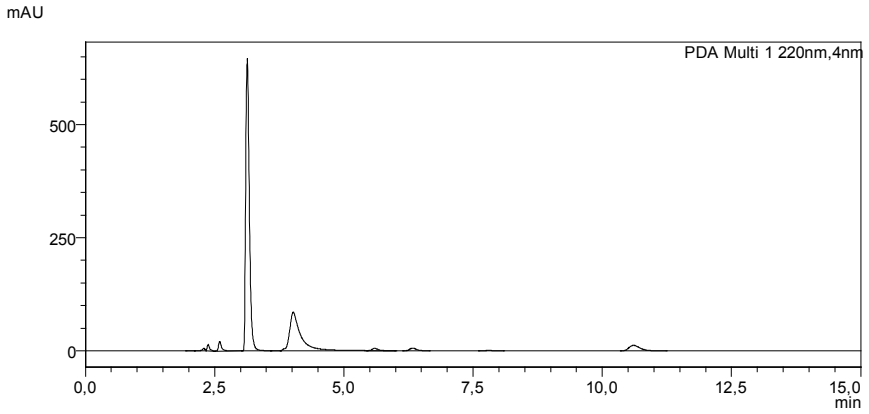

Figure 2. Representative chromatogram of PR, MT and IBU using HPLC-UV/DAD system

System suitability testing (SST) was investigated including resolution (R_s), tailing factor (T), capacity factor (*k'*), asymmetry factor (A_s), selectivity (α) and theoretical plate number (N) to decide optimal conditions for HPLC-UV/DAD method. Obtained SST results was shown in Table 1.

Table 1. System Suitability Testing results of applied method

System Suitability Testing				
Parameter	**IBU**	**MTP**	**PR**	**Recommended value**
Retention time (min)	10.727	3.977	3.149	-
Theorical plate number (N)	5114.1	2875.7	4174.8	N > 2000
Tailing factor (T)	1.406	1.92	1,478	T < 2
Asymmetry factor (A_s)	1.04	1.11	1	0.95 < As <1.2
Capacity factor (k')	3.732	0.754	0.389	k > 2
Resolution (R_s)	18.08	3.509	6.13	Rs > 2
Selectivity (α)	4.94	1.94		α > 1

Method validation

Applied HPLC-UV method was validated according to the International Conference on Harmonization guidelines ICH Q2(R1) to evaluate the quality of the analytical method [21] and also the method was validated for the linearity, LOD, LOQ, accuracy, precision, specificity and robustness. LabSolution software (Shimadzu Corporation) was used to monitor all data and integrated all of the chromatograms. All results were given as mean ± standard deviation for three replicates (n=3) of the samples. MS Excel 2007 was used for data analysis (Microsoft Corporation, USA) for data analysis.

Linearity, LOD and LOQ

The linearity of the IBU, MET and PR was evaluate using nine-point calibration point for each compound within the range of 0.5-80 μg mL^{-1}, 4.0-160 μg mL^{-1}, 7.0-210 μg mL^{-1} respectively. Three calibration sets were prepared and tested in triplicate for each compound. The obtained regression equation values are showed that good linear relationship has been achieved. Linearity, LOD and LOQ values are shown in Table 2. LOD and LOQ were determined using the 3.3 and 10 standard deviations (SD) of the achieved detector response (σ) to slope of the calibration curve (m), respectively. "σ" was calculated using the standard deviation of regression lines' y-intercepts.

Table 2. Linearity, range, LOD and LOQ results

Parameter	IBU	MTP	PR
Equation	y =25890x-4961.8	y =19562x-30960	y =43257x-83916
SE of intercept	184.972	496.366	2630.199
SE of slope	22.704	138.870	164.852
R^2	1.0000	0.9998	0.9996
Range (μg mL^{-1})	1-75	4-160	7-210
LOD (μg mL^{-1})	0.041	0.084	0.348
LOQ (μg mL^{-1})	0.124	0.439	1.053

Accuracy and precision

The method's accuracy was assessed using a recovery test, which compares the theoretical concentration of the chemicals with the experimental concentration. Three different concentrations of IBU (2, 200 and 50 μg mL^{-1}), MET (4, 20 and 80 μg mL^{-1}) and PR (7, 35 and 140 μg mL^{-1}) samples were prepared, and each was analyzed triplicate in same day. Recovery was calculated from the Eq (1).

$$Recovery\ (\%) = (Observed\ amount) - (original\ amount) / (Spiked\ amount) \times 100 \quad Eq\ (1)$$

To show precision of the applied method *intra-day* and *inter-day* variability studies were investigated. The precision data was shown with relative standard deviation (RSD%) which is calculated by Eq (2).

$$RSD\% = SD / Mean \times 100 \quad Eq\ (2)$$

All solutions were injected to the HPLC-UV/DAD system three times on the same day and, three consecutive days. According to the recovery results, the method was found accurate for IBU (98.29-104.17%), MET (97.27-119.85%), PR (98.52-117.70%). Results showed that a good accuracy and precision values are achieved (Table 3).

Table 3. Accuracy and precision results

Compound	Main value (µg mL^{-1})	*intra-day*			*inter-day*		
		Found value (µg mL^{-1})[a]	Precision (RSD %)[b]	Accuracy (recovery %)[c]	Found value (µg mL^{-1})[a]	Precision (RSD %)[b]	Accuracy (recovery %)[c]
IBU	2	2.088 ± 0,008	0.663	104.407	2.083 ± 0.003	0.278	104.17
	10	9.807 ± 0,004	0.076	98.067	9.829 ± 0.011	0.197	98.29
	50	50.202 ± 0,001	0.005	100.403	50.284 ± 0.042	0.145	100.56
MET	4	4.779 ± 0,006	0.234	119.486	4.794 ± 0.083	2.987	119.85
	20	19.427 ± 0,019	0.168	97.136	19.455 ± 0.018	0.157	97.27
	80	81.362 ± 0,608	1.294	101.703	81.159 ± 0.102	0.217	101.44
PR	7	8.222 ± 0,012	0.251	117.457	8.239 ± 0.010	0.211	117.70
	35	34.412 ± 0,061	0.309	98.319	34.482 ± 0.033	0.202	98.52
	140	137.575 ± 0,042	0.053	98.268	138.114 ± 0.223	0.343	98.65

[a] Mean ± Standard Error, [b] RSS, Relative Standard Deviation, [c] Recovery % = [(Observed amount) – (original amount) /Spiked)/] × 100, (n=6).

pKa determination of IBU

Standard stock solution of IBU was prepared as 10.0 µg mL^{-1} s for pKa determination with HPLC at the range of pH 3.00 – 6.50. Based on pH and capacity factor (k') of IBU, a sigmoidal curve was produced. The sigmoidal relationship was used to determine the pKa of IBU.

pKa determination of Ibuprofen using HPLC

Standard solution of 10.0 µg mL^{-1} IBU was investigated in different pH values (3.00, 3.50, 3.85, 4.20, 4.50, 4.85, 5.20, 5.50, 5.85, 6.20 and 6.50) with containing 20 mM PBS: ACN (55:45 v/v) mobile phase. Uracil was used to show dead volume in the analysis. Retention time of IBU was changed by changing pH of the PBS and obtained chromatograms of IBU is given in Figure 3.

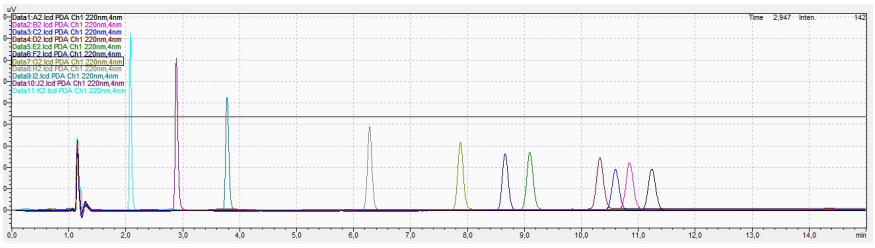

Figure 3. Obtained overlaid chromatograms using HPLC in different mobile phase pH

The data measured from sigmoidal curve for pKa determination were found using derivative of the graphic equation. A sigmoidal curve was constructed between the pH of the solutions and capacity factor (k') of IBU. The pKa of IBU was determined according to the sigmoidal relation.

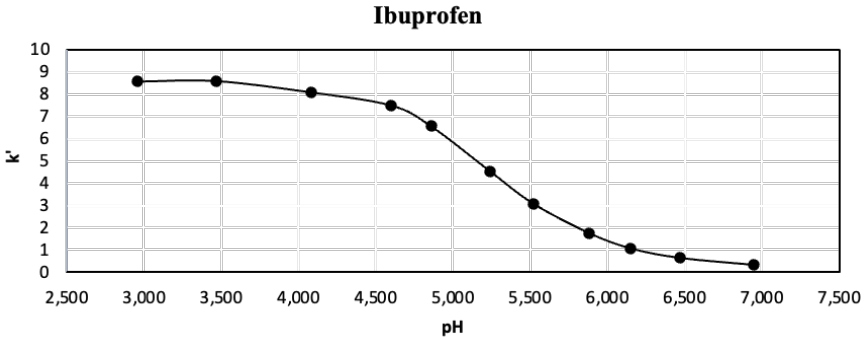

Figure 4. Obtained sigmoidal curve for determination of pK_a value of IBU

The sigmoidal curve was drawn (Figure 4) and pKa values of IBU was calculated as 5.20. Our pKa result is in accordance with the obtained results in the literature[22].

log kw determination

IBU was diluted to 5.0 μg mL^{-1} by using 20 mM PBS: MeOH (from 60:40 v/v to 30:70 v/v) solutions for log *kw* determination at pH 4.50, 5.50, 6.50 and 7.50. The relationship between log k' and methanol concentration in the mobile phase is well known in HPLC theory[20,23]. It is described with Equation 3 where k_w shows the k' value for a compound when aqueous phase is used as eluent, S is the slope of the regression curve, and φ is the volume percentage of methanol in the mobile phase. If the φ is zero, which means that there is no MeOH in the mobile phase and the mobile phase has consisted of only the phosphate buffer, the log k' will be equal to the log k_w.

$$\log k' = \log kw - S\varphi \quad (Eq.\ 3)$$

Intestinal absorption studies

Animals

All animals used in absorption studies were kept and handled according to Anadolu University's Committee on Animal Use and Care's regulations with the protocol number 2019/2. Before each trial, female Sprague Dawley albino rats (250-300 g) were fasted overnight (for around 12-18 h) with free access to tap water.

SPIP protocol and experimental groups

According to previously published research, an *in situ* single pass intestinal perfusion operation was performed[24-27]. It was decided to use pentobarbital (60 mg/kg) as an anesthetic and administer it intraperitoneally. A tiny midline incision (3-4 cm) was made in the abdomen, and 10 cm of the jejunum was isolated and carefully cannulated before the rest of the procedure was completed. The cannulation of the jejunal segment was accomplished using flexible PVC tubing (inlet tubing with an internal diameter (id) of 0.76 mm and exit tubing with an id of 1.70 mm). The perfusion pump was then connected to the tubings (Minipuls 3, USA). To remove any remaining debris, the jejunum was washed with blank perfusion media at a flow rate of 0.4 mL.min^{-1} for 15 minutes at room temperature. Perfusion tests were carried out at four different pH levels as Group 1; pH=7.4, Group 2; pH=5.9 (pH=pK_a+1), Group 3; pH=3.9 (pH=pK_a-1). An initial perfusion solution was perfused through the exposed segment at a flow rate of 0.2 mL min^{-1} for 60 min for each group, comprising the test drug (IBU) and the reference substances MET and PR.

Quantification of the absorption samples

The amounts of IBU and reference substances dissolved in the perfusion medium, that passed through the rat gut were determined using validated HPLC method. The peak areas of IBU, MET and PR were measured at two different wavelengths, 220 nm and 254 nm, respectively. The calibration curves for IBU (1-75 μg mL^{-1}), PR (7-210 μg mL^{-1}), and MET (4-160 μg mL^{-1}) were created by diluting stock solutions of compounds with mobile phase.

Data analysis

To determine the drug's effective permeability (Peff), the C_{out}/C_{in} ratio was corrected for water transport using Equation 4[28].

$$\left[\frac{C_{out}}{C_{in}}\right]' = \left[\left(\frac{C_{out}}{C_{in}}\right).\left(\frac{C_{in.Phenol.Red}}{C_{out.Phenol.Red}}\right)\right] \qquad \textit{(Eq. 4)}$$

$C_{in.Phenol.Red}$ indicates the PR concentration at the inlet, whereas $C_{out.Phenol.Red}$ indicates the PR concentration at the outflow. The effective permeability (P_{eff}) values of the medication were determined through the rat gut wall using the "plug flow" model described in Equation 5[29].

$$P_{eff} = \frac{-Q \ln\left[\frac{C_{out}}{C_{in}}\right]'}{2\pi r l} \qquad \textit{(Eq 5.)}$$

where Q is the flow rate of perfusion solution (mL.sec^{-1}); $\left[\frac{C_{out}}{C_{in}}\right]'$ is the corrected drug concentration ratio of the outlet to inlet concentration (Equation 3). "r" is the radius of the perfused intestinal segment (for jejunum r = 0.2 cm) and "l" is the length of the intestinal segment (cm)[30-32]

The net water flux (NWF) values in the *in-situ* perfusion studies (water absorption and efflux in the intestinal segment) were calculated based on inlet ($C_{in.Phenol.Red}$), outlet ($C_{out.Phenol.Red}$) concentrations of PR and (Q_{in}) the inlet perfusate flux using the following Equation 6:

$$NWF[\frac{\mu L}{h.cm}] = \frac{\left[1-\left(\frac{C_{out.Phenol.Red}}{C_{in.Phenol.Red}}\right)\right].Q_{in}}{l} \qquad (Eq.\ 6)$$

A negative net water flux indicates loss of fluid from the mucosal side (lumen) to the serosal side (blood). A positive net water flux indicates the secretion of fluid into the segment[29].

RESULTS and DISCUSSION

For determination of log kw values, IBU (10 µg mL^{-1}) and uracil solutions (5 µg mL^{-1}) diluted with water were analyzed using the mobile phase containing various ratios [70:30, 60:40, 50:50, 40:60;v/v] of MeOH and 20 mM phosphate buffer solutions (pH 4.5, 5.5. 6.5, and 7.5). The detection wavelength was 220 nm and the injections were performed triplicate. log k' values were extrapolated from binary eluents to 100% aqueous solutions to estimate the log kw values (Table 4). When the equation 1 was used, log kw values were found as 4.26, 3.74, 3.31, and 3.02. Based on the procedure described in experimental section, calculated P_{eff} values are shown in Table 5 and Figure 5.

Table 4. Obtained results for k' values

Mobile phase ratio (%*v-v*) (MeOH-PBS)		k' values			
		pH 4.5	pH 5.5	pH 6.5	pH 7.5
IBU/Uracil	70-30	2.97	1.4	0.97	0.74
	60-40	10.56	4.8	3.18	2.1
	50-50	35.8	14.82	8.6	5.90
	40-60	>85	-	-	-

Table 5. Obtained P_{eff} values of IBU and MTP from SPIP studies

	P_{eff} value(x10^{-4} cm/sn) (Mean±SD)		
Compounds	**pH 7.4**	**pH 5.9**	**pH 3.9**
MTP	0.564 ± 0.384	0.389 ± 0.210	0.277 ± 0.050
IBU	0.816 ± 0.501	1.821 ± 1.297	4.309 ± 0.328

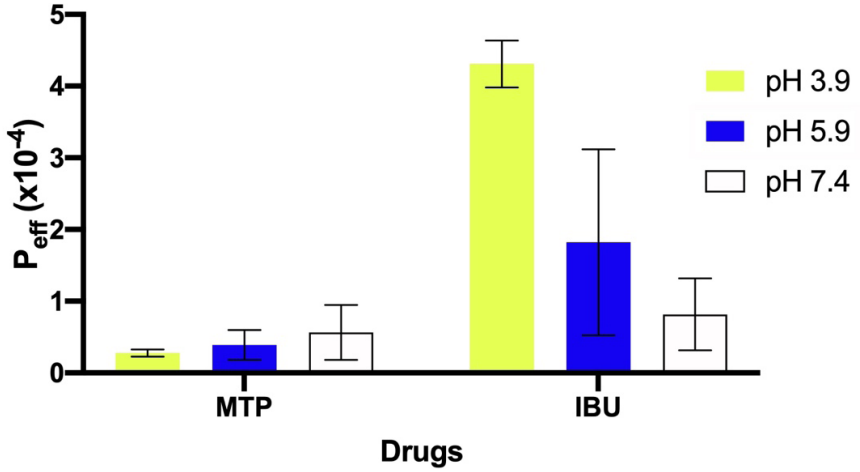

Figure 5. P_{eff} value of Intestinal permeability studies

The results of the *in vivo* intestinal permeability study performed at pH 3.9, 5.9 and 7.4 showed that there was a correlation between pH dependent solubility change of IBU and intestinal permeability values. The relationship was linear for both pH-log kw (y= -0.421x + 6.10, R^2=0.987 where x: pH and y: log kw) and pH-P_{eff} (y=-1.10x + 8.10, R^2=0.975 where x: pH and y: P*eff*) and this situation allowed us to suggest that the permeability of IBU as a result of passive diffusion was directly corelated with the pH of the medium. As it is reported by DrugBank [https://go.drugbank.com/], MET is a basic compound with a pKa value of 9.67 and IBU is an acidic drug and pKa value is 4.9. The reason why the trend of the permeability of IBU was increased and MET was decreased for pH 4.5 to 7.5, respectively can be easily explained by their pKa defining ionization percentage in a different medium having different pH values. The relationship between pH dependent lipophilicity of a compounds and pKa can be described with Log D which is distribution coefficient widely used to measure the lipophilicity of ionizable compounds. The acidic characteristics of IBU (pKa: 4.9) allow its lipophilicity to increase dramatically in acidic medium (pH

3.9), but above its pKa value, its lipophilicity decreases noticeably. This situation was confirmed in log kw studies, since log kw is related to the lipophilicity of a compound as reported in previous studies [33-36]. The basic characteristics of MET (pKa: 9.67) make MET behave the opposite of IBU based on pH changes of the medium. However, the pKa value of MET was relatively higher than the pH of the medium (pH 3.9, 5.9 and 7.4) we used in intestinal permeability studies. This situation caused the change of MET permeability relatively less than the permeability of IBU based on pH changes of the medium. The brief results of our study showed that *in-vitro* consideration of the lipophilicity of the drugs and/or drug candidates ascertains proper information on permeability related to passive diffusion. Since intestinal permeability may change dramatically as a result of the pH changes in the medium, some diseases like inflammatory bowel disease transforming the pH of the colon may cause noticeable changes in pharmacokinetic profiles of drugs. This situation must be considered especially for drugs having pKa values between 3.0 and 7.0.

In this study, the intestinal permeability of an NSAID, IBU, was evaluated where the pH of the perfusion medium varies from 3.9 to 7.4. The acidic pH of the perfusion mediums is to simulate a disease like inflammatory bowel disease causing a dramatic change in the colon in some cases. The results showed that the permeability of IBU changed considerably from pH 3.9 to 7.4. This situation was mainly associated with the changes in the lipophilicity of the IBU. The *in-vitro* results (log kw studies) also confirmed the changes of the lipophilicity using HPLC. Our data suggest that the pH changes in the colon as a result of the disease may cause a difference in the pharmacokinetic profiles of the drug and this situation related to the passive diffusion profile for the drug can be predicted using log kw experiments.

STATEMENT OF ETHICS

Ethical approval has been approved for this research. [All animals used in absorption studies were kept and handled according to Anadolu University Committee on Animal Use and Care's regulations with the protocol number as 2019/2.]

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS CONTRIBUTIONS

Mustafa Sinan Kaynak: Project administration, Funding acquisition Visualization, Formal analysis, Data curation, Investigation, Software, Conceptualization, Methodology, Writing- Original draft, Writing- Reviewing and Editing. Murat Soyseven, Berna Kaval, Mustafa Çelebier, Emrah Akgeyik, Selma Şahin, Göksel Arli: Visualization, Formal analysis, Data curation, Investigation, Validation, Software, Methodology, Writing- Original draft preparation, Writing-Reviewing and Editing.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Day RO, Graham GG. Non-steroidal anti-inflammatory drugs [NSAIDs]. BMJ, 2013;346:f3195. Doi: https://doi.org/10.1136/bmj.f3195

2. Rainsford K. Ibuprofen: pharmacology, efficacy and safety. Inflammopharmacology, 2009;17(6):275-342. Doi: https://doi.org/10.1007/s10787-009-0016-x

3. Zarghi A, Arfaei S. Selective COX-2 inhibitors: a review of their structure-activity relationships. Iran J Pharm Res: IJPR, 2011;10(4):655. Doi: https://doi.org/10.22037/ijpr.2011.1047

4. Moore RA, Derry S, Straube S, Ireson-Paine J, Wiffen PJ. Faster, higher, stronger? Evidence for formulation and efficacy for ibuprofen in acute pain. PAIN®, 2014;155(1):14-21. Doi: https://doi.org/10.1016/j.pain.2013.08.013

5. Bushra R, Aslam N. An overview of clinical pharmacology of Ibuprofen. Oman Med J, 2010;25(3):155. Doi: https://doi.org/10.5001/omj.2010.49

6. Green GA. Understanding NSAIDs: from aspirin to COX-2. Clin Cornerstone, 2001;3(5):50-59. Doi: https://doi.org/10.1016/S1098-3597(01)90069-9

7. Wells LK, Drum M, Nusstein J, Reader A, Beck M. Efficacy of ibuprofen and ibuprofen/acetaminophen on postoperative pain in symptomatic patients with a pulpal diagnosis of necrosis. J Endod, 2011;37(12):1608-1612. Doi: https://doi.org/10.1016/j.joen.2011.08.026

8. Raju NV, Bharadwaj RA, Thomas R, Konduri GG. Ibuprofen use to reduce the incidence and severity of bronchopulmonary dysplasia: a pilot study. J Perinatol, 2000;20(1):13-16. Doi: https://doi.org/10.1038/sj.jp.7200296

9. Moore N, Salvo F, Duong M, Blin P, Pariente A. Cardiovascular risks associated with low-dose ibuprofen and diclofenac as used OTC. Expert Opinion on Drug Safety. 2014;13(2):167-179. Doi: https://doi.org/10.1517/14740338.2014.846324

10. Pasqualetti P, Bonomini C, Dal Forno G, Paulon L, Sinforiani E, Marra C, et al. A randomized controlled study on effects of ibuprofen on cognitive progression of Alzheimer's disease. Aging Clin Exp Res, 2009;21(2):102-110. Doi: https://doi.org/10.1007/BF03325217

11. Harris RE, Beebe-Donk J, Doss H, Doss DB. Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade. Oncol Rep, 2005;13(4):559-583. Doi: https://doi.org/10.3892/or.13.4.559

12. Sinclair HK, Bond CM, Hannaford PC. Over–the-counter ibuprofen: how and why is it used? Int J Pharm Pract, 2000;8(2):121-127. Doi: https://doi.org/10.1111/j.2042-7174.2000.tb00996.x

13. Hall AH, Smolinske SC, Conrad FL, Wruk KM, Kulig KW, Dwelle TL, et al. Ibuprofen overdose: 126 cases. Ann Emerg Med, 1986;15(11):1308-1313. Doi: https://doi.org/10.1016/S0196-0644(86)80617-5

14. Öker EE, Hermann L, Baum CR, Fentzke KM, Sigg T, Leikin JB. Serious toxicity in a young child due to ibuprofen. Acad Emerg Med, 2000;7(7):821-823. Doi: https://doi.org/10.1111/j.1553-2712.2000.tb02278.x

15. Volans G, Monaghan J, Colbridge M. Ibuprofen overdose. Int J Clin Pract, 2003(135):54-60.

16. Mahmud SM, Franco EL, Turner D, Platt RW, Beck P, Skarsgard D, et al. Use of non-steroidal anti-inflammatory drugs and prostate cancer risk: a population-based nested case-control study. PloS one, 2011;6(1). Doi: https://doi.org/10.1371/journal.pone.0016412

17. Mannila A, Rautio J, Lehtonen M, Järvinen T, Savolainen J. Inefficient central nervous system delivery limits the use of ibuprofen in neurodegenerative diseases. Eur J Pharm Sci, 2005;24(1):101-105. Doi: https://doi.org/10.1016/j.ejps.2004.10.004

18. Fallingborg J. Intraluminal pH of the human gastrointestinal tract. Dan Med Bul, 1999;46(3):183-196.

19. Fallingborg J, Christensen LA, Jacobsen BA, Rasmussen SN. Very low intraluminal colonic pH in patients with active ulcerative colitis. DDS, 1993;38(11):1989-1993. Doi: https://doi.org/10.1007/BF01297074

20. Hong H, Wang L, Zou G. Retention in RP-HPLC: Lipophilicity determination of substituted biphenyls by reversed-phase high performance liquid chromatography. J Liq Chromatogr R T, 1997;20(18):3029-3037. Doi: https://doi.org/10.1080/10826079708006578

21. Guideline IHT. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1(20)5.

22. National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 3672 IRJ, 2022. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Ibuprofen

23. Braumann T. Determination of hydrophobic parameters by reversed-phase liquid chromatography: theory, experimental techniques, and application in studies on quantitative structure-activity relationships. J Chromatogr A, 1986;373:191-225. Doi: https://doi.org/10.1016/S0021-9673(00)80213-7

24. Sinan Kaynak M, Celebier M, Akgeyik E, Sahin S, Altınoz S. Application of HPLC to investigate the physicochemical properties and intestinal permeability of ketoprofen. Curr Pharm Anal, 2017;13(1):72-79. Doi: https://doi.org/10.2174/1573412912666160422151409

25. Müge A, Kaynak MS, Şahin S. Simultaneous determination of acyclovir, metoprolol and phenol red by a RP-HPLC method for intestinal perfusion studies. HUJPHARM, 2015(2):146-161.

26. Gökçe EH, Kaynak MS, Yurdasiper A, Üstündağ-Okur N, Şahin S. Comparison of intestinal permeability of nebivolol hydrochloride loaded solid lipid nanoparticles with commercial nebivolol tablet. Marmara Pharm J, 2018;22(4):578-586. Doi: https://doi.org/10.12991/jrp.2018.100

27. Zakeri-Milani P, Valizadeh H, Azarmi Y, Jalali MB, Tajerzadeh H. Simultaneous determination of metoprolol, propranolol and phenol red in samples from rat in situ intestinal perfusion studies. DARU J Pharm Sci, 2006;14(2):102-108. Doi: https://sid.ir/paper/275338/en

28. Krishna G, Moton A, Ma L, Medlock MM, McLeod J. Pharmacokinetics and absorption of posaconazole oral suspension under various gastric conditions in healthy volunteers. AAC, 2009;53(3):958-966. Doi: https://doi.org/10.1128/aac.01034-08

29. Fagerholm U, Johansson M, Lennernäs H. Comparison between permeability coefficients in rat and human jejunum. Pharm Res, 1996;13(9):1336-1342. Doi: https://doi.org/10.1023/A:1016065715308

30. Nagare N, Damre A, Singh K, Mallurwar S, Iyer S, Naik A, et al. Determination of site of absorption of propranolol in rat gut using in situ single-pass intestinal perfusion. Indian J Pharm Sci, 2010;72(5):625. Doi: https://doi.org/10.4103/0250-474X.78533

31. Komiya I, Park J, Kamani A, Ho NF, Higuchi WI. Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes. Int J Pharm, 1980;4(3):249-262. Doi: https://doi.org/10.1016/0378-5173(80)90140-4

32. Dahan A, Amidon GL. Segmental dependent transport of low permeability compounds along the small intestine due to P-glycoprotein: the role of efflux transport in the oral absorption of BCS class III drugs. Mol. Pharmaceutics, 2009;6(1):19-28. Doi: https://doi.org/10.1021/mp800088f

33. Braumann T, Weber G, Grimme LH. Quantitative structure—activity relationships for herbicides: reversed-phase liquid chromatographic retention parameter, log kw, versus liquid-liquid partition coefficient as a model of the hydrophobicity of phenylureas, s-triazines and phenoxycarbonic acid derivatives. J Chromatogr A, 1983;261:329-343. Doi: https://doi.org/10.1016/S0021-9673(01)87961-9

34. Potts RO, Guy RH. Predicting skin permeability. Pharm Res, 1992;9(5):663-669. Doi: https://doi.org/10.1023/A:1015810312465

35. Montanari M, Montanari C, Piló-Veloso D, Cass Q. Estimation of the RP-HPLC Lipophilicity parameters Log K', and Log KW, a comparison with the Hydrophobicity Index φ0. J Liq Chromatogr R T, 1997;20(11):1703-1715. Doi: https://doi.org/10.1080/10826079708006327

36. Tate PA, Dorsey JG. Column selection for liquid chromatographic estimation of the kw′ hydrophobicity parameter. J Chromatogr A, 2004;1042(1-2):37-48. Doi: https://doi.org/10.1016/j.chroma.2004.05.039

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Topical apigenin as a promising therapeutic agent for psoriasis: Evaluating efficacy alone and in combination with clobetasol in an imiquimod-induced model of psoriasis in mice

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ABSTRACT

Psoriasis is an immune-mediated skin disease characterized by excessive growth and abnormal differentiation of keratinocytes that were treated initially with steroids which showed severe side effects for long-term use, so, medicinal plants are being explored as a potential alternative to identify new and effective agents with fewer adverse effects, such as apigenin which is investigated alone and in combination with clobetasol in this study for their anti-psoriatic activity on imiquimod-induced psoriasis on sixty male Albino mice divided into 6 groups. Group I is the control group, while the rest of the groups were induced psoriasis by Imiquimod (IMQ) for 6 consecutive days and administered different interventions for each group for 8 consecutive days including topical apigenin and apigenin/clobetasol combination followed by a measurement of the clinical, histopathological, and laboratory effects and the findings demonstrated that apigenin effectively reduced the PASI score, enhanced the histopathology, downregulated the expression of TNF-α, IL-17, and VEGF, and significantly increased the IL10 level in mice's skin tissue homogenate which indicate that Apigenin alone and in combination with clobetasol hold promise for the management of psoriasis, offering a potential alternative or adjunct to current therapeutic approaches.

Keywords: psoriasis, apigenin, Imiquimod, Clobetasol, IL-10

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INTRODUCTION

Psoriasis is a chronic inflammatory, non-contagious illness characterized by multisystemic inflammation with reddish plaques and white scales that is more common in the knees, feet, hands, elbow, scalp, and sacral regions[1]. It is associated with the activation of the adaptive immune system, particularly T-cells, although its exact cause and development mechanism are unknown. An imbalance between T helper Type 1 and Type 2 cells, as well as cytokine generation, are among the leading potential causes. Epidermal differentiation disruption and impairment of the skin barrier are significant characteristics. Psoriasis-specific Th1/Th17 cytokine milieu impacts epidermal differentiation[2,4].

Imiquimod (IMQ) – which is a Toll-like receptor (TLR) 7/8 ligand dermatological application induces and possibly exacerbates psoriasis, in a treated mouse model it exhibits similarities to human plaque-type psoriasis in terms of skin erythema, thickening, scaling, epidermal alterations (acanthosis, parakeratosis), neo-angiogenesis, and inflammatory infiltrate comprising T cells, neutrophils, and dendritic cells[5]. Prior research has established the significance of various factors in this model, including the psoriasis area severity index (PASI) score, histological staining, and the involvement of inflammatory cytokines[6,9].

Phototherapy, systemic drugs such as methotrexate and cyclosporine, oral medications such as apremilast, and topical therapies are all options for treating moderate-to-severe psoriasis. Yet, even though many therapies are effective and well tolerated, people with psoriasis frequently do not achieve skin clearance[10]. Nevertheless, due to the adverse effects of anti-inflammatory medicines, investigations on natural substances to replace chemical pharmaceuticals have become increasingly active. As a result, it is critical to identify novel candidates for topical administration to decrease side effects and obtain improved therapeutic efficacy[10].

Apigenin, a flavone subclass, is one of the most frequent flavonoids discovered in plants[12]. The bioactive natural chemical is scientifically referred to as 4′,5,7 trihydroxy flavone (4′,5,7-trihydroxy flavone). It is a 270 Da flavonoid that is found in numerous fruits and vegetables, as well as fragrant plants such as chamomile (Figure 1)[13].

Figure 1. Chamomile flower Apigenin powder extract

Apigenin has a variety of physiological effects[14], including anti-inflammatory[15], antioxidant[16], anticancer[17], anti-viral properties[18] and hypoglycemic effects[19,20].

The current study attempts to investigate topical Apigenin's potential therapeutic effects as a new modality for the treatment of psoriasis symptoms alone and in combination with clobetasol on an imiquimod-induced model.

METHODOLOGY

Study design

Male BALB/c albino mice, weighing between 24 and 30 g and aged between 8 and 12 weeks, were split into six groups of ten mice each, for a total of 60 mice. Several body parts were marked to help identify the animals. The mice were acquired from the Al-Nahrain University – Biotechnology Research Center in Baghdad, Iraq. They were kept in polypropylene cages with a temperature control system (15–21°C) and an inverted light–dark cycle (12–12 hours). The mice were given seven days to acclimate before the experiment began at the same facility. The animals were fed a regular diet and were allowed unrestricted access to water. Prior the start of the experiment, the mice were checked for the presence of any skin lesions, and only mice with seemingly healthy skin and coats were included in the study. All of the study animals were shaved from the dorsal region to reveal an area of the back skin measuring approximately 1x2 cm using an electric razor followed by a hair removal cream application (Veet®, Reckitt Benckiser Pvt. Ltd., India). The experiment lasted 14 days in total from the first day. About the distribution of mice:

- G-1 (Apparently healthy group) remained with no intervention during the whole experimental duration.

- G-2 (Induction group) that received induction of psoriasis by a dose of 62.5 mg of topical Imiquimod cream 5% (Aldara® 5% Cream, Meda Pharmaceuticals, Solna, Sweden) once daily on the shaved back skin for 6 days until the appearance of a psoriatic lesion as mentioned by van der Fits et al. study[21], mice in this group received no further intervention.
- G-3 (Petrolatum group) received induction of psoriasis by a dose of 62.5 mg of topical Imiquimod cream 5% once daily on the shaved back skin for 6 days as mentioned by van der Fits et al. study and then on the 7th day received medicinal petrolatum jelly (Iraqi Federation of Industries, Baghdad, Iraq) topically twice daily 8 days duration.
- G-4 (Clobetasol group) received induction of psoriasis by a dose of 62.5 mg of topical Imiquimod cream 5% once daily on the shaved back skin for 6 days as mentioned by van der Fits et al. study and then on the 7th day received as standard of care for psoriasis topical Clobetasol propionate 0.05% ointment (Dermovate®, GlaxoSmithKline, Brentford, UK) once daily (at a dosage of 0.25 g/kg)[22] for 8 days duration.
- G-5 (Apigenin 2% group) received induction of psoriasis by a dose of 62.5 mg of topical Imiquimod cream 5% once daily on the shaved back skin for 6 days as mentioned by van der Fits et al. study and then on the 7th day received a preparation of topical apigenin ointment 2% w/w[23,24] (prepared from powder supplied by Hyperchem, Hangzhou, China) twice daily for 8 days duration.
- G-6 (Apigenin-clobetasol combination group) received induction of psoriasis by a dose of 62.5 mg of topical Imiquimod cream 5% once daily on the shaved back skin for 6 days as mentioned by van der Fits et al. study and then on the 7th day received a topical preparation of 0.025% Clobetasol propionate ointment combined with apigenin ointment 1% w/w twice daily for 8 days duration. Figure 2 illustrates the flow chart of the study.

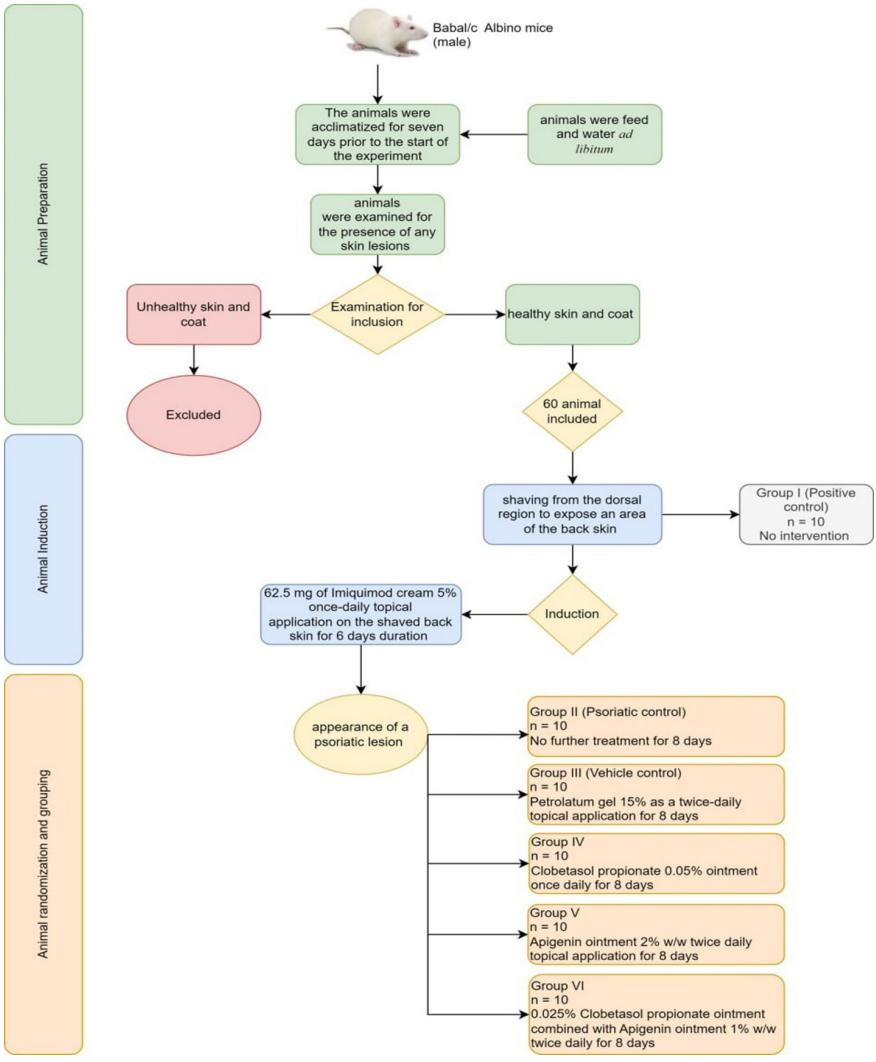

Figure 2. Study design

The Psoriasis Area Severity Index (PASI) score in mice was used to assess the success of the Imiquimod-induced psoriasis model[25]. The skin erythema, increased skin thickness, and scaling that were observed in the animals during the induction phase were considered successful outcomes since they appeared in the animals before day 6 of induction[26]. Each mouse's back skin was examined visually for three different characteristics: induration (thickness), desquamation (scale), and erythema (redness). The total score ranged from 0 to 12[25], with each characteristic being assigned between 0 and 4 (0=None, 1=Slight, 2=Moderate, 3=Marked, and 4=Very marked) (Figure 3).

Figure 3. Induction of Psoriasis in mice. (A) Mouse before induction (B) Mouse after induction by Imiquimod cream

Preparation of apigenin ointment 2% w/w, USP 37 – NF 32

Twenty-five grams of Petrolatum ointment of 2% apigenin was prepared by fusion technique based on USP 37 – NF 32 [27]. 18.75 g of petrolatum was melted in a water bath followed by the addition of the required amount of apigenin (0.5 g) then it was mixed using a stirrer until the mixture was uniform, the weight was completed by petrolatum to reach 25 g with stirring, the combination was then cooled until congealed.

Apigenin and clobetasol combination ointment, USP 37 – NF 32

Commercial clobetasol ointment (Dermovate®) containing 0.05% Clobetasol propionate (CP) was used with the prepared apigenin 2% ointment. An equal amount of both apigenin and clobetasol (half concentration of both) was taken and well mixed by a spatula to obtain the final concentration of 1% apigenin and 0.025% clobetasol combination ointment[27].

Outcome measures

Based on the PASI Score, the effectiveness of the treatments in the tested groups was assessed[25]. Intraperitoneally (IP) anesthesia was administered to all mice using 80 mg/kg of ketamine and 10 mg/kg of xylazine. All mice after being completely anaesthetized were terminated by exsanguination, a procedure appropriate for tissue harvesting and preservation[28]. Tissue samples from the dorsal shaved skin (2 mm) were obtained and split into two parts. The first

part was prepared for histopathological analysis by immersing it in liquid paraffin at a temperature range of 55-60°C after first being dehydrated. The slide was made using this technique, which has been covered in previous research[29].

The pathological changes of the mice's skin tissues on a scale of 0 – 10 were then evaluated under light microscopy (Genix, USA) using Baker's grading system, a histopathological grading system used for assessing the severity of inflammation as illustrated in Table 1. The parts were evaluated in a blinded manner by two distinct investigators, and the average score was recorded[30].

Table 1. Histopathological scoring of the severity of inflammation (Baker scoring system)[31]

Layers	Feature	Score
Keratin	Munro Abscess	2.0
	Hyperkeratosis	0.5
	Parakeratosis	1.0
Epidermis	Thinning over Papillae	0.5
	Rete ridges appearance	1.5
	Acanthosis	0.5
	Lack of granular layer	1.0
Dermis	Lymphocytic infiltrate	
	Mild	0.5
	Moderate	1.0
	Severe	2.0
	Papillary Congestion	0.5

The second piece of skin tissue was ready for biochemical analysis in order to measure TNF-α, IL-17, IL-10, and VEGF. The tissue was first thoroughly rinsed in ice-cold PBS (pH 7.2) to remove any excess blood, and then weighed using an electrical balance and minced into small pieces before being homogenized in fresh lysis buffer. 1mL of lysis buffer was added to the tissue sample using a glass homogenizer on ice, and the tissue was homogenized using an electrical tissue homogenizer machine (Electrical tissue homogenizer, Staruar®, England). Following that, the homogenates were centrifuged at 10,000×g for 5 minutes. Before being used for analysis using the sandwich ELISA technique and the ELISA Reader (ELISA reader, Diagnostic Automation / Cortez Diagnostics®, California, USA), the supernatants were collected and kept at ≤ -20°C. With regard to the ELISA kits, the mice analytical kit (SCA133Mu,

Cloud-Clone Corp.) was used to determine TNF-α, the mice analytical kit (SEA056Mu, Cloud-Clone Corp.) was used to determine IL-10, the mice analytical kit (HEA063Mu, Cloud-Clone Corp.) was used to determine IL-17, and the mice analytical kit (SEA143Mu, Cloud-Clone Corp.) was used to investigate VEGF.

Program G Power, which is based on Cohen's principles was used to compute the sample size[32,33]. The groupings were randomly constructed using a table of random integers. The animals were tagged with tails and kept in labelled containers to reduce miscommunication[34] .

Statistical analysis

All analyses were carried out using "GraphPad Prism version 10.0.0. The Kruskal-Wallis's test was applied to verify the significance of the difference between the studied groups, followed by the post hoc "two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (False discovery rate)"[35]. The differences between the groups were considered significant statistically when the p-value was less than or equal to 0.05[36].

RESULTS and DISCUSSION

The results of the current study revealed that the animal model treated with imiquimod exhibited a statistically highly significant increase in PASI score when compared to the healthy control group. The topical application of apigenin ointment and Clobetasol Propionate 0.05% and a combination of them showed a highly significant reduction in the PASI score compared to the induction group and petrolatum group as illustrated in Figure 4 and Table 2. Results also revealed that the same manner of improvement was obtained regarding Baker's score in which G-4, G-5, and G-6 were significantly lower than G-2, and G-3. It was observed that G-4, G-5, and G-6 were non-significantly different from each other regarding both Baker's and PASI scores.

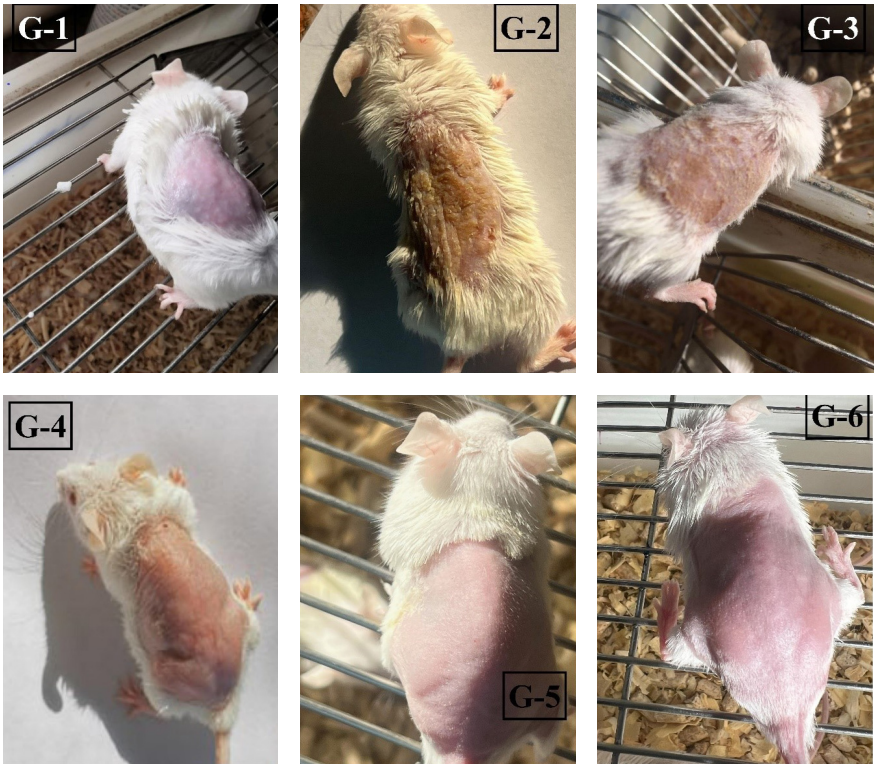

Figure 4. PASI score which includes erythema, thickness, and scales in the course of IMQ induction and treatment of psoriasis-like lesion; control group (G-1), induced non-treated group (G-2), petrolatum group (G-3), clobetasol propionate 0.05% (G-4), apigenin 2% group (G-5), apigenin 1%-clobetasol 0.025% (G-6)

Table 2. Assessment of skin psoriasis scores

Parameters	Baker's score	PASI score
G-1	-	-
G-2	9.00 ± 0.00[a]	11.70 ± 0.48[a]
G-3	7.60 ± 0.84[a]	9.30 ± 0.67[a]
G-4	2.15 ± 1.08[b]	3.00 ± 0.82[b]
G-5	1.85 ± 0.53[b]	1.70 ± 0.67[b]
G-6	1.80 ± 0.42[b]	1.50 ± 0.71[b]
p-value	<0.0001[#]	<0.0001[#]

Kruskal-Wallis's test [p-value≤0.05 indicates significant difference] with a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (False discovery rate) to calculate pair-wise p-value between each pair
Data presented as mean ± standard deviation SD: standard deviation, PSAI: psoriasis area and severity index

Histopathologic examination of the skin section of control group showed normal epidermal, dermal, and subcutaneous tissue layers (Figure 5[A]), while the histopathological examination of skin section of Induction group showed a multifocal (wide) area of sloughing, severe dense neutrophilic infiltration (the Munro's abscesses), and parakeratosis, hyperkeratosis, with lack of granular layer, acanthosis, increased rete ridges with papillary thinning. The dermis showed severe lymphocytic infiltration and vascular congestion (Figure 5[B]). The histopathological examination of the skin section of Petrolatum groups also showed epidermal hyperkeratosis and parakeratosis with focal Munro's abscesses with acanthosis and elongated rete ridges and papillary thinning with moderate to severe lymphocytic infiltration (Figure 5[C]).

The histopathological examination of the skin section of the standard treatment group (clobetasol group) shows hyperkeratosis, absence of (parakeratosis & Munro's abscess) and presence of epidermal granular layer with mild acanthosis, few rete ridges with mild thinning of papillae with mild lymphocytic infiltration of the dermis (Figure 5[D]). The skin of mice treated with apigenin 2% showed a mild keratosis with an absence of Munro's abscess and parakeratosis and epidermal mild acanthosis with few rete ridges. The dermis shows mild lymphocytic infiltrate (Figure 5[E]). The histopathological examination of the skin section of a combination treatment group showed mild epidermal thickness, with the absence of Munro's abscess and parakeratosis and epidermal mild acanthosis with absence of rete ridges and few lymphocytic dermal infiltrations (Figure 5[F]).

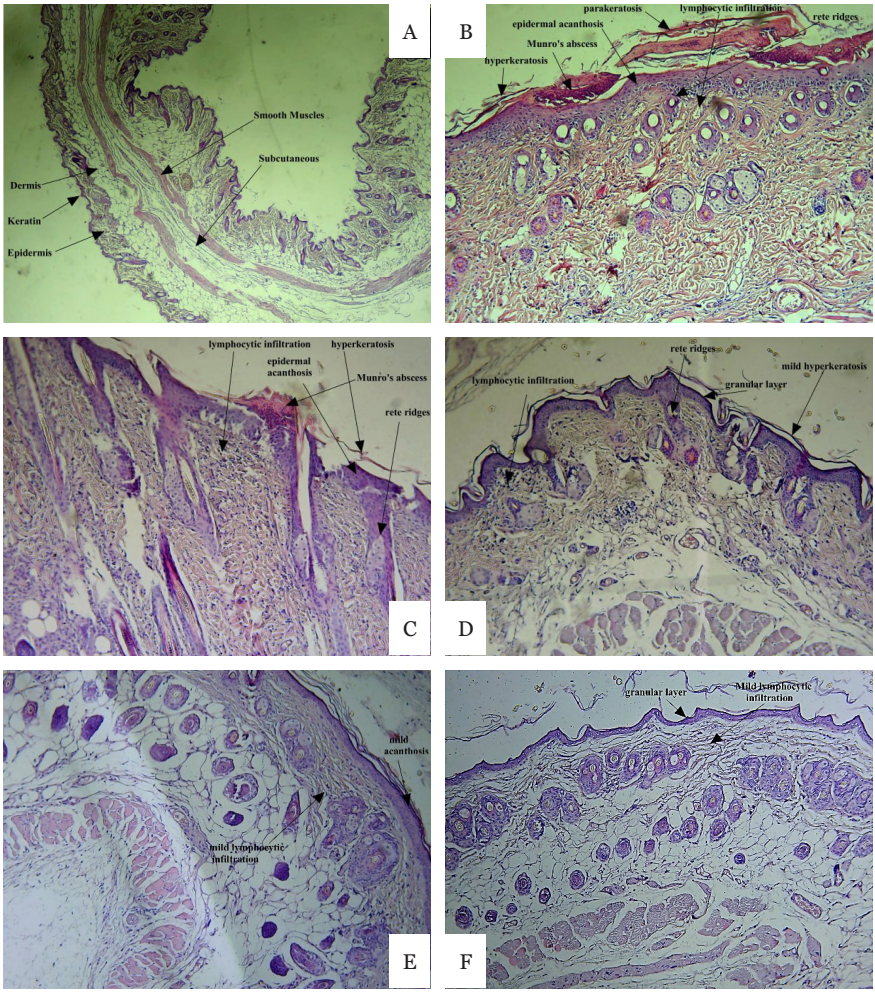

Figure 5. Histopathological section of mice skin: [A] healthy control group [B] induction group, [C] Petrolatum group [D] clobetasol control group [E] Apigenin 2% group [F] Apigenin – Clobetasol combination group; H&E stain (4X, & 10X)

Results obtained from the inflammatory markers revealed that G-2 showed the highest levels of TNF-α and was significantly higher than groups G-4, G-5, and G-6, whereas non-significant differences between G-2 and G3 and between G4, G5, and G-6 were observed, as illustrated in Table 3 and Figure 6. Results showed that G-2 demonstrated the highest levels of IL-17, which is significantly higher than the other groups (G-3 to G-6). Additionally, G-3 was significantly higher than G-4, G-5, and G-6, with non-significant differences between G-4, G-5, and G-6 as illustrated in Table 3 and Figure 6.

Moreover, G-2 showed the highest levels of VEGF which is significantly higher than all other groups (G-3 to G-6), and G-3 showed to be significantly higher than G-5 and G-6, whereas a non-significant difference between G-3 and G-4 and a non-significant difference between G-4, G5 and G-6 were obtained as illustrated in Table 3 and Figure 6.

G-2 showed the lowest levels of IL-10 which is significantly lower than the other groups (G-3 to G-6). On the other hand, there were non-significant differences between G-4, G-5 and G-6 which are significantly higher than G-2 and G-3, as illustrated in Table 3 and Figure 6.

Table 3. Assessment of biomarkers

Parameters	TNF-α	IL-17	VEGF	IL-10
G-2	807.13 ± 500.06[a]	553.04 ± 141.32[a]	552.20 ± 136.63[a]	31.83 ± 3.03[a]
G-3	281.79 ± 240.17[a]	278.52 ± 100.27[b]	209.56 ± 73.31[b]	92.50 ± 27.13[b]
G-4	65.37 ± 23.12[b]	165.07 ± 43.59[c]	134.57 ± 44.28[bc]	168.11 ± 57.07[c]
G-5	89.25 ± 18.76[b]	149.90 ± 63.30[c]	99.56 ± 17.42[c]	170.4 ± 12.65[c]
G-6	71.45 ± 17.83[b]	150.16 ± 21.19[c]	94.98 ± 7.37[c]	166.11 ± 47.80[c]
p-value	<0.0001[#]	<0.0001[#]	<0.0001[#]	<0.0001[#]

Kruskal-Wallis's test [p-value≤0.05 indicates significant difference] with a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (False discovery rate) to calculate pair-wise p-value between each pair
SD: standard deviation, IL= Interleukin; TNF-α= Tumor necrosis factor alpha; VEGF=Vascular endothelial growth factor

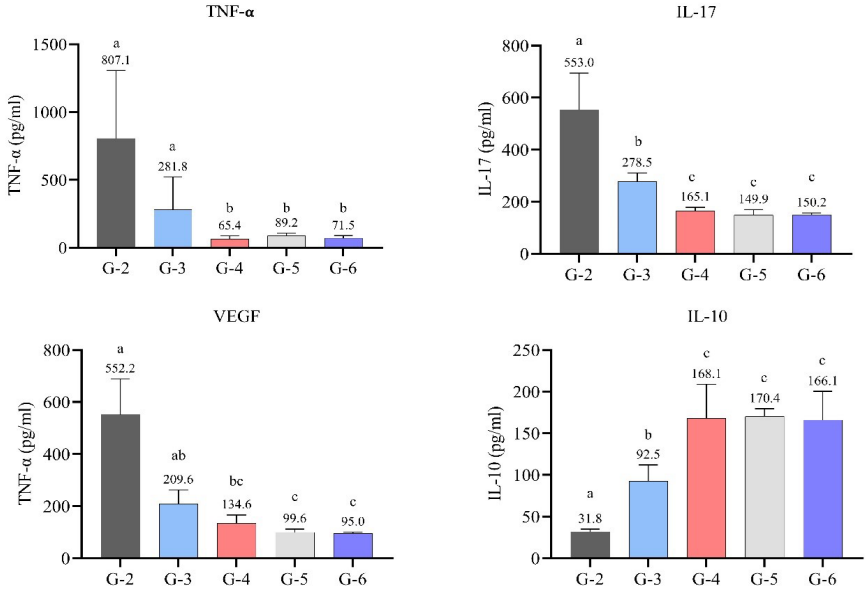

Figure 6. Histogram Comparison between all treated groups in terms of inflammatory markers (TNF-α, IL-17, VEGF and IL10); TNF-α= Tumor necrosis factor-alpha; IL= Interleukin; VEGF=Vascular endothelial growth factor

Psoriasis is a persistent autoimmune disorder distinguished by excessive proliferation of epidermal cells, expansion of dermal capillaries, infiltration of inflammatory cells, and anomalous growth of skin cells. The aforementioned factors are responsible for inducing various complications such as the development of erythematous plaques that are characterized by pruritus, inflammation, and discomfort[37]. Apigenin is a flavonoid that has caught attention in this study due to its potential as an immunomodulator. It is particularly noteworthy for its low intrinsic toxicity and lack of mutagenicity, distinguishing it from other flavonoids with similar structures[38] . In the current investigation, as shown in the results apigenin was responsible for a dramatic improvement in phenotypical observation and a highly significant reduction in cumulative (PASI) scores representing erythema, scaling, and thickness compared to the induction group. Present study findings also indicate a noteworthy improvement and a highly significant reduction in Baker score after treatment with topical apigenin in comparison to the IMQ-induced group.

This successful anti-inflammatory effect of apigenin in ameliorating psoriasis observational and histopathologic features attributed to its anti-inflammatory properties by its documented capacity to regulate the production of inflammatory mediators such as interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis

factor (TNF)-a, activator protein-1 (AP-1) factors, and cyclooxygenase (COX)-2. In addition to its antioxidant, anticancer, antiviral, antimutagenic, and antibacterial actions only some of the many therapeutic benefits associated with apigenin as documented in previous studies[39]. In addition, results from ELISA investigation demonstrated that apigenin causes a significant decrease in TNF-α, IL-17, and VEGF levels and an increase in the levels of IL-10, an anti-inflammatory cytokine. Various features of apigenin have been demonstrated in previous investigations to be the underlying mechanisms for its possible anti-psoriatic impact.

Apigenin has been found to mitigate the severity of inflammatory processes significantly through the inhibition of TNF-α and the augmentation of IL-10[40]. According to Kumar et al., apigenin lowers the level of pro-inflammatory cytokines including IL-1 and TNF-α, and increases IL-10 levels at the same time. This makes it abundantly evident that apigenin has a variety of functions in controlling immune response mediators, both pro-inflammatory and anti-inflammatory cytokines[41]. IL-10 is a potent anti-inflammatory cytokine that is essential for immune modulation in psoriasis[42].

Prior research has shown that apigenin enhances the production of IL10 by modulating the balance between Th1 and Th2 immune responses in Experimental Autoimmune Myocarditis (EAM). Apigenin has been found to inhibit the production of Th1-type cytokines, such as TNF-α, IFN-γ, and IL2, while simultaneously enhancing the Th2 response, leading to an upregulation of Th2-type cytokines, including IL10, IL4, and IL5[43] TNF-α is a crucial pro-inflammatory cytokine that promotes inflammation through multiple pathways. TNF-α enhances the expression of adhesion molecules and secondary mediators, which are implicated in the pathogenesis of psoriasis[44].

According to prior research, the anti-inflammatory function of apigenin is partially elucidated by its direct suppression of NF-KB by inhibiting the nuclear translocation of p65. The latter process leads to the deactivation of genes responsible for nitric oxide synthetase (NOS) and cycloxygenase-2 (COX-2), which plays a crucial role in inhibiting the inflammatory process and inducing cell-cycle arrest. Apigenin has been demonstrated to have a significant impact on the inflammatory process through its targeting and down-regulation of TNF-α and NF-kB, as evidenced by previous research[45]. This supports our findings that showed apigenin 2% ointment significantly decreased the level of TNF-α and significantly increased the level of (IL10) in skin tissue homogenate in comparison to the IMQ-induced group.

Apigenin modulated the function of dendritic cells (DCs) to affect naïve T cell differentiation, so, it can improve psoriasis symptoms by regulating inflammatory cytokine transcription through the Toll-like receptor 4 pathway[46]. The utilization of topical apigenin has been observed to enhance psoriasis by impeding the function of dendritic cells, subsequently leading to a reduction in the activation of T-helper cells. Prior research has indicated that apigenin significantly suppresses the activation of Th1 and Th17 cells in individuals with lupus erythematosus. Reduction in the protein expression levels of IL-17A and IFN-g was observed in the dermal tissue of mice treated with apigenin. In addition, the results of an in vitro experiment demonstrated that apigenin can inhibit the production of IL-6 by APCs. This is significant because IL-6 plays a crucial role in the development of Th17 cells and the suppression of Treg cells[47]. This is aligned with our results that showed apigenin significantly reduced the level of skin tissue (IL17) in comparison to the induction group.

The upregulation of VEGFA, which serves as the primary proangiogenic factor, has been observed in the cutaneous tissue of individuals with psoriasis and is positively associated with the severity of the disease[48,49]. It has been well established through studies that hypoxia is one of the primary stimulators of VEGF expression, which is mediated by the accumulation of HIF-1a[50]. Multiple investigations have demonstrated that apigenin inhibited angiogenesis in rheumatoid arthritis and cell hypoxia-reoxygenation injury by its ability to decrease the expression of various angiogenesis-related factors, such as HIF-1α and VEGF-A, in various types of human cancers, as evidenced by multiple sources[51]. The aforementioned findings indicate that apigenin has an anti-angiogenic effect which is crucial to the pathogenesis of psoriasis[52]. This is in line with our findings, which demonstrated a marked decline in the level of (VEGF) in comparison to the induction group.

Psoriasis is associated with oxidative stress, which triggers the activation of various signalling pathways, such as NF-KB and MAPK. This leads to the activation of Th1 and Th17 cells, secretion of proinflammatory cytokines, hyperproliferation of keratinocytes, infiltration of immune cells into the skin, and changes in blood vessel permeability due to lipid peroxidation. Therefore, the use of antioxidants is crucial in the treatment of psoriasis[53]. Prior studies have indicated that Apigenin exhibits antioxidant properties by enhancing the activity of antioxidant enzymes glutathione-synthase (GSH synthase), catalase (CAT), and superoxide dismutase (SOD). Apigenin exerts an impact on the production of cutaneous barrier components and the entry of calcium ions. Thus, it has the potential to be utilized in the treatment of inflammatory skin conditions and cancer[54]. This antioxidant characteristic has been proposed to contribute to the amelioration of induced psoriasis.

Furthermore, the skin treated with apigenin exhibited observable effects on skin barrier recovery. The application of apigenin ointment in our study resulted in an enhancement of the skin's state through augmentation of the hydration level of the stratum corneum. In mouse models, it was observed that apigenin had an impact on the production of skin structural proteins such as filaggrin, involucrin, and loricrin[55].

Utilizing a combination drug approach represents a viable strategy to enhance the synergistic efficacy of topical therapy. To optimize the therapeutic effect, a multi-target treatment approach utilizing synergistic combinations of two or more therapeutically relevant molecules that act through different mechanisms is recommended[56]. The combination of Apigenin 1% and clobetasol ointment 0.025 was administered in our investigation and the findings indicate that the co-administration of apigenin and clobetasol at reduced concentrations exhibited additive properties in the mitigation of psoriasis lesions. No significant difference in PASI and Baker scores between the combination group and the apigenin and clobetasol treated groups alone, in terms of inflammatory markers there was no significant difference between the combination and when the apigenin 2% ointment or clobetasol were used alone, This could be explained by the mechanisms of action of apigenin, a flavone with anti-inflammatory properties, and clobetasol, a potent corticosteroid that reduces inflammation and has immunosuppressive effects. If both act on the same inflammatory pathways, it could explain why their combination does not have a synergistic effect. Additionally, the concentration of clobetasol and apigenin in the combination preparation might not be optimized for the synergistic effect.

This study demonstrated that Apigenin's anti-inflammatory and antioxidant properties play a crucial role in reducing inflammation and oxidative stress and regulating Psoriasis progression. Topical apigenin improved the animals' anti-inflammatory and anti-oxidant status, maintained skin hemostasis, along with the modulation of key cytokines, reduction in proinflammatory cytokines (TNF-α, IL17) and VEGF, coupled with the increase in the anti-inflammatory cytokine (IL-10). Collectively contribute to the amelioration of psoriasis symptoms and supports the therapeutic potential of apigenin and its anti-psoriatic effect is comparable to that of clobetasol. The combined administration of apigenin and clobetasol exhibited additive properties compared to the individual use of either medication. Thus, the present study indicates that Apigenin alone and in combination with clobetasol hold promise for the management of psoriasis, offering a potential alternative or adjunct to current therapeutic approaches.

STATEMENT OF ETHICS

The study was approved by the "Research Ethics Committee at the College of Medicine, Al-Nahrain University" (Approval number: 2308, date: 1st November 2022).

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

Design – Hasan AM, Gatea FK; Acquisition of data – Hasan AM; Analysis of data – Hasan AM; Drafting of the manuscript – Hasan AM; Critical revision of the manuscript– Gatea FK; Statistical analysis– Hasan AM, Gatea FK; Technical or financial support– Hasan AM; supervision – Gatea FK.

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REFERENCES

1. Mascarenhas-Melo F, Carvalho A, Gonçalves MBS, Paiva-Santos AC, Veiga F. Nanocarriers for the topical treatment of psoriasis - pathophysiology, conventional treatments, nanotechnology, regulatory and toxicology. Eur J Pharm Biopharm, 2022;176:95-107. Doi: 10.1016/j.ejpb.2022.05.012

2. Armstrong AW, Read C. Pathophysiology, clinical presentation, and treatment of psoriasis: a review. JAMA, 2020;323(19):1945-1960. Doi: 10.1001/jama.2020.4006

3. Gondo GC, Koons S, Metcalf C, Bell SJ, Mehta NN. Viewing psoriasis as a systemic disease for better health outcomes. JID Innov, 2021;1(2):100007. Doi: 10.1016%2Fj.xjidi.2021.100007

4. Rikken G, van den Brink NJM, van Vlijmen-Willems I, van Erp PEJ, Pettersson L, Smits JPH, et al. Carboxamide derivatives are potential therapeutic ahr ligands for restoring il-4 mediated repression of epidermal differentiation proteins. Int J Mol Sci, 2022;23(3):1773. Doi:10.3390%2Fijms23031773

5. El Malki K, Karbach SH, Huppert J, Zayoud M, Reissig S, Schüler R, et al. An alternative pathway of imiquimod-induced psoriasis-like skin inflammation in the absence of interleukin-17 receptor a signaling. J Invest Dermatol, 2013;133(2):441-451. Doi: 10.1038/jid.2012.318

6. Mohammed SS, Kadhim HM, Al-Sudani IM, Musatafa WW. Study the topical effect of six days use of different lycopene doses on imiquimod-induce psoriasis-like skin inflammation in mice. Int J Health, Sc 2022;6(S3):171-185. Doi: 10.53730/ijhs.v6nS3.5241

7. Ahmed NH, Al-Zubaidy AAK, Qasim BJ. Effect of tadalafil in comparison with clobetasol ointment in induced psoriasis in mice male. Tikrit J Pharm Sci, 2022;16(1):9-18. Doi: 10.25130/tjphs.2022.16.1.2.9.18

8. Salman HR, Al-Zubaidy AA, Abbas AH, Zigam QA. The ameliorative effects of topical gemifloxacin alone or in combination with clobetasol propionate on imiquimod-induced model of psoriasis in mice. Naunyn Schmiedebergs Arch Pharmacol, 2024;397(1):599-616. Doi: 10.1007/s00210-023-02629-9

9. Salman HR, Alzubaidy AA, Abbas AH, Mohammad HA. Attenuated effects of topical vinpocetine in an imiquimod-induced mouse model of psoriasis. J Taibah Univ Med Sci, 2023;19(1):35-53. Doi: 10.1016/j.jtumed.2023.09.002

10. Kerdel F, Zaiac M. An evolution in switching therapy for psoriasis patients who fail to meet treatment goals. Dermatol Ther, 2015;28(6):390-403. Doi: 10.1111/dth.12267

11. Alalaiwe A, Lin CF, Hsiao CY, Chen EL, Lin CY, Lien WC, et al. Development of flavanone and its derivatives as topical agents against psoriasis: the prediction of therapeutic efficiency through skin permeation evaluation and cell-based assay. Int J Pharm, 2020;581:119256. Doi: 10.1016/j.ijpharm.2020.119256

12. Salehi B, Venditti A, Sharifi-Rad M, Kręgiel D, Sharifi-Rad J, Durazzo A, et al. The therapeutic potential of apigenin. Int J Mol Sci, 2019;20(6):1305. Doi: 10.3390%2Fijms20061305

13. Abid R, Ghazanfar S, Farid A, Sulaman SM, Idrees M, Amen RA et al. Pharmacological properties of 4', 5, 7-trihydroxyflavone (apigenin) and its impact on cell signaling pathways. Molecules, 2022;27(13):4304. Doi: 10.3390/molecules27134304

14. Xu K, Yang Y, Lan M, Wang J, Liu B, Yan M, et al. Apigenin alleviates oxidative stress-induced myocardial injury by regulating SIRT1 signaling pathway. Eur J Pharmacol, 2023;944:175584. Doi: 10.1016/j.ejphar.2023.175584

15. Ginwala R, Bhavsar R, Chigbu DI, Jain P, Khan ZK. Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin. Antioxidants (Basel), 2019;8(2):35. Doi: 10.3390%2Fantiox8020035

16. Clayton ZS, Hutton DA, Brunt VE, VanDongen NS, Ziemba BP, Casso AG, et al. Apigenin restores endothelial function by ameliorating oxidative stress, reverses aortic stiffening, and mitigates vascular inflammation with aging. Am J Physiol Heart Circ Physiol, 2021;321(1):H185-H196. Doi: 10.1152/ajpheart.00118.2021

17. Cheng Y, Han X, Mo F, Zeng H, Zhao Y, Wang H, et al. Apigenin inhibits the growth of colorectal cancer through down-regulation of E2F1/3 by miRNA-215-5p. Phytomedicine, 2021;89:153603. Doi: 10.1016/j.phymed.2021.153603

18. Xu RY, Xu XW, Deng YZ, Ma ZX, Li XR, Zhao L, et al. Resveratrol attenuates myocardial hypoxia/reoxygenation-induced cell apoptosis through DJ-1-mediated SIRT1-p53 pathway. Biochem Biophys Res Commun, 2019;514(2):401-406. Doi: 10.1016/j.bbrc.2019.04.165

19. Cazarolli LH, Kappel VD, Pereira DF, Moresco HH, Brighente IM, Pizzolatti MG, Silva FR. Anti-hyperglycemic action of apigenin-6-C-β-fucopyranoside from *Averrhoa carambola*. Fitoterapia,2012;83(7):1176-1183. Doi: 10.1016/j.fitote.2012.07.003

20. Liu H, Huang P, Wang X, Ma Y, Tong J, Li J, Ding H. Apigenin analogs as α-glucosidase inhibitors with antidiabetic activity. Bioorg Chem, 2024;143:107059.
Doi: 10.1016/j.bioorg.2023.107059

21. Van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J Immunol, 2009;182(9):5836-5845. Doi: 10.4049/jimmunol.0802999

22. Carvalho Mde F, Pereira CS, Fregnani JH, Ribeiro Fde A. Comparative histological study on wound healing on rat's skin treated with Mitomycin C or Clobetasol propionate. Acta Cir Bras, 2015;30(9):593-597. Doi: 10.1590/s0102-865020150090000002

23. Rajab AA, Al-Wattar WT, A Taqa GJJoAVS. The roles of apigenin cream on wound healing in rabbits model. J Appl Vet Sci, 2022;7(1):1-5. Doi: 10.21608/javs.2021.97151.1104

24. Singh VK, Sahoo D, Agrahari K, Khan A, Mukhopadhyay P, et al. Anti-inflammatory, antiproliferative and anti-psoriatic potential of apigenin in RAW 264.7 cells, HaCaT cells and psoriasis like dermatitis in BALB/c mice. Life Sci, 2023;328:121909. Doi: 10.1016/j.lfs.2023.121909

25. Fredriksson T, Pettersson U. Severe psoriasis-oral therapy with a new retinoid. Dermatologica, 1978;157(4):238-244. Doi: 10.1159/000250839

26. Baek JO, Byamba D, Wu WH, Kim TG, Lee MG. Assessment of an imiquimod-induced psoriatic mouse model in relation to oxidative stress. Arch Dermatol Res, 2012;304(9):699-706. Doi: 10.1007/s00403-012-1272-y

27. The United States pharmacopeia. The National formulary. Rockville, Md.: United States Pharmacopeial Convention, Inc.; 1979.

28. Kocsis D, Horváth S, Kemény Á, Varga-Medveczky Z, Pongor C, Molnár R, et al. Drug delivery through the psoriatic epidermal barrier-a "skin-on-a-chip" permeability study and ex vivo optical imaging. Int J Mol Sci, 2022;23(8):4237. Doi: 10.3390/ijms23084237

29. Cardiff RD, Miller CH, Munn RJ. Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harb Protoc, 2014;2014(6):655-658. Doi: 10.1101/pdb.prot073411

30. Mohammed SS, Kadhim HM, Al-Sudani IM, Musatafa WW. Anti-inflammatory effects of topically applied azilsartan in a mouse model of imiquimod-induced psoriasis. Int J Drug Deliv Technol, 2022;12:1249-1255. Doi: 10.25258/ijddt.12.3.53

31. Baker BS, Fry L. The immunology of psoriasis. Br J Dermatol, 1992;126(1):1-9. Doi: 10.1111/j.1365-2133.1992.tb08394.x

32. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods, 2007;39(2):175-191. Doi: 10.3758/bf03193146

33. Charan J, Kantharia ND. How to calculate sample size in animal studies? J Pharmacol Pharmacother, 2013;4(4):303-306. Doi: 10.4103%2F0976-500X.119726

34. Festing MF. Design and statistical methods in studies using animal models of development. ILAR J, 2006;47(1):5-14. Doi: 10.1093/ilar.47.1.5

35. Seaman MA, Levin JR, Serlin RC. New developments in pairwise multiple comparisons: some powerful and practicable procedures. Psychol Bull, 1991;110(3):577-586. Doi: 10.1037/0033-2909.110.3.577

36. Abdulamir HA, Aldafaay AAA, Al-Shammari AH. The role of liver function tests in monitoring the effect of enzyme replacement therapy in children with gaucher disease. Res J Pharm Technol, 2022; 15(8):3490-3496. Doi: 10.52711/0974-360X.2022.00585

37. Afonina IS, Van Nuffel E, Beyaert R. Immune responses and therapeutic options in psoriasis. Cell Mol Life Sci, 2021;78(6):2709-2727. Doi: 10.1007/s00018-020-03726-1

38. Tang D, Chen K, Huang L, Li J. Pharmacokinetic properties and drug interactions of apigenin, a natural flavone. Expert Opin Drug Metab Toxicol, 2017;13(3):323-330. Doi: 10.1080/17425255.2017.1251903

39. Majma Sanaye P, Mojaveri MR, Ahmadian R, Sabet Jahromi M, Bahramsoltani R. Apigenin and its dermatological applications: a comprehensive review. Phytochemistry, 2022;203:113390. Doi: 10.1016/j.phytochem.2022.113390

40. Palacz-Wrobel M, Borkowska P, Paul-Samojedny M, Kowalczyk M, Fila-Danilow A, Suchanek-Raif R, et al. Effect of apigenin, kaempferol and resveratrol on the gene expression and protein secretion of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) in RAW-264.7 macrophages. Biomed Pharmacother, 2017;93:1205-1212. Doi: 10.1016/j.biopha.2017.07.054

41. Alam W, Rocca C, Khan H, Hussain Y, Aschner M, De Bartolo A, et al. Current status and future perspectives on therapeutic potential of apigenin: focus on metabolic-syndrome-dependent organ dysfunction. Antioxidants (Basel), 2021;10(10):1643. Doi: 10.3390%2Fantiox10101643

42. Georgescu S-R, Tampa M, Caruntu C, Sarbu M-I, Mitran C-I, Mitran M-I, et al. Advances in understanding the immunological pathways in psoriasis. Int J Mol Sci, 2019;20(3):739. Doi: 10.3390/ijms20030739

43. Zhang S, Liu X, Sun C, Yang J, Wang L, Liu J, et al. Apigenin attenuates experimental autoimmune myocarditis by modulating th1/th2 cytokine balance in mice. Inflammation, 2016;39(2):678-686. Doi: 10.1007/s10753-015-0294-y

44. Chuang SY, Lin CH, Sung CT, Fang JY. Murine models of psoriasis and their usefulness for drug discovery. Expert Opin Drug Discov, 2018;13(6):551-562. Doi: 10.1080/17460441.2018.1463214

45. Asadi A, Goudarzi F, Ghanadian M, Mohammadalipour A. Evaluation of the osteogenic effect of apigenin on human mesenchymal stem cells by inhibiting inflammation through modulation of NF-κB/IκBα. Res Pharm Sci, 2022;17(6):697-706. Doi: 10.4103/1735-5362.359436

46. Yoon JH, Kim MY, Cho JY. Apigenin: a therapeutic agent for treatment of skin inflammatory diseases and cancer. Int J Mol Sci, 2023;24(2):1498. Doi: 10.3390/ijms24021498

47. Cao F, Cheng M-H, Hu L-Q, Shen H-H, Tao J-H, Li X-M, et al. Natural products action on pathogenic cues in autoimmunity: efficacy in systemic lupus erythematosus and rheumatoid arthritis as compared to classical treatments. Pharmacol Res, 2020;160:105054. Doi: 10.1016/j.phrs.2020.105054

48. Li X, Xi B, Miao Y, Ma X, Zhang J, Gao J, et al. Nintedanib ameliorates imiquimod-induced psoriasis in mice by inhibiting NF-κB and VEGFR2 signaling. Int Immunopharmacol, 2021;100:108129. Doi: 10.1016/j.intimp.2021.108129

49. Chen Y, Tai Z, Zhu C, Yu Q, Zhu Q, Chen Z. Vascular endothelial growth factor a vegfa inhibition: an effective treatment strategy for psoriasis. Int J Mol Sci, 2023;25(1):59. Doi: 10.3390%2Fijms25010059

50. Noda Y, Goshima S, Miyoshi T, Kawada H, Kawai N, Tanahashi Y, et al. Assessing chemotherapeutic response in pancreatic ductal adenocarcinoma: histogram analysis of iodine concentration and ct number in single-source dual-energy CT. AJR Am J Roentgenol, 2018;211(6):1221-1226. Doi: 10.2214/ajr.18.19791

51. Ikeda H, Kakeya H. Targeting hypoxia-inducible factor 1 (HIF-1) signaling with natural products toward cancer chemotherapy. J Antibiot (Tokyo), 2021;74(10):687-695. Doi: 10.1038/s41429-021-00451-0

52. Fu J, Zeng W, Chen M, Huang L, Li S, Li Z, et al. Apigenin suppresses tumor angiogenesis and growth via inhibiting HIF-1α expression in non-small cell lung carcinoma. Chem Biol Interact, 2022 1;361:109966. Doi: 10.1016/j.cbi.2022.109966

53. Pleńkowska J, Gabig-Cimińska M, Mozolewski P. Oxidative stress as an important contributor to the pathogenesis of psoriasis. Int J Mol Sci, 2020;21(17):6206. Doi: 10.3390/ijms21176206

54. Yoon JH, Kim MY, Cho JY. Apigenin: a therapeutic agent for treatment of skin inflammatory diseases and cancer. Int J Mol Sci, 2023;24(2):1498. Doi: 10.3390/ijms24021498

55. Li HJ, Wu NL, Lee GA, Hung CF. The therapeutic potential and molecular mechanism of isoflavone extract against psoriasis. Sci Rep, 2018;8(1):6335. Doi: 10.1038%2Fs41598-018-24726-z

56. Yadav K, Singh D, Singh MR, Pradhan M. Multifaceted targeting of cationic liposomes via co-delivery of anti-IL-17 siRNA and corticosteroid for topical treatment of psoriasis. Med Hypotheses, 2020 ;145:110322. Doi: 0.1016/j.mehy.2020.110322

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Formulation development and *in vivo* study of nanoemulgel of *Channa striata* and *Citrus limon* extract for caesarean wound treatment

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ABSTRACT

Empirically, snakehead fish with its high albumin content has been widely used in the healing of post-caesarean wounds. Lemon can also be used to prevent wound infections by inhibiting thegrowth of bacterial activities. This study was conducted to discover the best nanoemulgel formulafrom the combination of snakehead fish and lemon extract, then evaluate its healing activity for post-caesarean wounds. Nanoemulgel was characterized by pH, viscosity, spreadability, adhesion, particle size, and zeta potential to determine the best formula. Healing activity evaluation was performed on female Wistar rats which were divided into seven groups (n=3). Healing parameters evaluated were wound length closure, epidermal thickening, and tissue reconstruction. Wound length was measured every 5 days for 15 days. Subsequently, histopathological observations H&E staining were used to determine epidermal thickening and wound tissue reconstruction. The results showed that nanoemulgel I (NEG1) containing 7% snakehead fish extract, and 3% lemon extract had the best wound healing ability, with an averagewound closure of 84±2.1% on day 10, epidermal thickening of 2.31 ± 0.06 μm on day 15, and better tissue structure reconstruction than other groups. Thus, the nanoemulgel design can optimize

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the snakehead fish and lemon extract in accelerating the healing process of post- caesarean wounds.

Keywords: *Channa striata*, *Citrus limon*, nanoemulgel, reepithelialization, wound healing

INTRODUCTION

According to the World Health Organization (WHO), maternal perinatal mortality and morbidity can be prevented through caesarean section, with a percentage ranging from 10% to 15%[1,2]. The Cesarean Section Rate (CSR) initially was 5%, then increased to 12% in 2012, and further rose to 17% in 2017 in Indonesia. Ayuningtyas et al. (2018) reported that caesarean sectionhas become a trend, constituting up to 70% of deliveries in private hospitals[3]. The prevalence of pain and morbidity in mothers after caesarean section is higher compared to vaginal deliveries because it can lead to infections, the most common complication[4–6]. Although antibiotics are widely available to prevent infections, the risk of infection continues to rise[7,8]. Infections in post-caesarean operation wounds can be prevented by accelerating the reepithelization process[9].

The speed of the reepithelization process can be increased by elevating the albumin levels inthe body[10-12]. Therefore, the majority of mothers after caesarean section are recommended to consume snakehead fish due to its high albumin content[13]. This is considered less effective because it does not directly reach the target action, resulting in suboptimal efficacy. Currently, snakehead fish albumin is widely developed in capsule form, but oral administration has low bioavailability, need to undergo first-pass metabolism, chemical degradation, and enzymatic reactions, therefore topical administration is more recommended[14]. Therefore, Andrie and Sihombing used 5-10% snakehead fish extract on ointment formulation for wound healing. However, the healing acitivity only reached the inflammation stage[15]. Besides using albumin, the acceleration of healing in post-caesarean operation wounds can be assisted by using citric acid because it inhibits the growth of bacterial activities[16,17]. Sim et al. have proven that 3% citric acid has wound healing potential[18]. An acidic environment significantly influences wound healing as it acts as a natural physiological response to mediate various cellular processes internally to restore barriers which facilitate higher oxygenation levels in the wound, affect macrophage and fibroblast activities, as well as enzymatic activities which participate in wound healing[18].

The advancement in drug synthesis has led to the development of nanocarriers as a strategyto achieve efficient drug penetration, making them suitable for a

topical drug delivery system. Some researchers have claimed that nanoemulsions are a potential drug delivery system due to their high drug loading, solubility capacity, ease of manufacturing, stability, and controlled releasepatterns[19,20]. However, nanoemulsions have low viscosity, resulting in brief contact with the skinand suboptimal penetration of active substances. Therefore, to obtain an effective topical formulation, a nanoemulgel formulation was created to extend the contact time of the formulationwith the skin, providing high drug loading, penetration, and better diffusion compared to other formulations[21,22].

METHODOLOGY

Materials

Snakehead fish (*Channa striata*) and lemon (*Citrus limon*) were purchased from the local market in Solo, Central Java. Materials and solvents used for extraction included distilled water obtained from PT. Agung Jaya (Solo, Central Java), ether, sodium sulfite, sodium acetate, buffer solution, and $CaCl_2$ obtained from Merck (Darmstadt, Germany), then HCl 37%, H_2SO_4, NaOH 6M purchased from CV. Anugrah Jaya Kimia (Surabaya, East Java). Determination of albumin and citric acid content in the extract used standard bovine serum albumin (BSA) from Sigma Aldrich, biuret reagent (ROFA Lab), methylene blue, Sudan III, and citric acid anhydrous 99% from Merck (Darmstadt, Germany), deionized water (OneMed, Indonesia), and technical grade ethanol from PT. Agung Jaya (Solo, Central Java). The formulation of nanoemulsion and nanoemulgel used Tween 80, Propylene glycol, and olive oil from Merck (Darmstadt, Germany), carbopol 940 and triethanolamine (TEA) purchased from Petronas Chemical, Malaysia, and Dimethyloldimethyl (DMDM) hydantoin purchased from CV. Cipta Kimia (Sukoharjo, Central Java).

Methods

Snakehead fish extraction and albumin content determination

A total of 50 grams of finely minced snakehead fish meat was mixed with 125 ml of acetatebuffer solution, then centrifuged at a speed of 2500 rpm for one hour. The snakehead fish solution was separated from the meat sediment, and then 150 ml of ether and 30 ml of 25% sodium sulfitewere added. It was centrifuged again at 2500 rpm for two hours. The sediment (the bottom layer containing albumin) was separated from the supernatant (the upper layer) for analysis of the albumin content contained within it. The analysis of albumin levels in snakehead fish extract was using spectrophotometry with a wavelength range of 450-700 nm. The materials required for analysis include a standard albumin, BSA (bovine serum albumin), and biuret reagent[23].

Lemon extraction and citric acid content determination

A total of 75 ml of lemon juice was adjusted to a pH of 7.5-8 by adding 2M NaOH. Then, 37.5 ml of 10% $CaCl_2$ was added to the lemon juice, and it was heated on a hotplate until it reached a constant boiling temperature of 165°C. Subsequently, the solution was decanted, and the formedprecipitate was treated with 8.25 ml of 2M H_2SO_4. This mixture was heated and decanted again, similar to the previous process. The obtained precipitate was left to dry[24]. The analysis of citric acid levels in lemon extract was using spectrophotometry with a wavelength range of 190-280 nm. The materials required for analysis include citric acid standard, deionized water, and 0.25 Mof HCl[24,25].

Preparation of snakehead fish and lemon extract nanoemulsion

Two formulations of nanoemulsion (NE) with snakehead fish and lemon extracts were developed. NE1 consists of 30% propylene glycol, 25% tween 80%, 7% snakehead fish extract, 3% lemon extract, 5% olive oil, and distilled water. NE2 differs in the percentage of the two extracts, with 3% snakehead fish extract and 7% lemon extract. The homogenization anddispersion of nanoemulsion particles were carried out using a magnetic stirrer and ultra turrax at a speed of 12,000 rpm for 5 minutes[26].

Characterization of snakehead fish and lemon extract nanoemulsion

Nanoemulsion (NE) characterization was tested based on emulsion type criteria, kinetic andthermodynamic stability, pH, and particle size[27,28].

Preparation of gel base and incorporation of nanoemulsion in the gel base

The gel base is prepared by dispersing 2% carbopol in warm water, followed by adding 1.15% triethanolamine (TEA), 0.38% dimethyloldimethyl (DMDM) hydantoin, and aquadest to make a total of 100 grams of gel base. The incorporation of 25 grams of NE into 75 grams of thegel base is achieved through a combination of an ultra turrax at 10,000 rpm for 5 minutes and anultrasonicator probe with a 70% amplitude for 45 minutes[29].

Characterization of snakehead fish and lemon extract nanoemulgel

Nanoemulgel (NEG) characterization was tested based on pH, viscosity, spreadability, adhesion, particle size, and zeta potential[27,28,30].

In vivo study for wound healing activity of snakehead fish and lemon extract nanoemulgel

A total of 21 female rats aged 2-3 months underwent acclimatization for two weeks in the Animal Laboratory of Faculty of Medicine UNS. The rats were

then divided into seven treatmentgroups. Post-operative wounds were created through minor surgery on the abdominal skin of therats with an incision length of ±2 cm, stitched using plain catgut. After surgery, the rats were treated according to the assigned test group, with Group I as a placebo control, II with NEG1, III with NE2, IV as normal, V with commercial preparation, VI with NEG1 extract, and VI with NEG2 extract. The treatment was given for 15 days, and wound length examination was every 5 days[31].

Histopathology examination of wound tissue

Skin tissue from the abdomen, approximately 3x3 cm in size, was collected, washed with sodium chloride solution, and then immersed in 10% Neutral Buffered Formalin (NBF). The tissue was then stained with H&E (Hematoxylin and Eosin). Wound re-epithelialization was observed under a light microscope at 100× magnification[18].

Data analysis

The analysis methods involved the use of software such as MS Excel, SPSS, GraphPad Prism,and Image-J. The wound tissue re-epithelialization was analyzed with Image-J and the effectivityof the gel in wound healing was concluded through One-Way ANOVA analysis with a confidencelevel of 95%.

RESULTS and DISCUSSION

Determination of albumin content in snakehead fish extract

The extraction of snakehead fish (SF) was performed at the isoelectric point (pH 4,6) which is the pH range when protein solubility is lowest, making it easier to form precipitates[32]. Therefore, a pH 4.6 acetate buffer was used as the solvent. Separation of the extract from the SFmeat residue was carried out by centrifugation for one hour at 2500 rpm. Purification of the SF extract from non-protein components was done using ether with the "like dissolve like" principle.The precipitation of the SF extract containing albumin (AL) was achieved through the salting-out mechanism by adding sodium sulfite[33]. The yield of the extraction process shown in Table 1. Thepresence of AL content in SF extract was confirmed through the similarity of wavelengths and visible spectrum between the SF extract and BSA[34]. The maximum wavelength for BSA is 544 nm and the maximum wavelength for the extract sample is 543 nm, with the similiar spectrum asshown in Figure 1(a). Quantification showed that there was 77% AL content in the yield of the SFextract.

Table 1. Yield of the snakehead fish extraction

Sample	Weight (g)	Yield (%)
Snakehead fish meat	50	-
Snakehead fish extract	0.4	0.8
Albumin content	0.3	77

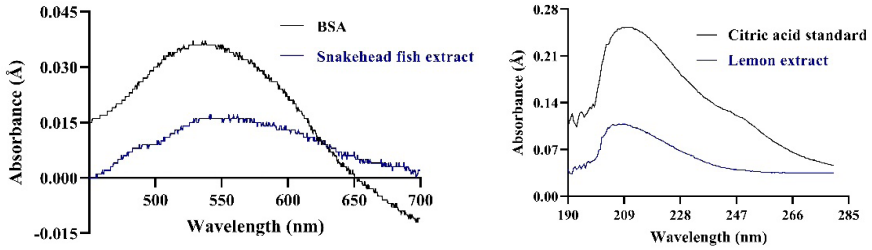

Figure 1. Visible spectrum of Bovine Serum Albumin (BSA) and snakehead fish extract (a); UV spectrum of citric acid standard and lemon extract (b)

Determination of citric acid content in lemon extract

The lemon extraction process begins by creating lemon juice in an alkaline environment through the addition of sodium hydroxide to form clearer and amorphous crystal precipitates[35]. Citric acid (CA) in the lemon reacts with the added calcium chloride, resulting in the formation of a salt precipitate. At this stage, two salts are formed: calcium citrate, which precipitates, and sodium chloride, which remains dissolved. Therefore, decantation is performed to separate them.The calcium citrate precipitate is then converted back into CA by adding sulfuric acid[24]. The yieldof the extraction process shown in Table 2. The presence of CA content in lemon extract was confirmed through the similarity of wavelengths and UV spectrum between the lemon extract andCA standard[34]. The maximum wavelength for CA standard is 210 nm and the maximum wavelength for the extract sample is 209,5 nm, with the similar spectrum as shown in Figure 1b.Quantification showed that there was 64% CA content in the yield of the lemon extract.

Table 2. Yield of the lemon extract

Sample	Weight (g)	Yield (%)
Lemon juice	75	-
Lemon extract	3.75	5
Citric acid content	2.39	64

Characterization of snakehead fish and lemon extract nanoemulsion

Nanoparticles (140-400 nm) can optimize the delivery of active ingredients due to their large particle surface area, allowing for an increased number of particles that can be incorporated into the hydrogel matrix[19,36]. Based on Table 3, the particle size of both formulations fell within the nanoparticle range, with 294.13 ± 1.63 nm for NE1 and 181.5 ± 0.78 nm for NE2. The particle size distribution of both formulations can be seen in Figure 2 and is classified as a monodisperse andhomogeneous system (PDI < 1.0) with a PDI value of 0.39 ± 0.01 for NE1 and 0.24 ± 0.03 for NE2[37].

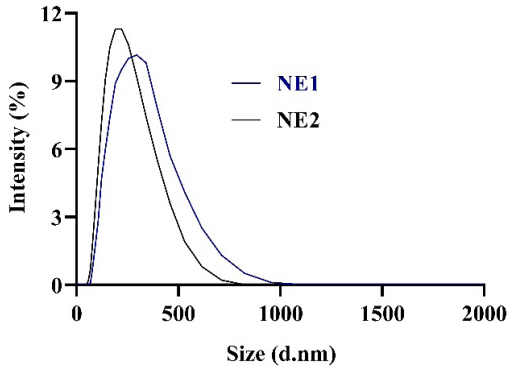

Figure 2. Particle size distribution of nanoemulsion formulations

The higher content of citric acid in NE2 compared to NE1 makes the pH of NE2 more acidic than NE1, as presented in Table 3. Both formulations show that they are O/W emulsion type which are more stable for incorporation into water-based gel formulations. Both NE formulationsare stable kinetically because after the kinetic stability test with centrifugation at 3800 rpm for 30 minutes, no creaming or flocculation occurred[38]. However, both NE formulations exhibited thermodynamic instability, as indicated by the reversible separation of the aqueous and oil phasesafter storage at -4°C and 40°C for 24 hours[39]. This is consistent with the findings of Ullah et al. that NE tend to be thermodynamically unstable but stable kinetically[27].

Table 3. Characterization of the snakehead fish and lemon extract nanoemulsion formulations (Mean ± SD, n=3)

Formulation code	pH	Emulsion type	Mean particle size (nm)	Polydispersity index	Stability	
					Kinetic	Thermodynamic
NE1	3.36 ± 0,09	M/A	294.13 ± 1.63	0.39 ± 0.01	√	√
NE2	2.69 ± 0,1	M/A	181.5 ± 0.78	0.24 ± 0.03	√	√

Characterization of snakehead fish and lemon extract nanoemulgel

Both nanoemulgels (NEGs) were within the nanometer particle size range that can easily penetrate the skin, which is 140-400 nm[36]. The droplet size for NEG1 and NEG2 are 223,27 ± 8,02 nm and 231,80 ± 10,58 nm with monodisperse system (PDI < 1,0)[37], as presented in Figure 3a. Figure 3b shows the zeta potential values of NEG1 and NEG2 are -22.4 ± 0.4 and -21.2 ± 0.6, indicating good stability (-30 mV hingga -20 mV)[40,41].

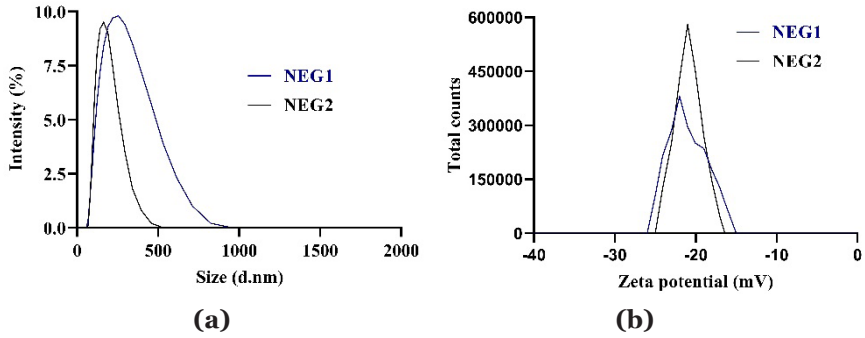

Figure 3. Particle size distribution of nanoemulgel formulations (a); zeta potential distribution of nanoemulgel formulations (b)

As presented in Table 4, the pH values of both formulations fall within the safe pH range forskin (5,0-8,0)[42]. NEG1 has higher viscosity than NEG2, indicating that NEG1 provides better adhesion, prolonging the contact time of the formulation with the skin[43]. On the other hand, NEG2, with lower viscosity, exhibits better spreadability compared to NEG1[44].

Table 4. Characterization of the snakehead fish and lemon extract nanoemulgel formulations (Mean ± SD, n=3)

Formulation code	Mean particle size (nm)	Polydispersity index	Zeta Potensial (mV)	pH	Viscosity (cPs)	Spreadability (cm)	Adhesiveness (s)
NEG1	223.27 ± 8.02	0.5 ± 0.04	-22.4 ± 0.4	6.83 ± 0.03	8038 ± 3	3.20 ± 1.42	17.41 ± 2.41
NEG2	231.80 ± 10.58	0.66 ± 0.01	-21.2 ± 0.6	6.77 ± 0.04	445 ± 3	4.30 ± 3.78	2.07 ± 0.78

In vivo wound healing activity

The wound healing pattern in post-operative rats in the in vivo study, as shown in Figure 4, illustrates the ability of the NEG formulation of SFL extract to aid in the healing of post-caesareansection wounds. Wound healing activity is assessed by the closure of the wound and the fading of scars.

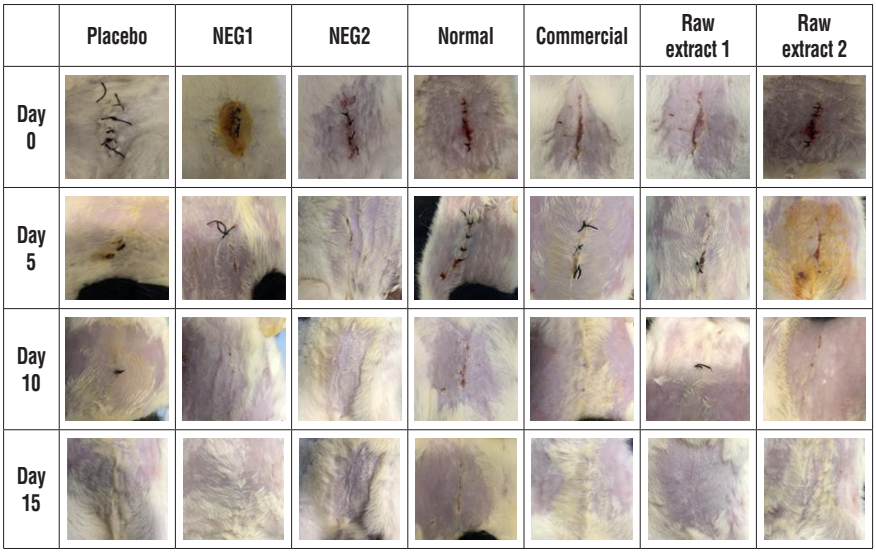

Figure 4. Representative image of wound healing progress in rat for 15 days

Furthermore, the length of wound closure was calculated every 5 days for all treatment groups, and the results are presented in Figure 4. Meanwhile, Figure 5(a) shows the percentage ofwound closure for all groups on the 10th day, which is significantly different (p-value < 0.05). Thegroups treated with NEG1 and NEG2 also exhibited significantly different percentages of woundclosure compared to the normal group, indicating that the NEG formulation of lemon and snakehead fish extracts has wound healing activity. NEG1 and NEG2 are

also found to have betterwound healing activity than commercial albumin formulations because the percentage of woundclosure in both of these groups significantly differs from the commercial formulation group. The percentage of wound closure in the NEG1 and NEG2 groups also significantly differs from the NEG1 extract and NEG2 extract groups, indicating that the NEG carrier can optimize the effectiveness of SFL extracts in wound healing. The NEG1 group differs significantly from the NEG2 group, with an average percentage of wound closure (%) in the NEG1 group of 84 ± 2.1 andin the NEG2 group of 81 ± 1.5. It can be concluded that NEG1 has better wound healing activity than NEG2.

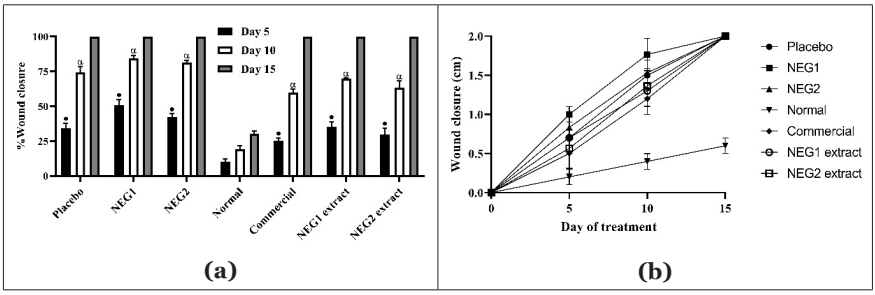

Figure 5. Wound closure percentage diagram (a); wound closure length from day 0 until day 15 (b). The significant difference ($p<0,05$) in comparison with normal group are expressed as • for day 5 and α for day 10.

Histopathology of wound tissue

Epidermal thickening accompanied by an abundance of sebaceous glands and hair follicles indicates better tissue regeneration of the skin[45]. Figure 6(c) shows that on the 15th day, the NEG1group has mature hair follicles, numerous sebaceous glands, and a more organized skin structure compared to the other groups. The placebo group, NEG2, commercial group, NEG1 extract, and NEG2 extract also displayed sebaceous glands and hair follicles, as seen in Figure 6. However, the skin structure and the maturation of the glands were not as advanced as in the NEG1 group. In the normal group, only undifferentiated glands were present.

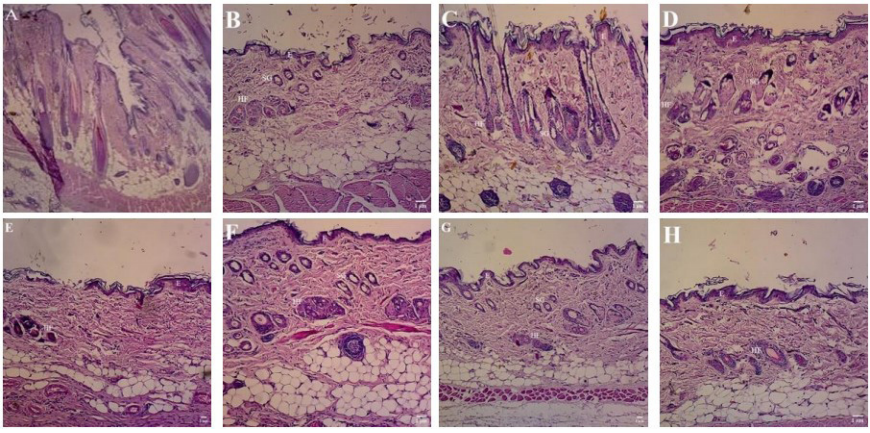

Figure 6. H&E staining photomicrographs of all groups: wound skin (A), placebo group (B), NEG1 group (C), NEG2 group (D), normal group (E), commercial product group (F), NEG1 extract group (G), and NEG2 extract group (H). Epidermis (E), sebaceous glands (SG), and hair follicles (HF) were marked.

Figure 7 illustrates the epidermal thickness from largest to smallest as NEG1 > NEG2 > Commercial > NEG1 extract > NEG2 extract > Placebo > Normal. The test results showed that only the epidermal thickness of the NEG1 and NEG2 groups differs significantly (p-value < 0.05)from the normal group. Therefore, it can be inferred that NEG1 and NEG2 are more effective in aiding wound healing compared to others. The epidermal thickness of NEG1 significantly differs from NEG2, with an average epidermal thickness of the NEG1 group at 2.31 ± 0.06 μm and the NEG2 group at 1.69 ± 0.22 μm. Thus, NEG1 containing 7% snakehead fish extract and 3% lemon extract can facilitate wound healing by stimulating epidermal thickening and skin structure reconstruction more rapidly than NEG2.

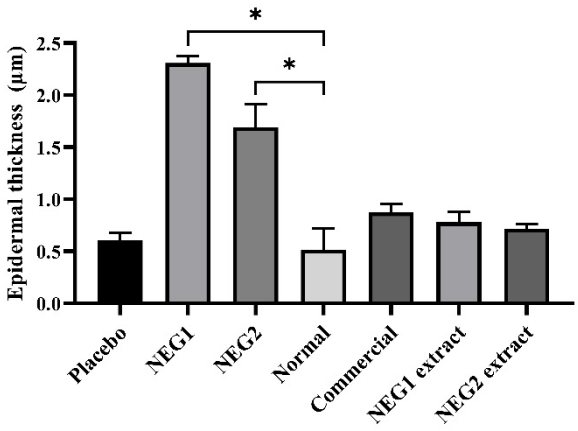

Figure 7. Diagram of the epidermal thickness on the 15th day of treatment. Symbol * expressed the significant difference ($p<0.05$) in comparison with normal group.

Based on the results of this study, it can be concluded that the nanoemulgel design has successfully optimized the activity of snakehead fish extract and lemon extract in wound healing. F1 nanoemulgel which contains 7% snakehead fish extract, and 3% lemon extract has better characteristics and effectiveness. The study revealed that F1 was histopathologically proven to stimulate post-cesarean section wound healing through re-epithelialization and reconstruction of skin tissue structure better than other formulas.

STATEMENT OF ETHICS

In vivo studies have been eligible for ethical eligibility based on the ethical eligibility letter issued by Moewardi Hospital, Surakarta, with letter number 1.220/VI/HREC/2023. In vivo testing procedures and animal welfare assurance have been approved by the head of the Experimental Animal Laboratory, Faculty of Medicine, Sebelas Maret University.

CONFLICT OF INTEREST STATEMENT

The authors report that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

AA, NAB, TASD designed the study. TASD, FAN worked on literature search. AA, NAB,TASD, FAN, SAR conducted the experimental work and collected the data. AA, NAB analyzed and interpreted the data. TASD, FAN, SAR wrote the draft of manuscript. All authors involvedin revising the final manuscript. AA supervised the study and proofread the manuscript.

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REFERENCES

1. Marmitt LP, Machado AKF, Cesar JA. Recent trends in cesarean section reduction in extreme south of Brazil: a reality only in the public sector? Ciênc saúde coletiva, 2022;27(8):3307-3307. Doi: 10.1590/1413-81232022278.05742022

2. Simamora DL, Sinaga EW. Systematic review relationship between cesarean section delivery and asphycia incidence. JUK-medifa, 2023;1(02).ISSN: 2963-993X.

3. Ayuningtyas D, Oktarina R, Misnaniarti M, Dwi Sutrisnawati NN. Health ethics in sectio caesarean delivery without medical indication. MKMI, 2018;14(1):9. Doi: 10.30597/mkmi.v14i1

4. Song Q, Yan J, Bu N, Qian Y. Efficacy and safety of broad spectrum penicillin with or without beta-lactamase inhibitors vs first and second generation cephalosporins as prophylactic antibiotics during cesarean section: a systematic review and meta-analysis. J Obstet Gynaecol, 2023;43(1):2195946. Doi: 10.1080/01443615.2023.2195946

5. Zhang T, Wang J, Hua Z, Yao X, Zhang F, Zhou Y. Effect of adjunctive prophylactic macrolides used at the caesarean section on endometritis and surgical site wound infection: a meta-analysis. Int Wound J, 2023;20(8):3307-3314. Doi: 10.1111/iwj.14211

6. Shi M, Chen L, Ma X, Wu B. The risk factors and nursing countermeasures of sepsis after cesarean section: a retrospective analysis. BMC Pregnancy Childbirth, 2022;22(1):696. Doi: 10.1186/s12884-022-04982-8

7. Li L, Cui H. The risk factors and care measures of surgical site infection after cesarean section in China: a retrospective analysis. BMC Surg, 2021;21(1):248. Doi: 10.1186/s12893-021-01154-x

8. Caputo WJ, Monterosa P, Beggs D. Antibiotic misuse in wound care: can bacterial localization through fluorescence imaging help? Diagnostics, 2022;12(12):3207. Doi: 10.3390/diagnostics12123207

9. Primadina N, Basori A, Perdanakusuma DS. Wound healing process from the aspects of cellular and molecular mechanisms. Qanun Medika, 2019;3(1):31. Doi: 10.30651/jqm.v3i1.2198

10. Hutapea TPH, Madurani KA, Syahputra MY, Hudha MN, Asriana AN. Albumin: source, preparation, determination, applications, and prospects. J Sci Adv Mater Devices, 2023;8(2):100549. Doi: 10.1016/j.jsamd.2023.100549

11. Fajrin DH, Lamana A, Ardiansyah F. Differences in the effect of boiled eggs and cork fishon the duration of perineal wound healing in postpartum women. J Ilm Obsgin, 2023;15(2):192-197. Doi: 10.36089/job.v15i2.1165

12. Sari SM, Anggraini A, Putri RD. Cork fish extract on perineal wound. JMM, 2020;4(4):305-311. Doi: 10.33024/jmm.v4i4.3332

13. Fitrianti E, Zulkarnain Z, Nurmayanti N. Effectiveness of cork fish extract albumin (*Channa striata*) on postoperative wound healing in domestic cats at UPTD Puskeswan Makassar. Filogeni, 2023;3(2):79-84. Doi: 10.24252/filogeni.v3i2.34747

14. Yulianda D, Maharani L, Suryoputri MW. Use of oral albumin and injection albumin in liver cirrhosis patients at RSUD Prof. Dr. Margono Soekarjo Purwokerto. Acta Pharm Indo, 2020;8(1):8. Doi: 10.20884/1.api.2020.8.1.2437

15. Andrie M, Sihombing D. The effectiveness of snakehead (*Channa striata*) extract containing ointment on healing process of acute stage II opened wound on male Wistar rats. Pharm Sci Res, 2017;4(2):88. Doi: 10.7454/psr.v4i2.3602

16. Dewi KEK, Habibah N, Mastra N. Inhibition test of various concentrations of lemon juice against *Propionibacterium acnes* bacteria. JST, 2020;9(1):86-93. Doi: 10.23887/jstundiksha .v9i1.19216

17. Rohani S. Isolation and characterization of wound healing compounds from chloroform extract of Binahong leaves (*Anredera cordifolia* (Ten.) Steenis). Magnamed, 2021;8(1):40. Doi: 10.26714/magnamed.8.1.2021.40-59

18. Sim P, Strudwick XL, Song Y, Cowin AJ, Garg S. Influence of acidic pH on wound healing in vivo: a novel perspective for wound treatment. IJMS, 2022;23(21):13655. Doi: 10.3390/ijms232113655

19. Donthi MR, Munnangi SR, Krishna KV, Saha RN, Singhvi G, Dubey SK. Nanoemulgel: a novel nano carrier as a tool for topical drug delivery. Pharmaceutics, 2023;15(1):164. Doi: 10.3390/pharmaceutics15010164

20. Az-zahra AP, Wijayanti FT, Ramadhanti L, Faizal IA. Formulation and evaluation of eel fish oil nanoemulsion using sonication method. Pharmaqueous, 2022;4(2):25-34. Doi: 10.36760/jp.v4i2.448

21. Indalifiany A, Malaka MH, Sahidin, Fristiohady A, Andriani R. Formulation and physical stability test of nanoemulgel containing *Petrosia* sp. ethanolic extract. JFSP, 2021;7(3):321-331. Doi: 10.31603/pharmacy.v7i3.6080

22. Akram S, Anton N, Omran Z, Vandamme T. Water-in-oil nano-emulsions prepared by spontaneous emulsification: New insights on the formulation process. Pharmaceutics, 2021;13(7):1030. Doi: 10.3390/pharmaceutics13071030

23. Suardi S, Bahri S, Khairuddin, Sumarni NK, Rahim EA. Comparison of cork fish (*Channa striata*) albumin content from boiling and steaming process by using biuret test. Kovalen, 2020;6(1):67-73. Doi: 10.22487/kovalen.2020.v6.i1.12699

24. Kanse NG, Deepali M, Kiran P, Priyanka B, dan Dhanke PA. Review on citric acid production and its applications. Int J Curr Adv Res, 2017;6(9):5880-5883. Doi: 10.24327/ijcar.2017.5883.0825

25. Krukowski S, Karasiewicz M, Kolodziejski W. Convenient UV spectrophotometric determination of citrates in aqueous solutions with applications in the pharmaceutical analysis of oral electrolyte formulations. J Food Drug Anal, 2017;25(1):717-722. Doi: 10.1016/j.jfda.2017.01.009

26. Noriega-Peláez EK, Mendoza-Muñoz N, Ganem-Quintanar A, Quintanar-Guerrero D. Optimization of the emulsification and solvent displacement method for the preparation of solid lipid nanoparticles. Drug Dev Ind Pharm, 2011;37(2):160-166. Doi: 10.3109/03639045.2010.501800

27. Ullah N, Amin A, Farid A, Selim S, Rashid S. Development and evaluation of essential oil- based nanoemulgel formulation for the treatment of oral bacterial infections. Gels, 2023;9(3):1-21. Doi: 10.3390/gels9030252

28. Dawoud MHS, Abdel-Daim A, Nour MS, Sweed NM. A quality by design paradigm for albumin-based nanoparticles: formulation optimization and enhancement of the antitumor activity. J Pharm Innov, 2023;1(1):1-20. Doi: 10.1007/s12247-022-09698-y

29. Zheng N, Wang L, Sun Z. The effects of ultrasonication power and time on the dispersion stability of few-layer graphene nanofluids under the constant ultrasonic energy consumption condition. Ultrason Sonochemistry, 2021;80(1):1-7. Doi: 10.1016/j.ultsonch.2021.105816

30. Rahmawati DA, Setiawan I. The formulation and physical stability test of gel fruit strawberry extract (*Fragaria x ananassa* Duch.). JNHM, 2019;2(1):38-46. Doi: 10.23917/jnhm.v2i1.8046

31. Anwar K, Widodo DF, Nurlely, Triyasmono L, Sudarsono, Nugroho AE. Activity of ethanol extract gel of alum ut (*Ampelocissus rubiginosa* L.) root tubers on healing of incisional wounds in Wistar rats. Trad Med J, 2018;23(1):30-39. Doi: 10.36387/jiis.v4i1.245

32. Asfar M, Tawali AB, Mahendradatta M. Extraction of snakehead fish (*Channa striata*) albumin at its isoelectric point. J Agercolere, 2019;1(1):6-12. Doi: 10.37195/jac.v1i1.55

33. Dahal YR, Schmit JD. Ion specificity and nonmonotonic protein solubility from salt entropy. Biophys J, 2018;114(1):76-87. Doi: 10.1016/j.bpj.2017.10.040

34. Irnawati, Sahumena MH, Dewi WON. Analysis of hydroquinone in facial whitening cream using the UV-Vis spectrophotometric method. Pharmacon, 2016;5(3):229-237. Doi: 10.35799/pha.5.2016.15074

35. Puspadewi R, Anugrah R, Sabila D. The ability of *Aspergillus wentii* to produce citric acid. Kartika J Ilm Farm, 2017;5(1):15-20. Doi: 10.26874/kjif.v5i1.83

36. Saani MS, Abdolalizadeh J, Heris SZ. Ultrasonic/sonochemical synthesis and evaluation ofnanostructured oil in water emulsions for topical delivery of protein drugs. Ultrason Sonochemistry, 2019;55:86-95. Doi: 10.1016/j.ultsonch.2019.03.018

37. Algahtani M, Ahmad M, Nourein I, Albarqi H. Preparation and characterization of curcuminnanoemulgel utilizing ultrasonication technique for wound healing: *in vitro*, *ex vivo*, and *in vivo* evaluation. Gels, 2021;7(4):1-17. Doi: 10.3390/gels7040213

38. Souto EB, Cano A, Martins-Gomes C, Coutinho TE, Zielińska A, Silva AM. Microemulsions and nanoemulsions in skin drug delivery. Bioengineering, 2022;9(4):158. Doi: 10.3390/bioengineering9040158

39. Ribeiro RC, Barreto SM, Ostrosky EA, da Rocha-Filho PA, Veríssimo LM, Ferrari M. Production and characterization of cosmetic nanoemulsions containing *Opuntia ficus-indica* (L.) mill extract as moisturizing agent. Molecules, 2015;20(2):2492-509.Doi:10.3390/molecules20022492

40. Rezaei A, Abdollahi H, Derikvand Z, Hemmati-Sarapardeh A, Mosavi A, Nabipour N. Insights into the effects of pore size distribution on the flowing behavior of carbonate rocks: linking a nano-based enhanced oil recovery method to rock typing. Nanomaterials, 2020;10(5):972-980. Doi: 10.3390/nano10050972

41. Maruno M, Rocha-Filho PA. O/W nanoemulsion after 15 years of preparation: a suitable vehicle for pharmaceutical and cosmetic applications. J Dispers Sci Technol, 2009;31(1):17-22. Doi: 10.1080/01932690903123775

42. Sanaji JB, Krismala MS, Liananda FR. The effect of Tween 80 concentration as a surfactant on nanoemulgel Ibuprofen's physical characteristics. Indonesian J Med Sci, 2019; 6(2):1-9. ISSN: 2623-0038

43. Yati K, Jufri M, Gozan M, Mardiastuti Dwita LP. The effect of varying concentrations of hydroxy propyl methyl cellulose (HPMC) on the physical stability of tobacco extract gel (*Nicotiana tabaccum* L.) and its activity against *Streptococcus mutans*. Pharm Sci Res, 2018;5(3):133-141. Doi: 10.7454/psr.v5i3.4146

44. Dantas MG, Reis SA, Damasceno CM, Rolim LA, Rolim-Neto PJ, Carvalho FO, et al. Development and evaluation of stability of a gel formulation containing the monoterpene borneol. Sci World J, 2016;7394685:1-4. Doi:10.1155/2016/7394685

45. Balderas-Cordero D, Canales-Alvarez O, Sánchez-Sánchez R, Cabrera-Wrooman A, Canales-Martinez MM, Rodriguez-Monroy MA. Anti-inflammatory and histological analysis of skin wound healing through topical application of Mexican propolis. Int J Mol Sci, 2023;24(14):1-19. Doi: 10.3390/ijms241411831

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Thermosensitive gel of pomegranate peel extract as alternative for the treatment of local infections

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ABSTRACT

Studies on the use of herbal extracts in the treatment of local diseases are increasing day by day. To maintain activity of extract and provide convenient usage, ideal formulation must be developed for extracts. In this study, we aimed to develop thermosensitive gel of pomegranate peel extract and investigate its antimicrobial activity. The total phenolic content and antioxidant capacity of extract was found 397.00 ± 9.36 mg GAE/100 g, and 10750.00 ± 132.29 mg TE/100 g respectively. The gelation temperature of thermosensitive was measured as 25.3 ± 1.5˚C. The developed gel formulation showed antimicrobial activity against *Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida albicans* as similar with extract. As a result, the developed thermosensitive gel formulation of pomegranate peel extract could be an alternative for the treatment of local infections by its ease of usage and efficacy in future clinical trials after detailed characterization.

Keywords: pomegranate peel, extract, thermosensitive gel, antimicrobial activity

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INTRODUCTION

Pomegranate peel is an important resource with its bioactive compounds such as tannins, phenolic acids, and flavonoids. Thanks to its content, pomegranate peel provides antioxidant, anti-inflammatory, anticancer, and antimicrobial effects. However, its usage has been limited and it is mostly disposed of as agricultural waste. To turn it into a medicine, studies carried out are increasing day by day[1-6].

On the other hand, these studies are limited to the application of the extract as a solution form. In the drug development process, formulation development study is one of the critical steps to maintain activity of drug candidate and provide convenient usage for patient. Some of the development studies cover the application of pomegranate peel extract with the developed gel formulations. Mittal et al. evaluated the efficacy of the gel they prepared gel using carboxy-methyl cellulose. At the end of the study, they observed that only the gel containing pomegranate peel extract from the three gels they compared was effective enough to reduce the number of *Enterococcus faecalis*[7]. In another study, the extract obtained from peel of pomegranate was formulated as a carboxymethylcellulose-based gel[8]. Gel formulation for pomegranate peel extract, developed by Vasconcelos et al., consisting of carbopol, water, and triethanolamine, and efficacy was demonstrated on various microorganisms[8]. Similarly, carbopol gel formulation was also used to evaluate the effectiveness of pomegranate peel extract in the healing of diabetic wounds, and the results of the study showed that wound healing improved[9]. Additionally, chitosan/gelatin gels containing pomegranate peel extract were prepared by Bertolo et al.[10].

Unlike the gels that are generally formed with carbopol, carboxy methyl cellulose and chitosan, thermosensitive gels offer an important opportunity for local administration of pomegranate peel extract, because formulation is liquid at room temperature and gel at body temperature. With these feature, thermosensitive gel formulation is an attention-grabbing alternative to provide long-term retention and controlled drug release of extract in the application area with easy usage[11-12]. In this study, a thermosensitive gel formulation containing pomegranate peel extract was developed to provide a new alternative to the use of pomegranate peel extract in the treatment of local infections. For this purpose, ethanol extract of pomegranate peel was obtained, total phenolic content and antioxidant activity of the obtained extract were evaluated to standardize the properties of the extract. Then poloxamer 407 based gel formulation of pomegranate peel extract was prepared, gelation temperature was determined, and antimicrobial activity tests were performed against *Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*.

METHODOLOGY

Materials

Fruit of *Punica granatum* L. was obtained a local manufacturer from Bilecik, Turkey. Poloxamer 407 and Polyether sulfone (PES) syringe filter were purchased from BASF and Merck, respectively. All chemicals and microbial growth mediums were also obtained from Merck. pH meter (MW801, Milwaukee, Portable Meter), precision balance (TW423L model, Shimadzu Corp.), incubator (En120) was used in the experiments.

Preparation pomegranate peel extract

150 grams of fresh pomegranate peel was ground with a knife grinder, and 1000 ml of ethanol/water (70/30) mixture cooled to +4 °C was added to the peel[13]. The resulting suspension was shaken at 150 rpm for 2 hours at room temperature in a dark room. The resulting supernatant was filtered and concentrated under vacuum at +40 °C. The obtained extract was filtered through a 0.22 μm PES syringe filter and stored at -20°C. The solids content of the obtained extract was determined after all water had been removed.

Determination of total phenolic component and antioxidant activity

Total phenolic component and antioxidant activity were determined to standardize the properties of the extract. The Folin–Ciocalteu method was used to quantitatively determine the phenolic substances contained in the pomegranate peel. This method, which is widely used to measure plant-derived phenols, was carried out by Singleton and Rossi's method[14,15]. Briefly, 0.2 N folin reagent was added to the samples and then incubated by adding sodium carbonate solution and calculated by absorbance measured at 765 nm. The result is expressed as mg GAE (Gallic Acid Equivalent)/100 grams of sample. The 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging is a frequently used method to determine antioxidant activity. The free radical scavenging activity of the samples was determined according to DPPH method. In this method, 4 mL of 0.004% (w/v) methanolic DPPH solution and extract solutions were mixed, and the absorbance of the samples was measured at 517 nm after they were incubated for 30 minutes at room temperature in the dark[16,17]. Using Equation 1, the DPPH scavenging activity (%) of the samples was calculated. The DPPH radical scavenging activities of the extracts were calculated as trolox equivalents (mg TEs/g).

Equation 1:

$$\textit{The DPPH Scavenging activity (\%)} = \frac{\textit{Absorbance of Control - Absorbance of Sample}}{\textit{Absorbance of Control}} \text{ x100}$$

Preparation of extract containing thermosensitive gel and determination of gelation temperature

Thermosensitive gel formulation containing pomegranate peel extract was prepared with cold method[18,19]. Briefly after pomegranate peel extract concentrate, cooled down to 4˚C and poloxamer 407 (18% w/v) was added slowly and mixed. The mixture was stored overnight at 4˚C to obtain a clear solution. For the determination of the solid content originating from the extract, the extract was completely dried, and its weight was weighed. Based on this weight, the gel concentration was prepared to be 39,6 mg/mL. For the determination of gelation temperature, 10 ml of gel was added into a 30 R vial and cooled to 4˚C. Then, while the vial was stirred continuously at 150 rpm, the temperature was increased to 1˚C per minute and the point at which magnetic stirrer bar stopped turning was determined as the gelation temperature.

Antimicrobial activity of extract containing gel

In antimicrobial activity tests, 2 Gram (+) bacterial cells, 1 Gram (-) bacterial cell and 1 yeast cell obtained from the American Type Culture Collection were used. Information on the microorganisms used is given in Table 1.

Table 1. Microorganisms and media used in antimicrobial activity tests (MHA: Mueller Hinton Agar, SDA: Sabouraud Dextrose Agar)

Microorganism		Medium
Enterococcus faecalis ATCC 2942	Gram (+) bacteria	MHA
Staphylococcus aureus ATCC 29213	Gram (+) bacteria	MHA
Pseudomonas aeroginosa ATCC 27853	Gram (-) bacteria	MHA
Candida albicans ATCC 24433	Yeast	SDA

In order to test the antimicrobial activities of the obtained formulation, the agar well diffusion method[20], which is similar to the Disk Diffusion, NCCLS, 2003 method, was used. Mueller Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for yeasts were used. Bacterial strains used in the studies were adjusted according to Mc Farland 0.5 (1.5×10^8) and yeast strains

were adjusted according to Mc Farland 2 (6 x 10^8) and inoculated into media autoclaved at 121°C for 15 minutes. Only ethanol-impregnated discs were used as negative control and commercial antibiotics (Azithromycin (0.97 µg/L) and Voriconazole (3.90 µg/L)) were used as positive control. After adding test samples, bacteria were incubated at 37°C for 24 hours and yeasts at 30°C for 48 hours. At the end of the incubation period, it was observed whether inhibition zones were formed around the discs and the inhibition zones formed around the discs were measured using a millimetric ruler. Trials were carried out under aseptic conditions and in 3 parallels, and the tests were repeated twice to determine their accuracy[21]. ANOVA followed by Fisher's LSD post hoc test was used to compare more than two groups with Minitab®16 (Minitab Inc.; State College, PA, USA). When p-value was <0.05 (*), the difference between groups was considered statistically significant.

RESULTS and DISCUSSION

Determination of total phenolic component and antioxidant activity of pomegranate peel extract

The total phenolic content and antioxidant capacity of the pomegranate peel extract were determined as 397.00 ± 9.36 mg GAE/100 g and 10750.00 ± 132.29 mg TE/100 g, respectively. When this result was compared with previous literature results, Gozlekci et al. analyzed 4 pomegranate cultivars grown in Turkey ("Lefan," "Katirbasi," "Cekirdeksiz-IV," and "Asinar"). In their examination, they determined that the highest phenolic content was in the peel extract for all cultivars. They determined that this value varies between 1775.4-3547.8 mg GAE/L, depending on the pomegranate cultivars[22]. The type of pomegranate grown in Inhisar, Bilecik is the "Devedisi" cultivar, which is different from these four. On the other hand, the reason for the slightly higher value we obtained could be related to the step of preparing the pomegranate peel for extraction. In our study, pomegranate peel was not dried in the sun or in the oven, so higher activity could be preserved. In order to show the effect of drying, Marchi et al. examined the differences between the pomegranate peel by drying them in an oven and a lyophiliser[23]. At the end of the study, they showed that drying with temperature reduces the antioxidant capacity. While 595.7 µmol Trolox/g activity was determined in lyophilized samples, this value decreased to 351.3 µmol Trolox/g.[23]. Also, the evaluation of the solvent used, which is another critical point in the extraction method, was carried out by Malviya et al. In the extraction study performed with methanol, ethanol, water and their combinations, it was determined that the extracts obtained with 70 ethanol: 30 water or 100% water had the highest activity and phenolic con-

tent[24]. This finding also explains the high activity obtained with the 70 ethanol: 30 water ratio used in our study. Within the scope of our study, thanks to the fact that the products were used without drying and the extraction process was carried out at a temperature not exceeding +40°C, relatively higher total phenolic content and antioxidant capacity were obtained.

Preparation of extract containing thermosensitive gel and determination of gelation temperature

As the temperature increases, poloxamer 407 copolymer molecules assembly as spherical micelles. Its structure includes a dehydrated polypropylene oxide core and an outer core composed of hydrated polyethylene oxide chains. In cases where the concentration is sufficient, these micelles form the gel structure (Figure 1)[25].

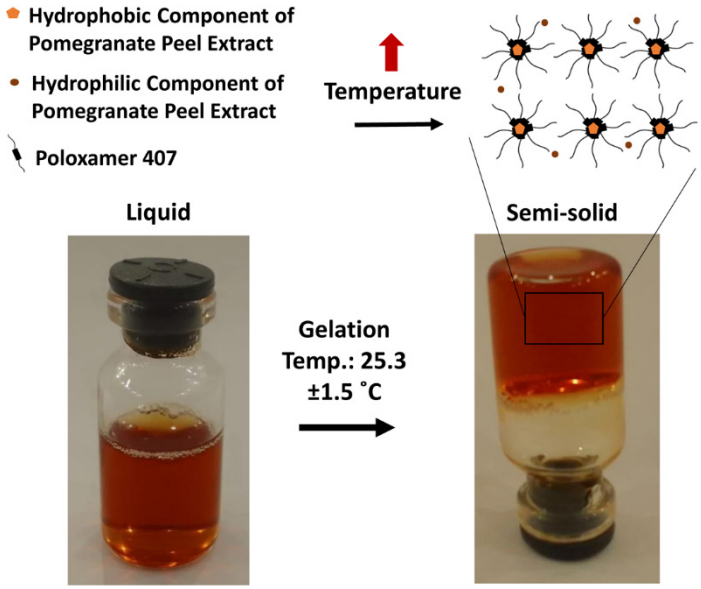

Figure 1. Schematic representation of extract containing thermosensitive gel and determination of gelation temperature

By this way, it can become gel form solution at body temperature. Especially thanks to its hydrophobic core, it offers an important opportunity to transport therapeutics, limited solubility in water. Although the ethanol is removed and the extract is filtered after ethanol extraction, the nano-sized precipitated substances can exist in extract. Another advantage of this gel prepared with Poloxamer 407 is that it can dissolve these precipitates and form a homogeneous solution. In this way, effectiveness of these substances could be increase[26]. In our study, a simple and industrially applicable formulation was developed by adding polymer

directly to the extract, which was concentrated and cooled. Since the gelation temperature of the obtained gel was measured, this value was found to be 25.3 ± 1.5˚C and it was determined that the gel obtained was reversible. This value obtained shows that the purpose of instant gelation at body temperature, which is in solution in cold, is achieved. When other studies conducted were examined, the gelation temperature of the gel developed by Cetin et al. for wound healing was found to be 28˚C[27]. There are studies showing that the gelation temperature prepared with poloxamer 407 can affect by other substances in the medium[25, 28,29]. So, the finding we obtained in our study is compatible with the literature. On the other hand, it is clearly seen in the literature that the gels prepared using 18% w/v poloxamer 407 have a fluid liquid consistency below the determined gelation temperature. This has been clearly shown in our previous studies. It has also been shown in our previous studies that the viscosity of the gel prepared with poloxamer 407 at this concentration has the expected spreadable properties[18,27,28]. In this study, based on our previous experiences, we primarily focused on the gelation temperature and activity, which are the main variable properties of the gel. A similar approach has previously been used by other groups developing gel formulations for pomegranate peel extract. Mittal et al. evaluated the efficacy of the gel; they prepared gel using carboxy-methyl cellulose[7]. In this study, gel was prepared, and antimicrobial assessment was performed directly as methodology. In another study, gel formulation for pomegranate peel extract, developed by Vasconcelos et al., consisting of carbopol, water, and triethanolamine. Similar methodology was used in this study[8]. Additionally, chitosan/gelatin gels containing pomegranate peel extract were prepared by Bertolo et al.[10]. In this study, rheological evaluation, total phenolics content, and antioxidant activity were measured as major parameters without antimicrobial activity tests. All these studies showed that each study can determine its methodology based on its own focused target without doing all the analysis. Since it has been clearly demonstrated in the literature that poloxamer thermosensitive gels can be easily applied to the application site, have long-term retention, and provide controlled release properties. So, *in vitro* antimicrobial activity tests were carried out to show that the obtained gel's efficacy rather than performing *in vivo* studies.

Antimicrobial activity of extract containing gel

Agar well diffusion method was used to determine antimicrobial activity of 4 different test microorganisms. The formed zone diameters after 24 and 48 hours of incubation were measured for bacteria and yeast, respectively. Inhibition zone diameters were found as 25, 21 ± 1.41, 23.5 ± 0.7, 15.5 ± 0.7 against *Enterococcus faecalis* (Figure 2), *Staphylococcus aureus* (Figure 3), *Pseudomonas aeruginosa* (Figure 4) *and Candida albicans* (Figure 5), respectively.

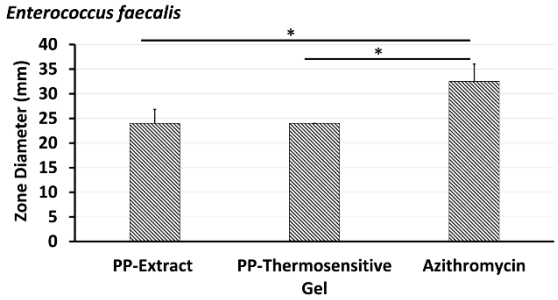

Figure 2. Inhibition zone diameters of PP-Extract (pomegranate peel extract), PP-Thermosensitive Gel (pomegranate peel extract containing thermosensitive gel) and Azithromycin (0.97 µg/L) against *Enterococcus faecalis,* *p<0.05

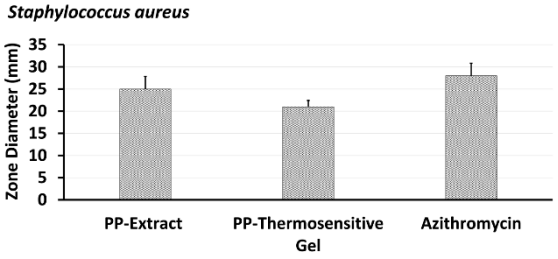

Figure 3. Inhibition zone diameters of PP-Extract (pomegranate peel extract), PP-Thermosensitive Gel (pomegranate peel extract containing thermosensitive gel) and Azithromycin (0.97 µg/L) against *Staphylococcus aureus* *p<0.05.

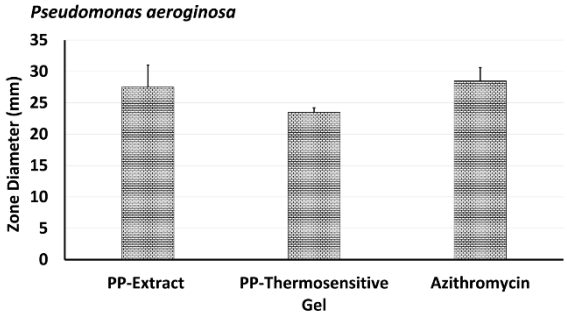

Figure 4. Inhibition zone diameters of PP-Extract (pomegranate peel extract), PP-Thermosensitive Gel (pomegranate peel extract containing thermosensitive gel) and Azithromycin (0.97 µg/L) against *Pseudomonas aeruginosa,* *p<0.05

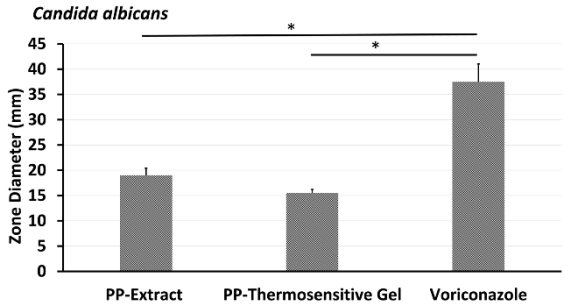

Figure 5. Inhibition zone diameters of PP-Extract (pomegranate peel extract), PP-Thermosensitive Gel (pomegranate peel extract containing thermosensitive gel) and Voriconazole (3.90 µg/L) against *Candida albicans*, *p<0.05

In addition, solid Nutrient Agar medium was prepared, and *Staphylococcus aureus* was inoculated into the whole petri dish with a smear. Then, a line dividing the petri dish in half was drawn and the gel obtained from pomegranate extract was applied to half of it as a thin layer and left to incubate in an oven at 37 ºC for 24 hours. At the end of the incubation, bacterial growth was observed in the non-gel-applied part of the petri dish, while very intense bacterial growth was not observed in the gel-applied part (Figure 6).

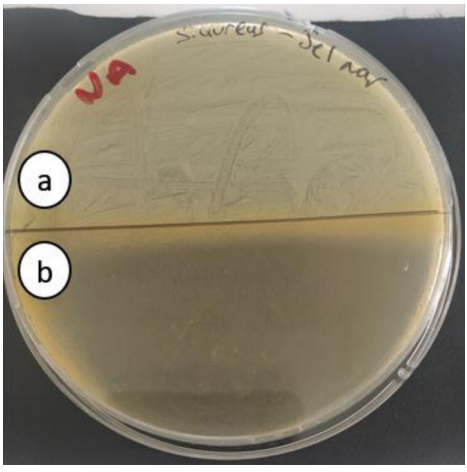

Figure 6. (a) Non-gel-applied part of *Staphylococcus aureus* inoculated petri dish. (b) Pomegranate peel extract containing thermosensitive gel applied part of *Staphylococcus aureus* inoculated petri dish.

When results are examined, it is observed that both the ethanol extract of pomegranate peel and its thermosensitive formulation show antimicrobial activity against to all tested organisms. Difference between these two groups is not statistically significant. On the other hand, slightly decreased activity shown in gel group. After addition of gel formulation to microorganisms, gel form occurs and extract release occurs with controlled release manner. Also, *in vitro* condition, water is limited when compared the vaginal, oral, and wound application sides. In the absence of water, it will take time for the gel to dissolve and the drug release from the gel will be limited. This may be the likely reason for this limited decrease in activity. Since the release in the application area cannot be fully imitated due to the need to provide a sink condition in the release study to be carried out in the *in vitro* environment, the medium in the petri dish, which would have the lowest water content, was tested as a worst-case scenario. Also, azithromycin and voriconazole activity showed, successfully as positive control groups. Although the antimicrobial activity of the extract and the gel obtained with this extract seems to be lower than the control groups, it is clearly understood that this effect will be related to the concentration of the positive controls and extract used[30]. And antimicrobial activity results of empty gel were not included in the results because no activity was observed in the empty gel group.

In another study, pomegranate peel was dried at 33˚C for 7 days and ground, then extracted and formulated as a carbopol-based gel. Here 0.5 ml extract was obtained from 540 mg powder. It has been stated that 1:64 dilution is effective for *Candida albicans*. In this method, due to drying, oxidation and degradation could be occurred. Because of difference in methods, studies are not comparable. On the other hand, antifungal activity was successfully demonstrated in both studies[8]. Conducting antimicrobial activity studies with pomegranate peel extracts, Demir et al. determined that the inhibition zone diameters were 21.00 and 18.50 mm for *Staphylococcus aureus* and *Enterococcus faecalis*, respectively[31]. In our new formulation have equal or greater effect against these organisms. It was examined that the ethanol extracts of pomegranate peel obtained in this study showed antibacterial activity against both gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus)* and gram-negative (*Pseudomonas aeruginosa)* organisms. The results obtained because of antimicrobial activity experiments are compatible with previous studies using pomegranate peel ethanol extracts and these effects were successfully maintained in thermosensitive gel formulation.

Pomegranate peel is an important resource with its antioxidant, anticancer, and antimicrobial effects. However, its usage has been limited and it is mostly disposed of as agricultural waste. The studies carried out to turn it as a drug with its bioactive compounds. As a result, developed thermosensitive gel formulation of pomegranate peel extract could be an alternative for the treatment of oral, vaginal, dermal antifungal and antibacterial infections by its ease of usage, and efficacy.

STATEMENT OF ETHICS

Not applicable.

CONFLICT OF INTEREST STATEMENT

None.

AUTHORS CONTRIBUTIONS

Adem Sahin: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition.

Ülküye Dudu Gül: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. Gizem Bayazıt: Investigation, Formal analysis, Writing - review & editing. Mustafa Sinan Kaynak: Supervision, Writing - review & editing.

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REFERENCES

1. Xiang Q, Li M, Wen J, Ren F, Yang Z, Jiang X, et al. The bioactivity and applications of pomegranate peel extract: a review. J Food Biochem, 2022;46(7):e14105. Doi: 10.1111/jfbc.14105

2. Giri NA, Gaikwad NN, Raigond P, Damale R, Marathe RA. Exploring the potential of pomegranate peel extract as a natural food additive: a review. Curr Nutr Rep, 2023 Jun;12(2):270-289. Doi: 10.1007/s13668-023-00466-z

3. Kumar N, Daniloski D, Pratibha Neeraj, D'Cunha, NM, Naumovski N, Petkoska AT. Pomegranate peel extract – a natural bioactive addition to novel active edible packaging. Curr Nutr Rep, 2022;156:111378. Doi: 10.1016/j.foodres.2022.111378

4. Teniente AL, Flores-Gallegos C, Esparza-González SC, Campos-Múzquiz LG, Nery-Flores SD, Rodríguez-Herrera R. Anticancer effect of pomegranate peel polyphenols against cervical cancer. Antioxid, 2023;12(1). Doi: 10.3390/antiox12010127

5. Chen J, Liao C, Ouyang X, Kahramanoğlu I, Gan Y, Li M. Antimicrobial activity of pomegranate peel and its applications on food preservation. J Food Qual, 2020;2020. Doi: 10.1155/2020/8850339

6. Tito A, Colantuono A, Pirone L, Pedone E, Intartaglia D, Giamundo G, et al. Pomegranate peel extract as an inhibitor of Sars-Cov-2 spike binding to human ACE2 receptor (*in vitro*): a promising source of novel antiviral drugs. Front in Chem, 2021;9:638187. Doi: 10.3389/fchem.2021.638187

7. Mittal A, Tejaswi S, Shetty S, Uk A. Comparison of antibacterial activity of calcium hydroxide, *Azadirachta indic*a (neem), *Ocimum tenuiflorum* (tulsi) and *Unica granatum* (pomegranate) gels as intracanal medicaments against *Efaecalis*: an *in-vitro* study. Pharmacogn J, 2021;13:988. Doi: 10.5530/pj.2021.13.127

8. Vasconcelos LC, Sampaio FC, Sampaio MC, Pereira MS, Higino JS, Peixoto MH. Minimum inhibitory concentration of adherence of *Punica granatum* Linn (pomegranate) gel against *S. mutans, S. mitis* and *C. albicans*. Braz Dent J, 2006;17(3):223-227. Doi: 10.1590/s0103-64402006000300009

9. Karim S, Alkreathy HM, Ahmad A, Khan MI. Effects of methanolic extract based-gel from Saudi pomegranate peels with enhanced healing potential on excision wounds in diabetic rats. Front Pharmacol, 2021;12:704503. Doi: 10.3389/fphar.2021.704503.

10. Bertolo MRV, Martins VCA, Horn MM, Brenelli LB, Plepis AMG. Rheological and antioxidant properties of chitosan/gelatin-based materials functionalized by pomegranate peel extract. Carbohydr Polym, 2020;228:115386. Doi: 10.1016/j.carbpol.2019.115386

11. Chen Y, Lee JH, Meng M, Cui N, Dai CY, Jia Q, et al. An overview on thermosensitive oral gel based on poloxamer 407. Mater, 2021;14(16). Doi: 10.3390/ma14164522

12. Fan R, Cheng Y, Wang R, Zhang T, Zhang H, Li J, et al. Thermosensitive hydrogels and advances in their application in disease therapy. Polym, 2022;14(12). Doi: 10.3390/polym14122379

13. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. AJEV, 1965. Doi: 10.5344/ajev.1965.16.3.144

14. Lamuela-Raventós, RM. Measurement of antioxidant activity and capacity: Recent Trends and Applications. Wiley; 2017. 107-115. Doi: 10.1002/9781119135388.ch6

15. Cuendet M, Hostettmann K, Potterat O, Dyatmiko W. Iridoid glucosides with free radical scavenging properties from fagraea blumei. Helv Chim Acta, 1997;80(4):1144-1152. Doi: 10.1002/hlca.19970800411

16. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature, 1958;181(4617): 1199-1200. Doi: 10.1038/1811199a0

17. Timur SS, Şahin A, Aytekin E, Öztürk N, Polat K H, Tezel N, Gürsoy R N, Çalış S. Design and *in vitro* evaluation of tenofovir-loaded vaginal gels for the prevention of HIV infections. Pharm Dev Techn, 2018;23(3):301-310. Doi: 10.1080/10837450.2017.1329835

18. Machado HA, Abercrombie JJ, You T, Deluca P, Leung KP. Release of a wound-healing agent from PLGA microspheres in a thermosensitive gel. Biomed Res Int, 2013;387863. Doi: 10.1155/2013/387863

19. Gül Ü, İrdem E, Yavuz Ş, Ilhan S. Determination of dye biosorption capacity of lichens and reusability of wastes as antimicrobial agents. JTA, 2020;112:1-9. Doi: 10.1080/00405000.2020.1797263

20. Prakash J W, Antonisamy JM, Jeeva S. Antimicrobial activity of certain fresh water microalgae from Thamirabarani River, Tamil Nadu, South India. Asian Pac J Trop Biomed, 2011;1(2, Supplement):170-173. Doi: 10.1016/S2221-1691(11)60149-4

21. Gözlekçi S, Saraçoğlu O, Onursal E, Ozgen M. Total phenolic distribution of juice, peel and seed extracts of four pomegranate cultivars. Pharmacogn Mag, 2011;7(26):161-164. Doi: 10.4103/0973-1296.80681

22. Marchi L, Monteiro A, Mikcha J, Santos A, Menconi Chinellato M, Marques DR at al. Evaluation of antioxidant and antimicrobial capacity of pomegranate peel extract (P*unica granatum* L.) under different drying temperatures. Chem Eng Trans, 2015;44:121-126. Doi: 10.3303/CET1544021

23. Malviya S, Arvind Jha A, Hettiarachchy N. Antioxidant and antibacterial potential of pomegranate peel extracts. JFST, 2014;51(12):4132-4137. Doi: 10.1007/s13197-013-0956-4

24. Dumortier G, Grossiord JL, Agnely F, Chaumeil JC. A review of Poloxamer 407 pharmaceutical and pharmacological characteristics. Pharm Res, 2006;23(12):2709-2728. Doi: 10.10 07/s11095-006-9104-4

25. Cafaggi S, Russo E, Caviglioli G, Parodi B, Stefani R, Sillo G, et al. Poloxamer 407 as a solubilising agent for tolfenamic acid and as a base for a gel formulation. EUFEPS, 2008;35(1-2):19-29. Doi: 10.1016/j.ejps.2008.05.010

26. Cetin N, Menevse E, Celik ZE, Ceylan C, Rama ST, et al. Evaluation of burn wound healing activity of thermosensitive gel and PLGA nanoparticle formulation of quercetin in Wistar albino rats. J J Drug Deliv Technol, 2022;75:103620. Doi: 10.1016/j.jddst.2022.103620

27. Fakhari A, Corcoran M, Schwarz. Thermogelling properties of purified poloxamer 407. Heliyon, 2017;3(8):e00390. Doi: 10.1016/j.heliyon.2017.e00390

28. Dumortier G, El Kateb N, Sahli M, Kedjar S, Boulliat A. Chaumeil JC. Development of a thermogelling ophthalmic formulation of cysteine. Drug Dev Ind Pharm, 2006;32(1):63-72. Doi: 10.1080/03639040500390934

29. Gormez A, Bozari S, Yanmis D, Gulluce M, Sahin F, Agar G. Chemical composition and antibacterial activity of essential oils of two species of *Lamiaceae* against phytopathogenic bacteria. Pol J Microbiol, 2015;64(2):121-127. Doi: 10.33073/pjm-2015-018

30. Demir T, Akpınar Ö, Kara H, Gungor H. *In vitro* antidiabetic, antiinflammatory, cytotoxic, antioxidant and antimicrobial activities of pomegranate (*Punica granatum* L.) Peel. Akademik Gıda, 2019;61-71. Doi: 10.24323/akademik-gida.544647

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Impact of structured patient education provided by clinical pharmacist on disease prognosis, adherence and quality of life in Type 2 Diabetes patients: An interventional study

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Type II Diabetes Mellitus (TIIDM) is a metabolic disease characterized by hyperglycemia. Patient adherence to treatment is important for the success of treatment and quality-of-life (QoL). This study aimed to evaluate the effect of patient education provided by a clinical pharmacist (CP) on treatment adherence, QoL, and disease prognosis. In this prospective pre-post intervention study, patients aged 18-65 years who applied to the Internal Medicine Outpatient Clinic of a university hospital in Istanbul and used SGLT-2 inhibitors for the treatment of type 2 diabetes in the last 24 months were included. The benefits of patient education provided by a CP to adherence and QoL was investigated. Adherence was measured by the Medication Adherence Report Scale (MARS) and QoL by EuroQol 5D-3L. As a result of the 6-month pre-post intervention, patient education provided by CP had positive effects on adherence, QoL, weight, and body mass index (p=0.038). Although HbA1c decreased, changes in fasting blood glucose, lipid profile, and blood pressure

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were not statistically significant. Following intervention, treatment adherence increased (p=0.012), along with an improvement in QoL (p=0.013). The contribution of CP in enhancing adherence and treating TIIDM was beneficial.

Keywords: clinical pharmacy, diabetes mellitus, patient education, treatment adherence, quality of life

INTRODUCTION

Type 2 Diabetes Mellitus (TIIDM) is a serious and widespread health problem that affects millions of people and can cause micro and macro complications. In type 2 diabetes, even if insulin can be produced in sufficient quantities, the sensitivity of cells to insulin and therefore the process of insulin action is impaired[1]. Although type 2 diabetes is generally seen in adults, it can also be seen in children and adolescents due to lifestyle changes, inactivity, unbalanced nutrition, and obesity in recent years. In Turkey, there are over 9 million people with diabetes and this number is estimated to increase to over 13 million in 2045. Approximately 4 million people worldwide die annually due to TIIDM and related diseases[2].

The treatment of TIIDM is complex, involving both pharmacological and non-pharmacological therapy. Effective management of TIIDM is medically complex, resources and time-sensitive and requires a multidisciplinary approach[3]. Treatment includes several elements such as continuous patient education, preventing acute complications, and reducing the risk of chronic complications[4,5]. Increased adherence to treatment for TIIDM is associated with improved glycemic control, reduced emergency department visits and hospitalizations, and lower health costs[6]. It has been shown that HbA1c, total cholesterol, blood pressure, body mass index, and adherence to medication regimens are improved when a clinical pharmacist (CP) provide pharmaceutical care to the treatment of diabetes to help patients, provide them with information regarding TIIDM management, encourage them to achieve therapeutic and lifestyle goals and support their adherence to medication regimens[7].

Clinical pharmacy is a field of pharmacy that applies pharmaceutical sciences such as pharmacology, biochemistry, and toxicology in a patient-oriented manner based on rational drug use. The main goal of clinical pharmacy is to ensure that the patient receives the right treatment correctly and effectively, in the most cost-effective and convenient manner. For this purpose, a CP takes the role of a consultant, especially in medication treatment, by transferring theoretical and practical knowledge to the healthcare system by communicating with physicians and other health personnel. CP not only connects the patient

with appropriate, effective, and safe treatments but also strives for outcomes that enhance the patient's Quality of Life (QoL)[7].

The duties of the CP include providing information about the medications used in TIIDM treatment, counseling the physician and patient about treatment when necessary, ensuring that treatment is administered rationally and safely, providing patient education, improving self-care, and monitoring the treatment. However, the role of the CP in the healthcare system is much broader than providing patient care. The role of the CP includes developing treatment guidelines and policies, advising on medication-related expenditures, advising other health professionals on special conditions, and providing necessary training[8].

Patient education is an important CP service aimed to increase the patient's knowledge about the patient's disease and the prescribed and over the counter medications used, the patients' adherence with the treatment, and thus the success of the treatment. The treatment goals may not be achieved if the treatment is not performed appropriately. The course of treatment, the dosage, frequency, route of administration, common side effects, potential drug-drug and drug-nutrient interactions of prescription and non-prescription medications, if any, are among the fundamental information for patient education. In addition, recommendations such as lifestyle changes, diet, and exercise can be provided depending on the patient's current health status[7].

Providing patient education has an important effect on adherence. Adherence, which can also be defined as the patient's acceptance of the treatment, refers to the patient's participation within the treatment. Due to the complex nature of the treatment and the fact that it is a chronic disease, poor adherence is common in people with TIIDM. Since poor adherence can lead to treatment failure and serious complications and morbidity, the role of CPs in tailoring the treatment plans and improving adherence becomes even more important[7]. Increased adherence to treatment for TIIDM is associated with improved glycemic control, reduced emergency department visits and hospitalizations, and lower health expenditures[6]. Patient education can be done verbally in a face-to-face manner, in written form such as a brochure, or through audiovisual techniques. Depending on the patient's conditions and the disease, one or more appropriate methods may be used.

A pharmacist-led team has been shown to significantly improve treatment adherence and reduce hospitalizations in the intervention group as a result of medication therapy management interventions for a large proportion of patients[9]. It has also been demonstrated that CP interventions significantly reduce HbA1c and fasting blood glucose levels in TIIDM patients; in addition, CP increases the level of knowledge of patients about their disease and medi-

cations, improve adherence with treatment, and contribute to better performance of diabetes self-care activities[10].

Although it is possible to measure it with different methods, the patient's general health status and laboratory tests can provide general information about treatment adherence. Patients who have the necessary and sufficient information about the disease and its treatment tend to take more responsibility for their treatment and cooperate more with health professionals, having more knowledge about what to expect.

Successful treatment depends as much on the patient's ability to perform self-care activities correctly as on the right medication. Moreover, self-care activities play an important role in preventive health. It is important to understand and apply self-care activities correctly to prevent a complication that may develop due to an existing disease and to maintain and improve the current state of health. Hygiene, nutrition, and lifestyle form the basis of self-care activities and have a wide place in patient education. It is recognized that the success of treatment in many diseases is closely related to self-care[2].

Enhancing QoL is a primary objective of both treatment in general and specifically in the context of managing TIIDM. Due to its progressive nature, patients with TIIDM are exposed to various acute and chronic complications that affect life. In light of all these circumstances, successful treatment and the associated improvement in QoL are closely related to adherence to current treatment, patient education, and self-care activities, as well as the application of the correct treatment regimen[7].

This study aimed to investigate the effects of patient education provided by the CP on treatment adherence and QoL in TIIDM patients using SGLT-2 inhibitors.

METHODOLOGY

Design, sample size and participants

This study was designed as a prospective and cross-sectional study. Patients with TIIDM who were admitted to a university hospital in Istanbul as outpatients between September 2022 and May 2023 and who met the inclusion criteria and gave written informed consent were included. This study, which was conducted to measure the effect of CP intervention on treatment adherence and QoL, was reported to the STROBE (Strengthening of The Reporting of Observational Study in Epidemiology) guideline[11]. The effect size (ρ) was calculated as 0.82, considering the reference studies[12]. Alpha (α) was taken as 0.05 and beta (β) as 0.95 and it was calculated that the power of the study would be 95% with the inclusion of at least 22 participants. This study was approved by the local clinical research ethics committee with decision number 13/3.

The study included 18-65 years old patients with type 2 diabetes mellitus who had started an antidiabetic medication containing SGLT 2 inhibitor within the last 24 months and signed the consent form. Patients under 18 and over 65 years of age, pregnant women, people with oncologic diseases, people with a GFR below 60, people with systemic inflammatory diseases, and people with cognitive disorders were excluded.

Data collection

Patients participating in the study had face-to-face interviews with the CP after a routine interview with their physicians and written informed consent was obtained. During the first interviews, demographic data of the patients (educational status, marital status, employment status, etc.) were recorded. Each interview lasted an average of 20 minutes. At the first interview, 68 patients who met the inclusion criteria were reached. During the first interview, the CP provided structured standardized patient education about TIIDM, medications used in treatment, as well as treatment adherence, QoL, and self-care activities. The participants were informed about the complications that may occur if the treatment is not adhered to and the disease is not managed properly, the correct use of the medications they use for the treatment of TIIDM and the issues to be considered during the use of drugs, important points in the diet, the complementary role of exercise in the treatment and the benefit of reducing stress in disease management[13]. In this context, a patient education brochure, which was prepared in advance and contains general information about the TIIDM and diabetes medications, was disseminated to the patients. At the second interview, 34 patients continued to be participated the study. The process of including patients in the study is shown in Figure 1.

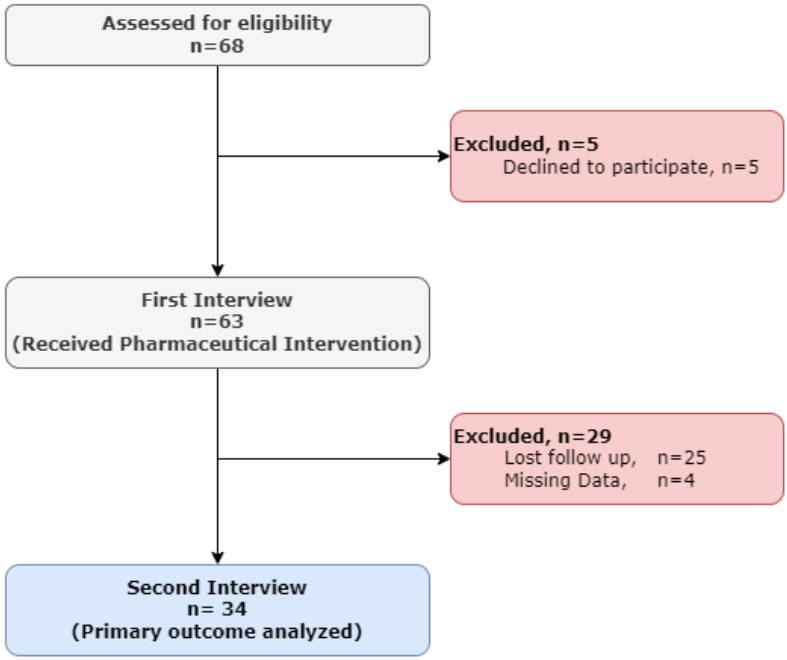

Figure 1. Study flowchart

To avoid biases during the study, the same CP administered the treatment adherence (MARS) questionnaire to all patients in both interviews[14] and the quality-of-life test (EQ 5D-3L)[15] and structured written and oral patient education was provided by the same CP.

Clinical pharmacist intervention and patient education

To examine the role of the CP in diabetes treatment, demographic data (age, gender, marital status, educational status, occupation, smoking, and alcohol use), Hemoglobin A1c (HbA1c) level, fasting blood glucose value, weight, body mass index, eGFR value, adherence level, QoL level were documented. Following the first interview, structured written and oral patient education was provided by the CP. The content of the education given by the CP at the first interview consisted of information about the TIIDM, and participants' other diseases, treatment processes, complications, medications used, diet program, and exercise. The Medication Adherence Report Scale (MARS)[14] and the quality-of-life test (EQ 5D-3L)[15] were used to measure participants' treatment adherence. In the second interview conducted six months after the first interview, the MARS and EQ (EuroQol) 5D-3L test were administered for the second time to determine the changes in treatment adherence and QoL.

The MARS test was applied in face-to-face interviews to determine the medication adherence of TIIDM patients who participated in the study. This scale, developed by Thompson et al. in 2000 to measure patients' adherence to their medications, consists of 10 questions, and answers are given as Yes/No. The questions investigate aspects such as carelessness in taking medication and forgetting to take it on time, discontinuation of medication if the patient feels well or unwell, opinions about medication use, whether the patient believes that taking medication protects from illness, whether the patient feels tired, sluggish, and strange after taking medication. Each 'Yes' answer to the question earns 1 point, while a 'No' answer corresponds to 0 points. Thus, a higher the score on a 10-point scale, indicates greater adherence to medication treatment.

In 1987, the EuroQol team convened with the aim of creating a standardized, non-disease-specific tool to assess health-related quality of life (HRQoL). They developed several tests to fulfill this objective. One of these, the EQ 5D-3L test, was developed in 1990[15].

The EQ 5D-3L scale is a tool with 5 sub-dimensions (domains) and a 3-point Likert scale. The first part of the EQ 5D-3L test, which consists of two parts, is descriptive; the second part consists of a visual analog scale in which the patient evaluates his/her general condition. The first part, the descriptive part, includes 5 domains: mobility, self-care, daily activities, pain/discomfort, and depression/anxiety. Participants have three different options for their answers on these five domains: no problems, some problems, and a lot of problems. For each question, the participant is asked to choose the option they feel closest to in terms of these options. Depending on the answers, patients receive different scores.

In the second part of the scale, the participant is asked to give himself/herself a score between 0 and 100 according to his/her health condition on that day and mark it on the scale. Thanks to this test, which measures the patient's QoL from different perspectives, the areas where the patient has problems about the disease can be identified and interventions can be planned accordingly.

Main outcome measure

The independent variables of the study were age, gender, marital status, educational status, occupation, smoking, and alcohol use, and the dependent variables were Hemoglobin A1c (HbA1c) level, fasting blood glucose value, weight, body mass index, eGFR value, patient's medication adherence level and patient's QoL level.

Statistical analysis

Demographic data were expressed as percentages and ratios. Variables presented as mean, standard deviation, median, and interquartile distribution. Data distribution was determined by Kolmogorov-Smirnov, Shapiro-Wilk tests, and histogram analysis. Data with parametric distribution were analyzed using Student's t-test; for those with nonparametric distribution, the Mann-Whitney U test was used. Chi-Square or Fisher Exact tests were applied to analyze discrete variables. Pearson correlation analysis or Spearman correlation analysis was used to determine the level of relationship between quantitative variables. $p<0.05$ was accepted as the statistical significance level.

RESULTS and DISCUSSION

TIIDM is a lifelong disease that often involves complex treatment. In addition to the correct administration of appropriate drug therapy, lifestyle changes, and self-care activities constitute an important part of the treatment. Treatment requires continuous follow-up; patient education should be provided to prevent acute complications, reduce the risk of chronic complications, and improve overall QoL. The importance of patient education and treatment adherence is especially evident when the incidence of acute complications such as hypoglycemia and chronic complications such as neuropathy, retinopathy, and nephropathy are considered. Managing diabetes treatment with a multidisciplinary team has positive effects on the course of the disease, patient adherence with treatment, improving QoL, and reducing health expenditures[16]. In such a team, CPs play a key role not only in administering the right treatment but also in providing patient education and improving self-care activities[7,12].

As in many diseases with complex treatment plans, TIIDM patients often experience treatment failure due to lack of adherence. By choosing the right treatment plan and adequate patient education, it is possible to increase treatment adherence and thus ensure better management of the disease. Considering its chronic nature, the impact of successful type 2 diabetes treatment on patients' QoL is significant. At this point, communication, cooperation, and harmony between the physician, CP, and patient are highly effective in increasing the success of treatment, preventing the development of side effects, and complications; and reducing the cost of treatment[7-10].

Sociodemographic data

Sociodemographic data of the patients participating in the study were collected at the first interview. Of the 34 patients included in the study to measure treatment adherence and QoL, 16 (47%) were female and 18 (53%) were male. The

mean age of the patients included in the study was 51.8 ± 9.06 years and 30 (88.2%) were married and 4 (11.8%) were single. While 15 (44.1%) participants were employed, 19 (55.9%) were retired or unemployed. Out of 34 patients 28 (82.35%) had family history of diabetes. The sociodemographic characteristics of the participants are shown in Table 1.

Table 1. Sociodemographic characteristics of participants

Parameters	**n=34**
Gender (n, %)	
Female	16 (47%)
Male	18 (53%)
Age (Mean ± SD)	51.8 ± 9.06
Marital Status (n, %)	
Married	30 (88.2%)
Single	4 (11.8%)
Education Level (n, %)	
Primary School	20 (58.8%)
Middle School	5 (14.7%)
High School	8 (23.5%)
University	1 (3%)
Occupation (n, %)	
Employed	15 (44.1%)
Unemployed	19 (55.9%)
Duration of diabetes (years) (Mean ± SD)	11.8 ± 7.4
Herbal Product Use (n, %)	
Yes	7 (21%)
No	27 (79%)
Smoking (n, %)	
Yes	6 (18%)
No	28 (82%)
Alcohol Use (n, %)	
Yes	1 (3%)
No	33 (97%)

Family History of Diabetes	
Yes	28 (82%)
No	6 (18%)
Comorbidities (n, %)	
Hyperlipidemia	17 (50%)
Hypertension	14 (41.18%)
Coronary artery disease	9 (26.47%)
Asthma	4 (11.75%)
Neuropathy	3 (8.82%)
Other	8 (23.53%)
SD: Standard Deviation	

The mean body weight of the patients at the first interview was 84.9 ± 15.8 kg. The mean height of the patients was 166.9 ± 9.7 cm. The mean duration of diabetes mellitus was 11.8 ± 7.4 years. The baseline clinical characteristics of the participants in the first and second interviews are shown in Table 2.

Table 2. Clinical characteristics of the participants

Parameters (Mean ± SD)	**1st Interview**	**2nd Interview**	**p-value**
Body Weight (kg)	84.9 ± 15.8	83.7 ± 15.0	**0.038**
Height length (cm)	166.9 ± 9.7		NA
Body Mass Index	30.4 ± 4.8	30 ± 4.6	**0.048**
Systolic Blood Pressure Value	122 ± 6.7	121.4 ± 4.9	>0.05
Diastolic Blood Pressure Value	80.7 ± 7.4	79.8 ± 6.0	>0.05
HbA1c (mmol/L)	7.77 ± 1.1	7.55 ± 1.1	>0.05
Fasting Blood Glucose (mg/dl)	159.6 ± 41.6	148.4 ± 35.5	>0.05
HDL Cholesterol (mg/dL)	44.4 ± 8.5	44.06 ± 8.3	>0.05
LDL Cholesterol (mg/dL)	114.6 ± 35.7	109.6 ± 39.8	>0.05
Triglycerides (mg/dL)	202.4 ± 114.7	198.5 ± 39.8	>0.05
eGFR (ml/min/1.73m2)	92 ± 14.4	95 ± 15.1	>0.05

eGFR: Estimated Glomerular Filtration Rate, HbA1c: Hemoglobin A1C, HDL: High-Density Lipoprotein, LDL: Low-Density Lipoprotein

Body weights were measured, and body mass index was calculated after the first and second interviews. Accordingly, there was a significant decrease in body weight (p=0.038) and body mass index (p=0.048) in the second interview. A significance was not observed in body mass index in our study which was consistent by literature[17]. Some other studies showed no significant reduction in body mass index in patients after CP intervention[10,18]. However, in a study investigating the effect of pharmacist education on type 2 diabetes patients in a community pharmacy setting in Türkiye, a significant decrease in body weight and body mass index of the participants was obtained[12]. In another study, similar to our study, a significant difference was observed in the body mass index of patients after CP intervention[7].

In our study, although statistically significant results were not obtained (p>0.05), there was a nominal decrease in the mean HbA1c values measured pre and post intervention (first measurement 7.77 mmol/L; second measurement 7.54 mmol/L). Similarly, fasting blood glucose levels decreased at the second interview (first measurement 160 mg/dl; second measurement 148 mg/dl), however this decrease was not statistically significant (p>0.05). Laboratory data (such as HbA1c, fasting blood glucose, lipid profile, and eGFR) recorded after the first and second interviews of the patients are presented in Table 2.

Unlike our findings, there are studies in which CP intervention and patient education practice had a significant effect on HbA1c and fasting blood glucose levels[7,12,17-20]. In the study conducted by Wishah et al. in Jordan, a significant decrease in HbA1c and fasting blood glucose levels was observed in the intervention group after CP interventions[10]. The study conducted by Wu et al. in the United States had similar results to our study and no significant change was found in HbA1c levels[16]. We think that the lack of a significant change in HbA1c and fasting blood glucose in our study is due to the relatively low number of participants.

In our study, no significant change was observed in the lipid profile of the patients after the first and second interviews (Table 2). The mean HDL cholesterol levels of the patients were increased, while the mean LDL cholesterol and triglyceride levels decreased, but these changes were not statistically significant. Similar to our results, some studies did not show a significant difference in LDL cholesterol and other lipid profiles[10,16,18]. Chan et al. observed a significant decrease in LDL cholesterol, but not in HDL cholesterol and triglyceride levels[17].

After CP intervention an increase observed in patients eGFR levels, but this increase was not statistically significant (Table 2). In comparison of the blood pressure measurements before and after CP intervention no significant difference recorded. Two different studies conducted in the USA and China that evaluated the beneficial effects of a CP within the multidisciplinary healthcare team on blood pressure did not show a significant reduction in blood pressure, similar to our study[16,17]. However, there are studies reported significant reductions in blood pressure after CPs involvement into the team[7,12,21,22].

Patients' level of treatment adherence

In our study, the answers given to the MARS by the patients after the first interview, averaged 7.38 points out of 10. Among the MARS items, the item with the highest score was Question 5 ('*I only take my medication when I feel sick*') with an average score of 0.91 and the item with the lowest score was Question 1 ('*Have you ever forgotten to take your medication?*') with an average score of 0.5. In the second interview, the patients' responses to the MARS had an average score of 8.06 out of 10. In the second interview, the item with the highest score was item 9 with an average score of 0.94, while the lowest score was item 1 with an average score of 0.67. The responses of the patients to the MARS for the first and second interviews are presented in Table 3.

Table 3. Patients' responses to the medication adherence rating scale test at the first and second interview (n=34)

Medication Adherence Rating Scale (MARS)	1st Interview		2nd Interview		p-value
	Yes	No	Yes	No	
Do you ever forget to take your medication?	17	17	11	23	**0.041**
Are you careless at times about taking your medication?	16	18	10	24	**0.020**
When you feel better, do you sometimes stop taking your medication?	9	25	4	30	>0.05
Sometimes if you feel worse when you take the medication, do you stop taking it?	6	28	6	28	>0.05
I take my medication only when I am sick.	4	31	4	31	>0.05
It is unnatural for my mind and body to be controlled by medication.	14	20	14	20	>0.05
My thoughts are clearer on medication.	23	11	25	9	>0.05
By staying on medication, I can prevent getting sick.	30	4	30	4	>0.05
I feel weird, like a 'zombie' on medication.	5	29	2	32	>0.05
Medication makes me feel tired and sluggish.	4	30	31	3	>0.05
Total	**128**	**213**	**137**	**204**	**0.012**

MARS: Medication Adherence Rating Scale

As a result of the CP intervention, a significant increase was observed in patients' QoL ($p<0.05$) and treatment adherence ($p=0.012$). When evaluated on item basis, the items that showed a statistically significant difference were '*Do you ever forget to take your medication?*' ($p=0.041$) and '*Are you careless at times about taking your medication?*' ($p=0.02$). Although there was an increase in the number of answers regarding adherence at other items, however this difference was not statistically significant. For example, compared to the first interview, more patients reported that they continued to take their medication regularly even when they feel better (first interview-25 patients, second interview-30 patients). Similarly, the number of patients who felt tired and sluggish after taking their medication was lower compared to the first interview ($p>0.05$). According to these results, it is seen that the items on taking medications regularly and correctly, which constitute an important part of patient education, have influential effect on the patients and they started to take their medications more regularly.

Considering studies conducted in different countries and different healthcare settings, the involvement of CP in to the healthcare team has been shown to beneficial on fasting blood glucose and HbA1c levels, QoL, healthcare expenditures, and incidence of adverse events[7,19]. In the study by Erku et al., it was observed that treatment adherence increased significantly in the group in which the CP was included in the treatment compared to the control group[9].

In another study, researchers showed that TIIDM treatment managed by a healthcare team including a CP resulted in higher adherence, and significantly lower HbA1c and LDL cholesterol compared to the control group. The risk of cardiovascular disease was also significantly reduced in the intervention group. However, there was no significant difference in HDL cholesterol, triglycerides, total cholesterol, blood pressure, and body mass index during the study period[17]. Another study conducted in Malaysia investigated the effect of patient education provided by a CP on HbA1c value, treatment adherence, and QoL. The study results showed that patient education significantly decreased HbA1c value and significantly increased treatment adherence compared to the control group[20]. In the study by Wishah et al. held in Jordan, there was a significant decrease in HbA1c and fasting blood glucose in the intervention group after 6 months of follow-up, as well as an increase in patients' knowledge about diabetes, treatment adherence, and self-care activities compared to the control group. However, there was no significant difference in lipid profile and body mass index between the two groups[10]. According to the results of a Chinese study, a statistically significant reduction in HbA1c and fasting blood glucose were obtained in the intervention group after patient education provided by the CP, while there was no significant difference between the two groups in body mass index and lipid profile[18]. In this study, a significant increase in treatment adherence was observed in the intervention group because of training.

Patients' level of quality of life

The response to the EQ 5D-3L test applied in the first interview to determine the QoL of the patients was 8.24 ± 1.35 out of 10. In the second interview, this score increased to 8.56 ± 1.26. The domain with the highest score (1.88 points) in the first interview was the self-care domain, while the domain with the lowest score (1.12 points) was the anxiety/depression. In the second interview, the item with the highest score (1.91) was self-care activities and daily activities, while the lowest score for anxiety/depression domain (1.18). Table 4 shows the average scores obtained by domains in the EQ-5D-3L test applied to determine the level of QoL in the first and second interviews.

Table 4. Mean scores of European Quality of Life 5 Dimensions 3 Level Version (EQ5D-3L) questions at the first and second interviews

EQ5D-3L Domains	1st Interview	2nd Interview	p-value
Mobility	1.71	1.82	>0.05
Self-Care	1.88	1.91	>0.05
Usual Activities	1.85	1.91	>0.05
Pain / Discomfort	1.68	1.74	>0.05
Anxiety/Depression	1.12	1.18	>0.05
Total	8.24	8.56	0.013

EQ5D-3L: European Quality of Life 5 Dimensions 3 Level Version

In our study, there was a significant increase in the total score obtained from the EQ 5D-3L test applied to measure the QoL of TIDM patients after the CP intervention in second interview (p=0.013) (Table 4). However, when evaluated on a domain-by-domain basis, the scores of the patients from each question increased, however this increase was not statistically significant. The domains that patients scored high in both interviews were '*Ability to take care of oneself*' and '*Ability to do daily tasks*'. The domain with the lowest common score in both interviews was anxiety/depression.

There are various studies to measure the QoL of diabetic patients. In a study, the EQ5D-3L test was used to measure QoL as in our study, and in this study, found no superiority in the intervention group over the control group in terms of the effect of patient education on QoL. However, there were significant changes in mobility and anxiety domains in the intervention group when the baseline and endpoints were compared[20].

A study conducted in Northern Cyprus, Körceğez et al. revealed that the healthcare team with a CP involved had positive effects on the treatment of TIIDM. As a result of the CP's interventions such as adjusting medication therapy and providing patient education to increase treatment adherence and improve self-care activities, the intervention group had a decrease in HbA1c level compared to the control group, systolic and diastolic pressure, body mass index and waist circumference, and an increase in self-care activities such as foot care, diet and home blood glucose measurement[7].

It is concluded that addition of the CP into the healthcare team increases QoL and treatment adherence, and also has a positive impact on certain treatment outcomes. Therefore, we strongly recommend collaboration between healthcare providers, especially physicians and CPs to establish effective communication with patients, manage diabetes treatment, enhance medication adherence and QoL, and prevent acute and chronic complications of TIIDM. Although various studies have found different results on the effects of CP on adherence and QoL cannot be standardized due to the lack of gold standard to measure these improvements[20].

All healthcare professionals involved in the treatment of diabetes, especially physicians and CPs, should take the initiative to increase the level of knowledge of patients; structured treatment plans according to patient needs by focusing on the patient's conditions and keep the communication channels open with patients and their relatives. This communication is crucial to determine the patient's status, evaluate treatment outcomes, and make necessary changes when needed. As shown in our study, proper education with patients and patient education can result to an increase in clinical outcomes, adherence, and QoL. In this context, our study demonstrated the contribution of physicians and CPs working in collaboration with an interdisciplinary approach to the treatment outcomes and QoL of patients in the management of type 2 diabetes.

STATEMENT OF ETHICS

The study received ethical approval from the Bezmialem Vakif University local Ethics Committee, with a decision number of 13/3.

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

Conceptualization: SD, MYB, and FVI; methodology: SD, MYB, AS, and FVI; formal analysis and investigation: SD and MYB; writing-original draft preparation: SD and MYB; writing-review and editing: SD and MYB; resources: SD, MYB, and AS; supervision: MYB, AS, and FVI.

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REFERENCES

1. Türkiye Diyabet Vakfı. Diyabet tanı ve tedavi rehberi. 9th edition. İstanbul: Türkiye Diyabet Vakfı, 2019.

2. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. IDF diabetes atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res Clin Pract, 2022;183:109-119. Doi: 10.1016/j.diabres.2021.109119

3. American Diabetes Association. Standards of medical care in diabetes-2014, Diabetes Care, 2014;37(1):14-80. Doi: 10.2337/dc14-S014

4. Norris SN, Lau J, Smith SJ, Schmid CH, Engelgau MM. Self-management education for adults with type 2 diabetes: a meta-analysis of the effect on glycemic control. Diabetes Care, 2002;25(7):1159-1171. Doi: 10.2337/diacare.25.7.1159

5. American Diabetes Association. Standards of medical care in diabetes-2015 abridged for primary care providers. Clin Diabetes, 2015;33(2):97-111. Doi: 10.2337/diaclin.33.2.97

6. Capoccia K, Odegard PS, Letassy N. Medication adherence with diabetes medication: a systematic review of the literature. Diabetes Educ, 2016;42(1):34-71. Doi: 10.1177/0145721715619038

7. Korcegez EI, Sancar M, Demirkan K. Effect of a pharmacist-led program on improving outcomes in patients with type 2 diabetes mellitus from northern Cyprus: a randomized controlled trial. J Manag Care Spec Pharm, 2017;23(5):573-582. Doi: 10.18553/jmcp.2017.23.5.573

8. Onatade R, Appiah S, Stephens M, Garelick H. Evidence for the outcomes and impact of clinical pharmacy: context of UK hospital pharmacy practice. Eur J Hosp Pharm, 2018;25(e1):e21-e28. Doi: 10.1136/ejhpharm-2017-001303

9. Erku DA, Ayele AA, Mekuria AB, Belachew SA, Hailemeskel B, Tegegn HG. The impact of pharmacist-led medication therapy management on medication adherence in patients with type 2 diabetes mellitus: a randomized controlled study. Pharm Pract (Granada), 2017;15(3):1026. Doi: 10.18549/PharmPract.2017.03.1026

10. Wishah RA, Al-Khawaldeh OA, Albsoul AM. Impact of pharmaceutical care interventions on glycemic control and other health-related clinical outcomes in patients with type 2 diabetes: randomized controlled trial. Diabetes Metab Syndr, 2015;9(4):271-276. Doi: 10.1016/j.dsx.2014.09.001

11. von Elm E, Altman DG, Egger M, Pocock SJ,Gøtzsche PC, Vandenbroucke JP. Strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. BMJ, 2007;335(7624):806-808. Doi: 10.1136/bmj.39335.541782.AD

12. Turnacilar M, Sancar M, Apikoglu-Rabus S, Hursitoglu M, Izzettin FV. Improvement of diabetes indices of care by a short pharmaceutical care program. Pharm World Sci, 2009;31(6):689-695. Doi: 10.1007/s11096-009-9333-9

13. Türkiye Endokrinoloji ve Metabolizma Derneği. TEMD diabetes mellitus komplikasyonlarının tanı, tedavi ve izlem kılavuzu. 15th edition. Ankara: Türkiye Endokrinoloji ve Metabolizma Derneği, 2022.

14. Chan AHY, Horne R, Hankins M, Chisari C. The Medication Adherence Report Scale: a measurement tool for eliciting patients' reports of nonadherence. Br J Clin Pharmacol, 2020;86(7):1281-1288. Doi: 10.1111/bcp.14193

15. Rabin R, de Charro F. EQ-5D: a measure of health status from the EuroQol Group. Ann Med, 2001;33(5):337-343. Doi: 10.3109/07853890109002087

16. Wu WC, Taveira TH, Jeffery S, Jiang L, Tokuda L, Musial J, et al. Costs and effectiveness of pharmacist-led group medical visits for type-2 diabetes: a multi-center randomized controlled trial. PLoS One, 2018;13(4):e0195898. Doi: 10.1371/journal.pone.0195898

17. Chan CW, Siu SC, Wong CK, Lee VW. A pharmacist care program: positive impact on cardiac risk in patients with type 2 diabetes. J Cardiovasc Pharmacol Ther, 2012;17(1):57-64. Doi: 10.1177/1074248410396216

18. Shao H., Chen G, Zhu C, Chen Y, Liu Y, He Y, et al. Effect of pharmaceutical care on clinical outcomes of outpatients with type 2 diabetes mellitus. Patient preference and adherence, 2017;11:897-903. Doi: 10.2147/PPA.S92533

19. Desse TA, Vakil K, Mc Namara K, Manias E. Impact of clinical pharmacy interventions on health and economic outcomes in type 2 diabetes: a systematic review and meta-analysis. Diabet Med, 2021;38(6):e14526. Doi: 10.1111/dme.14526

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Predictive value of lipid profile in predicting the resistance to insulin in apparently healthy adults

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ABSTRACT

Insulin resistance (IR) progression can lead to the development of metabolic syndrome and type 2 diabetes mellitus. Objective of the current work was to elucidate how useful lipid profile components were in predicting IR in healthy individuals in cross-sectional study conducted on 100 euglycemic, non-diabetic adults (aged ≥ 45 years). All individuals had their triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL), cholesterol, fasting blood sugar (FBS), fasting insulin and glycosylated hemoglobin (HbA1c) levels tested. The predictive values of lipid profile components separately or as ratios, as well as two obesity-related indicators: lipid accumulation products and visceral adiposity index, were evaluated in relation to the HOMA-IR using the receiver operating characteristic (ROC) curve. Results revealed that thirty (30%) of 100 apparently healthy subjects developed IR. The triglycerides/high density lipoprotein (TG/HDL) ratio had the best predictive value with an area under the curve (AUC) of 0.728, 95% CI = 0.617-0.838, $p<0.001$. The test had 77% sensitivity and 67% specificity, respectively. The optimal TG/HDL ratio cutoff value was 2.96, suggesting that

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among healthy adults, the TG/HDL ratio might be utilized as a routine screening test to detect IR.

Keywords: insulin resistance, lipid profile, homeostasis model assessment, healthy adults

INTRODUCTION

Insulin resistance (IR) refers to a diminished ability of target tissues, such as adipose tissue, muscle, and liver, to respond effectively to insulin stimulation. It causes a reduction in the ability of the body to handle glucose, that lead to an induction of insulin production from β-cell to compensate and hyperinsulinemia[1]. Hypertension, visceral obesity, endothelial dysfunction, dyslipidemia, elevated inflammatory markers, Hyperglycemia, and a prothrombotic state are among the metabolic consequences of IR[2]. However, the primary consequence of IR is the development of T2DM, with IR often preceding it by 10 to 15 years[3].

A large amount of data points to the possibility that IR may be linked to the buildup of fatty acid metabolites, or diacylglycerols, in many organs, most notably the skeletal muscles. Conversely, protecting against IR & preventing lipid buildup in muscles is achieved by blocking lipid entry into muscle through the elimination of lipoprotein lipase or other proteins involved in fat transport[4].

The homeostasis model assessment of insulin resistance (HOMA-IR) is now considered the most reliable method for quantifying IR. HOMA-IR was used to quantify IR and β-cell function based on insulin (or C-peptide) and fasting glucose levels[5]. The HOMA-IR model has proved to be a robust clinical and epidemiological tool for the assessment of IR. However, this approach is difficult to apply clinically, primarily due to cost, accessibility, and replicability issues[6]. Therefore, it is of paramount importance to find a practical alternative. Many attempts have been made to find a simple, non-expensive, and feasible alternative for this model. Triglyceride-glucose (TG/Glu) ratios, TG/HDL ratios, triglyceride-glucose indexes, McAuley's indexes, fasting glucose to insulin ratios (FG-I Ratio), and fasting C-peptide indexes are some of the alternatives, with sometimes controversial findings[7,8].

The objective of the current work was to assess the predictive value of lipid profile components and two lipid-rated parameters, visceral adiposity index (VAI) and lipid accumulation products (LAP), in predicting IR in healthy Iraqi adults.

METHODOLOGY

The study population

A cross-sectional study was conducted at Al Nahrain university/ college of medicine, Al Bayan University and Al-Mustafa University College, Baghdad, Iraq from May 2022 to March 2023. The study included 100 apparently healthy adult subjects of both sexes. Subjects enrolled in the study, are euglycemic, non-diabetic adults, ≥45 years old with HbA1C < 5.7%. The exclusion criteria of the volunteers who subjected to the current study are as the following:

- Subjects with diabetes
- Subjects with other severe chronic diseases, such as renal and cardiac diseases,
- Subjects who administered medications that may affect lipid metabolism or IR (such as anti-hyperlipidemic drugs corticosteroids,
- pregnant and breast-feeding women.

Demographic information was obtained from all subjects by direct interview which include age, sex, family history of diabetes, smoking status, and place of residence. All participants also had their body weight (kg), height (m), body mass index (BMI), waist circumference (WC), hip circumference (HC), and waist-hip ratio (WHR) assessed.

Sample collection and laboratory investigations

Fasting participants for eight hours before the collection of blood samples were subjected to the current research. On the next morning, five milliliter of blood was collected from the vein of the subject. FBS, HbA1c, a lipid profile the include the levels of VLDL, LDL, HDL, TC, TG, and fasting insulin were measured using a ready to-use commercial kit for each one (Inear Chemicals/ Spain). Also, HOMA-IR was calculated by multiplying FBS by fasting insulin divided by 405. According to a previous study,[7] the cutoff value of HOMA-IR was determined to be 3.1, beyond which subjects were considered to have IR. LAP and VAI were calculated for all participants according to the following formula[9].

"LAP = [waist circumference (cm) - 58] × triglycerides (mmol/l)."

"VAI = (WC (cm)/(39,68+(1.88*BMI) *(TG/1.03)*(1.31/HDL) for men" and

"VAI = (WC (cm)/(36,58+(BMI *1.89) *(TG/0.81)*(1.52/ HDL) for women."

Statistical analysis

All statistical analysis were performed by The SPSS software, version 25.0 (SPSS, Chicago). The mean and standard deviation of non-numerical data were displayed, and a student t-test was used for analysis. With the Chi-square test, numerical variables that were reported as percentages and numbers were examined. The predictive value of the components of the lipid profile in predicting IR was assessed using the receiver operating characteristic curve (ROC)[10]. For a difference to be considered statistically significant, a p-value of less than 0.05 was used.

RESULTS and DISCUSSION

Baseline characteristics of the patients

The mean age of the included subjects was 53.81 ± 8.78 years, ranging 45-80 years. Males represented 46% of them. Smoking and family history were relatively common and accounted for 39% and 52% of the subjects, respectively. The individuals' mean weight was 82.39 ± 12.4 kg, and their mean height was 167.64 ± 9.16 cm. The average BMI was therefore 29.38 ± 4.29 kg/m^2. Lastly, the average hip and waist circumferences were 104.76 ± 21.68 cm and 91.17 ± 18.91 cm, respectively (Table 1).

Table 1. Patients' baseline characteristics

NON-NUMERICAL VARIABLES		COUNT	%
Sexes	Male Female	46 54	46% 54%
Smoking	Never Ex/current	61 39	61% 39%
Family history of DM	No Yes	48 52	48% 52%
NUMERICAL VARIABLES		**MEAN ± SD**	**RANGE**
Weight (kg)		82.39 ± 12.4	58-112
Age, years		53.81 ± 8.78	45-80
Height (cm)		167.64 ± 9.16	146-198
Hip circumference (cm)		104.76 ± 21.68	64-152
Waist circumference (cm)		91.17 ± 18.91	59-144
WHR		0.88 ± 0.08	0.67-1.01
BMI (kg/m^2)		29.38 ± 4.29	18.37-42.22

Insulin resistance rate

Out of 100 subjects that appeared healthy, 30 subjects (30%) had insulin resistance, while the remaining 70 patients (70%), according to the HOMA-IR model, were insulin sensitive (Figure 1).

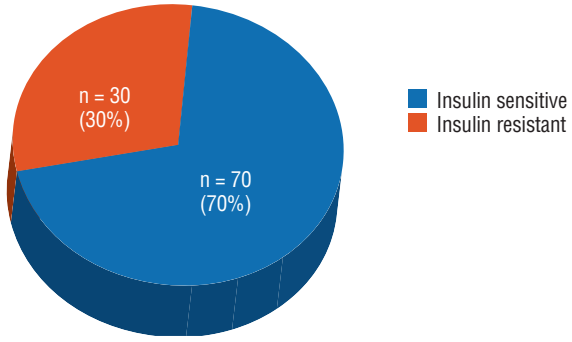

Figure 1. Insulin resistance rate

Association of demographic factors with insulin resistance

The insulin-resistant group had a significantly higher average age than the insulin-sensitive group (56.5 ± 10.44 years versus 52.67 ± 7.74 years). Likewise, females were more common among the insulin resistance group, with a significant difference (p=0.009). Other demographic characteristics did not significantly differ between the two groups; they were comparable (Table 2).

Table 2. Association of demographic factors with insulin status

Variables	Insulin sensitive (70)	Insulin resistance (30)	p-value
Age (years)	52.67 ± 7.74	56.5 ± 10.44	0.045*
Sex			
Male	38(54.2%)	8(26.67%)	0.009*
Female	32(45.71%)	22(73.33%)	
Smoking			
Never	44(62.86%)	17(56.67%)	0.358
Ex/current	26(37.14%)	13(43.33%)	
Family history of DM			
No	37(52.86%)	11(36.67%)	0.102
Yes	33(47.14%)	19(63.33%)	
Weight (kg)	80.97 ± 12.11	85.70 ± 12.64	0.080
Height (cm)	166.99 ± 9.1	169.17 ± 9.28	0.277
Waist circumference	89.31 ± 15.79	96.83 ± 24.22	0.068
Hip circumference	104.09 ± 20.51	106.33 ± 24.52	0.637
BMI (kg/m^2)	28.84 ± 4.22	30.59 ± 4.31	0.062

The association of between lipid profile and insulin status

Results illustrated in Table 3 showed that there was non-significant difference in the levels of TC, VLDL, and VAI between the two studied groups. On the other hand, insulin resistance group showed significantly higher levels of TG and HDL than insulin sensitive group (167.7 ± 56.13mg/dL versus 133.13 ± 40.04mg/dL and 49.66 ± 4.39mg/dL versus 47.22 ± 4.67mg/dL, respectively). Moreover, LDL and LAP were significantly higher in insulin resistance when compared to the insulin-sensitive group (103.9 ± 22.02mg/dL versus 94.22 ± 21.28mg/dL and 34.19 ± 6.63 versus 30.70 ± 7.98, respectively), according to Table 3.

Table 3. Lipid Profile association with insulin status

Variables	Insulin sensitive (n=70)	Insulin resistance (n=30)	p-value
Total cholesterol, mg/dl	178.75 ± 25.74	187.30 ± 29.22	0.147
Triglycerides, mg/dl	133.13 ± 40.04	167.7 ± 56.13	0.002*
HDL, mg/dl	49.66 ± 4.39	47.22 ± 4.67	0.041*
LDL, mg/dl	94.22 ± 21.28	103.9 ± 22.02	0.042*
VLDL, mg/dl	35.40 ± 14.75	34.48 ± 9.51	0.769
LAP	30.70 ± 7.98	34.19 ± 6.63	0.009*
VAI	4.44 ± 1.56	4.95 ± 1.94	0.170

FBS and HbA1c association with insulin status

Fasting blood glucose and fasting insulin, as components of the HOMA-IR equation, were much higher in the insulin-resistance group (92.45 ± 7.94 mg/dL and 17.77 ± 3.49 mIU/L, respectively) than in the insulin-sensitive group (88.06 ± 7.53 mg/dL and 8.57 ± 2.51 mIU/L, respectively) with highly significant differences. Further, HbA1c and HOMA-IR were higher in the insulin-resistant subjects relative to the insulin-sensitive subjects (5.37 ± 0.64% and 4.01 ± 0.78 versus 5.04±0.50% and 1.86 ± 0.54, respectively) as indicated in Table 4.

Table 4. FBS and HbA1c association with insulin resistance

Variables	Insulin sensitive (70)	Insulin resistance (30)	p-value
Fasting insulin, mIU/L	8.57 ± 2.51	17.77 ± 3.49	<0.001*
FBS, mg/dL	88.06 ± 7.53	92.45 ± 7.94	0.010*
HbA1c, %	5.04 ± 0.50	5.37 ± 0.64	0.007*
HOMA-IR	1.86 ± 0.54	4.01 ± 0.78	<0.001*

Diagnostic value of TG, HDL, LDL, and LAP in detecting insulin status

Insulin resistance was detected by evaluating the diagnostic value of LDL, TG, HDL, and LAP using the receiver operating characteristic (ROC) curve. HDL's area under the curve (AUC) was 0.643, 95% CI = 0.524-0.762, p=0.024. The test's results showed that its sensitivity was 63% and its specificity was 57%. For HDL, the optimal cutoff value was 48.5 mg/dL. The AUC for TG was 0.694, p=0.002, 95% CI = 0.584-0.805. The test's sensitivity and specificity were 63% and 68%, respectively. For TG, the optimal cutoff value was 144.5 mg/dL.

The AUC of LDL was 0.596, 95% CI = 0.473-0.720, p=0.048. The test's results showed 57% sensitivity and 57% specificity, respectively. For LDL, a cutoff value of 100.3 mg/dL was ideal. The AUC of LAP was 0.665, 95% CI = 0.553-0.777, and p=0.009. The test's results showed that its sensitivity was 60% and its specificity was 59%. The optimal LAP cutoff value was 30.76, as Figure 2 illustrates.

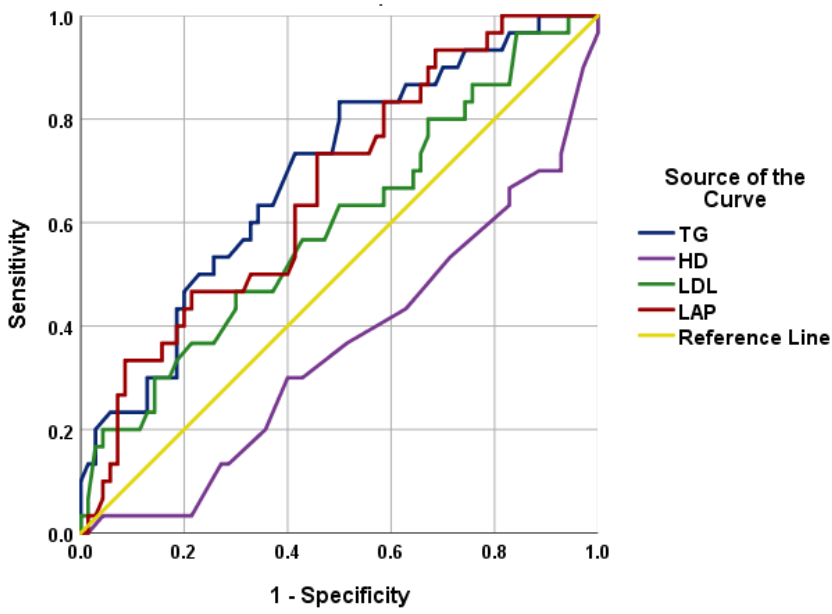

Figure 2. Receiver operating characteristic curve for HDL, TG, LDL, and LAP in detection of insulin status in healthy subjects

Diagnostic value of derived ratios

Two derived ratios were calculated from available data and examined for their diagnostic value of IR. These were the ratios of TG to HDL and TG to FBS. The AUC was 0.728, 95% CI = 0.617-0.838, p <0.001 for the TG/HDL ratio. The test had 77% sensitivity and 67% specificity, respectively. For the TG/HDL ratio, 2.96 was the ideal cutoff value.

The AUC was 0.646, 95% CI = 0.530-0.672, p=0.021 for the TG/FBS ratio. The test's results showed that its sensitivity was 67% and its specificity was 56%. The TG/FBS ratio's ideal cutoff value was 1.5. as Figure 3 illustrates.

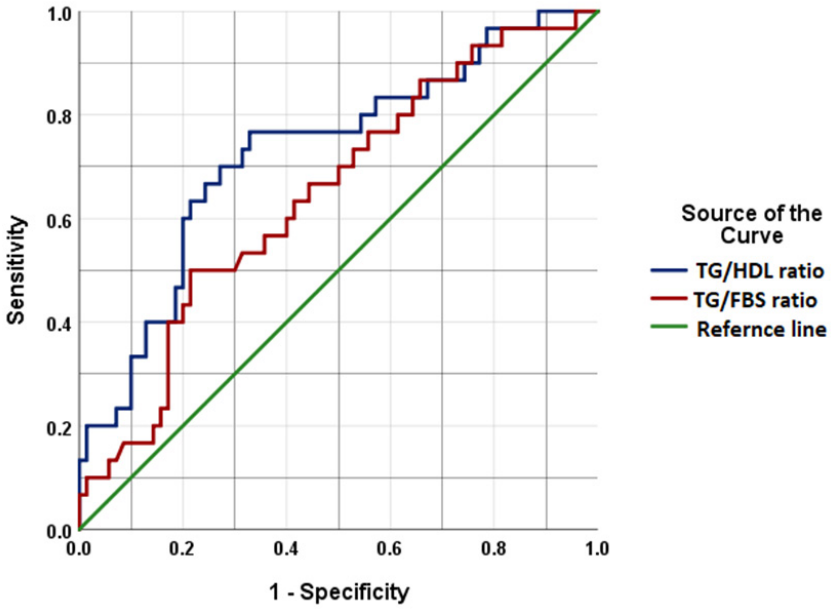

Figure 3. Receiver operating characteristic curve for TG/HDL and TG/FBS ratios in detection of insulin resistance in healthy subjects

The present research has identified a significant correlation between insulin resistance (IR) and advanced age which is consistent with other earlier investigations. Kim et al. discovered a significant relationship between age and IR in the Korean population[11]. In addition, Bermudez et al. discovered that the incidence of IR rose with age and was significantly greater in those aged 30 or older who were obese[12]. Decreased insulin sensitivity in the elderly can be attributed to various mechanisms, including reductions in insulin-stimulated whole-body glucose oxidation, age-related impairments in insulin action at the receptor and post-receptor levels, impaired insulin-mediated glucose uptake, an inability to suppress hepatic glucose output, and reductions in the β-cell response to glucose[13].

Female was also significantly associated with IR in the present study. There have been mixed results from earlier research assessing sex differences in insulin action. While some have observed no difference between men and women[14], others have stated that women are less likely than men to have IR[15]. In the meanwhile, the Greenhill study discovered that women have a lower incidence of IR resistance than men of comparable age prior to menopause[16]. Nevertheless, this protective effect fades after menopause, and the incidence of T2DM and IR in men and women converges, indicating that estrogen may have a preventive role.

Levels of HDL were inversely correlated with IR people in the current study. Multiple tissues' glucose metabolism is modulated by HDL, as demonstrated by Siebel et al.[17]. According to Chiang et al. there is significant variance in TG and HDL between people with and without IR; neither TC nor LDL show the same kind of correlation[18]. In a Chinese study, 1608 adult participants had nearly identical results[19]. Low HDL-C concentrations are hypothesized to be caused by IR through a number of different ways. First, IR is linked to increased exchange of VLDL for cholesterol esters from HDL particles and TG from chylomicrons, which lowers HDL-C. This process is controlled by the cholesteryl ester transfer protein (CETP)[20]. Second, lower hydrolysis of TG from chylomicrons and VLDL is caused by decreased lipoprotein lipase (LPL) activity, which may further restrict the contribution of TG-rich lipoprotein-derived HDL particles[21,22]. Third, higher HDL clearance and consequently lower HDL-C concentrations are linked to elevated HTGL (hepatic triglyceride lipase) activity in IR conditions[23]. Fourth, decreased apo A-I production and release from the liver and intestine may also be the cause of low HDL-C concentrations[24].

According to the result of the current study, LDL level was significantly associated with IR, Mykkänen et al. has been determined that there is a strong correlation between insulin resistance and an abundance of small, dense LDL particles[24]. This can be explained by the fact that the impact of IR on LDL metabolism seems to be less noticeable. In addition to its known ability to increase LDL receptor activity, insulin may be crucial for the metabolism of atherogenic small dense LDL particles[25]. Dannecker et al. found a significant positive correlation between C-peptide-based estimations of insulin secretion and fasting LDL values. Their findings suggested that increased LDL cholesterol levels might encourage pancreatic β cells to secrete more insulin[26]. However, Natali et al. did not discover any connection between LDL-c and insulin secretion[27].

TG levels had a significant correlation with IR people in the current investigation. The 517 Chinese participants in the Ma et al. study was split into four groups: those with normal lipid and glucose levels; those with dyslipidemia alone; those with dysglycemia alone; and those with both dyslipidemia and dysglycemia[28]. They discovered that in people with dyslipidemia alone, TG was connected with both IR and islet β-cell function, as well as the negative impact of hypertriglyceridemia on insulin sensitivity and islet β-cell activity. A different study by Riediger et al. discovered that in Canadian populations, a fasting TG may be helpful as a clinical predictor of IR and the onset of diabetes[29]. The fact that TG builds up in the liver and muscle with hypertriglyceridemia, suggesting lipid ectopic deposition, helps to explain the positive correlation between TG and IR[30]. Hepatocytes and skeletal myocytes produce TG metabo-

lites, such as fatty acyl CoA, diacylglycerol, and ceramides, which can affect insulin signaling and inhibit insulin-induced activation of glycogen synthase and insulin receptor substrate-1-associated PI 3-kinase[31]. Increases in hepatic TG lipase (HTGL) have also been linked to IR, and it has recently been shown that this activity plays a key role in controlling insulin clearance[32]. Since TG is not thought to be a signaling lipid, it is more likely that diacylglycerol, which is TG's synthetic precursor, ceramide, and other lipids are involved in the pathogenesis of hepatic IR through a variety of mechanisms, such as decreased insulin-stimulated glycogen synthase activity, reduced insulin receptor tyrosine kinase activity, and destabilization of the insulin receptor[33,34].

Lipid accumulation product (LAP) level was shown to be significantly correlated with IR in the current investigation. TG levels and WC are combined to create the LAP index, which represents lipid accumulation[35]. In addition to being seen as a sign of central fat accumulation, LAP has also been linked to poor glucose homeostasis, metabolic syndrome, T2DM, cardiovascular disease, and insulin resistance[36]. The body's fat buildup, which is accompanied by elevated TG and assessed WC levels, can be used to explain this substantial variation in LAP[32]. Consequently, elevated LAP can be a sign of lipid overabundance and ectopic lipid deposition[37]. Xia et al. showed that in non-diabetic subjects from China, there was close association between LAP and IR and exhibited stronger predictability of IR than WC and BMI[35]. Bermu´dez et al. showed LAP had a higher predictive capacity and association with IR in the Venezuelan population than VAI[38].

The TG/HDL ratio shown a strong predictive value for IR in the current investigation. The TG/HDL ratio has been reported to be useful in previous research for IR detection[7]. In a cross-sectional study, 258 non-diabetic overweight or obese people were included; 87% of these people were non-Hispanic white people[19].They found that in overweight individuals, the TG/HDL ratio may accurately predict IR. In another study, Gong et al. investigated the association of the TG/HDL ratio with IR in 49696 healthy American populations[39]. Moriyama, proposed a relationship between IR and the TG/HDL-C ratio in a sample of 1068 Japanese subjects in good health[40]. Rodríguez-Gutiérrez et al. also reported findings similar to this one[41]. However, it has been shown that the TG levels and TG–HDL ratio are not accurate indicators of IR in African Americans[42]. Therefore, although not African Americans, the TG/HDL ratio may be a useful diagnostic for identifying IR people of Aboriginal, Chinese, and European ancestry[43]. It is yet unknown how the TG/HDL ratio predicts IR. Nonetheless, adipose tissue is trapped and less fatty acid is retained in the IR by high TG and low HDL. More free fatty acids were therefore carried to

the liver, where they were converted into more TG and TG-containing VLDL. Additionally, as the plasma TG concentration rises, the TG of TG-containing VLDL and the cholesteryl ester of HDL exchange. This process creates the readily catabolized TG-rich HDL. As a result, IR patients had low HDL, high TG, and high TG/HDL[44].

The prevalence of IR among healthy Iraqi adults is 30%. Older ages more than 45 years and female are significantly associated with an increased risk of IR. Higher serum levels of TG, LDL and a lower level of HDL can enhance the development of IR in healthy subjects. The TG/HDL-C ratio has a good predictive value for IR.

STATEMENT OF ETHICS

The study received approval from the "Institute Review Board (IRB) of Al-Nahrain University/College of Medicine" on January, 2022 (58/2022).

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

Design – Hashim ZH, Al-Mayah QS, Al-Matubsi H; Acquisition of data – Khalid SH; Analysis of data – Khalid SH, Hashim ZH, Al-Mayah QS; Drafting of the manuscript – Khalid SH, Hashim ZH, Al-Mayah QS; Critical revision of the manuscript – Hashim ZH, Al-Mayah QS, Al-Matubsi H; Statistical analysis – Khalid SH, Hashim ZH, Al-Mayah QS; Technical or financial support – Khalid SH, Hashim ZH, Al-Mayah QS, Al-Matubsi H; Supervision – Hashim ZH, Al-Mayah QS, Al-Matubsi H.

REFERENCES

1. Brown JC, Harhay MO, Harhay MN. The value of anthropometric measures in nutrition and metabolism: comment on anthropometrically predicted visceral adipose tissue and blood-based biomarkers: a cross-sectional analysis. Nutr Metab Insights, 2019;12:1178638819831712. Doi: 10.1177/1178638819831712

2. Deacon CF. Physiology and pharmacology of DPP-4 in glucose homeostasis and the treatment of type 2 diabetes. Front Endocrinol, 2019;10:80. Doi: 10.3389/fendo.2019.00080

3. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes, 2015;6(3):456-480. Doi: 10.4239/wjd.v6.i3.456

4. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. Lancet, 2010;26;375(9733):2267-2277. Doi: 10.1016/S0140-6736(10)60408-4

5. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care, 2004;27(6):1487-1495. Doi: 10.2337/diacare.27.6.1487

6. Espinel-Bermúdez MC, Robles-Cervantes JA, del Sagrario Villarreal-Hernández L, Villaseñor-Romero JP, Hernández-González SO, González-Ortiz M, et al. Insulin resistance in adult primary care patients with a surrogate index, Guadalajara, Mexico, 2012. J Investig Med, 2015;63(2):247-250. Doi: 10.1097/JIM.0000000000000130

7. Ghani ZA, Qaddori H, Al-Mayah Q. Triglyceride/high-density lipoprotein ratio as a predictor for insulin resistance in a sample of healthy Iraqi adults. J Med Life, 2023;16(5):668-674. Doi: 10.25122/jml-2022-0239

8. Anoop S, Jebasingh FK, Rebekah G, Kurian ME, Mohan VR, Finney G, et al. The triglyceride/glucose ratio is a reliable index of fasting insulin resistance: observations from hyperinsulinaemic-euglycaemic clamp studies in young, normoglycaemic males from southern India. Diabetes Metab Syndr, 2020;14(6):1719-1723. Doi: 10.1016/j.dsx.2020.08.017

9. Bullen AL, Katz R, Kumar U, Gutierrez OM, Sarnak MJ, Kramer HJ, et al. Lipid accumulation product, visceral adiposity index and risk of chronic kidney disease. BMC Nephrol, 2022;23(1):401. Doi: 10.1186/s12882-022-03026-9

10. Mandrekar JN. Receiver operating characteristic curve in diagnostic test assessment. JTO, 2010;5:1315-1316. Doi: 10.1097/JTO.0b013e3181ec173d

11. Kim SG, Lim HS, Cheong HK, Kim CS, Seo HJ. Incidence and risk factors of insulin resistance syndrome in 20-59 year-old Korean male workers. J Korean Med Sci, 2007;22(6):968-972. Doi: 10.3346/jkms.2007.22.6.968

12. Bermudez V, Salazar J, Martínez MS, Chávez-Castillo M, Olivar LC, Calvo MJ, et al. Prevalence and associated factors of insulin resistance in adults from Maracaibo city, Venezuela. Adv Prev Med, 2016;2016:9405105. Doi: 10.1155/2016/9405105

13. Paolisso G, Marrazzo G, de Riu S, Sgambato S, Varricchio M, d'Onofrio F, et al. Effects of mild-to-moderate hyperglycaemia per se on glucose production and uptake in the elderly. Eur J Med, 1992;1(1):6-12.

14. Kim SH, Reaven G. Sex differences in insulin resistance and cardiovascular disease. J Clin Endocrinol Metab, 2013;98(11):E1716-E1721.Doi: 10.1210/jc.2013-1166

15. Moran A, Jacobs DR Jr, Steinberger J, Steffen LM, Pankow JS, Hong CP, et al. Changes in insulin resistance and cardiovascular risk during adolescence: establishment of differential risk in males and females. Circulation, 2008;117(18):2361-2368. Doi: 10.1161/CIRCULATIONAHA.107.704569

16. Greenhill C. Sex differences in insulin resistance. Nat Rev Endocrinol, 2018;14(2):65. Doi: doi10.10:38/nrendo.2017.168

17. Siebel AL, Heywood SE, Kingwell BA. HDL and glucose metabolism: current evidence and therapeutic potential. Front Pharmacol, 2015;6:258.Doi: doi:10.3389/fphar.2015.00258

18. Chiang JK, Lai NS, Chang JK, Koo M. Predicting insulin resistance using the triglyceride-to-high-density lipoprotein cholesterol ratio in Taiwanese adults. Cardiovasc Diabetol, 2011;10:93. Doi: 10.1186/1475-2840-10-93

19. Yang Y, Wang B, Yuan H, Li X. Triglycerides to high-density lipoprotein cholesterol ratio is the best surrogate marker for insulin resistance in nonobese middle-aged and elderly population: a cross-sectional study. Int J Endocrinol, 2021 Apr 30;2021:6676569. Doi: 10.1155/2021/6676569

20. de Vries R, Borggreve SE, Dullaart RP. Role of lipases, lecithin: cholesterol acyltransferase and cholesteryl ester transfer protein in abnormal high density lipoprotein metabolism in insulin resistance and type 2 diabetes mellitus. Clin Lab, 2003;49(11-12):601-613.

21. Bjornstad P, Eckel RH. Pathogenesis of lipid disorders in insulin resistance: a brief review. Curr Diabetes Rep, 2018;18:1-8. Doi: 10.1007/s11892-018-1101-6

22. Garg A, Haffner SM. Insulin resistance and atherosclerosis: an overview. Diabetes Care, 1996;19(3):274-274. Doi: 10.2337/diacare.19.3.274

23. Brinton EA, Eisenberg S, Breslow JL. Human HDL cholesterol levels are determined by apoA-I fractional catabolic rate, which correlates inversely with estimates of HDL particle size. Effects of gender, hepatic and lipoprotein lipases, triglyceride and insulin levels, and body fat distribution. Arterioscler Thromb Vasc Biol, 1994;14(5):707-720. Doi: 10.1161/01.ATV.14.5.707

24. Mykkänen L, Haffner SM, Rainwater DL, Karhapää P, Miettinen H, Laakso M. Relationship of LDL size to insulin sensitivity in normoglycemic men. Arterioscler Thromb Vasc Biol, 1997;17(7):1447-1453. Doi: 10.1161/01.ATV.17.7.1447

25. Gerber PA, Thalhammer C, Schmied C, Spring S, Amann-Vesti B, Spinas GA, et al. Small, dense LDL particles predict changes in intima media thickness and insulin resistance in men with type 2 diabetes and prediabetes--a prospective cohort study. PLoS One, 2013;8(8):e72763. Doi: 10.1371/journal.pone.0072763

26. Dannecker C, Wagner R, Peter A, Hummel J, Vosseler A, Häring HU, et al. Low-density lipoprotein cholesterol is associated with insulin secretion. J Clin Endocrinol Metab, 2021;106(6):1576-1584. Doi: 10.1210/clinem/dgab147

27. Natali A, Baldi S, Bonnet F, Petrie J, Trifirò S, Tricò D, et al. Plasma HDL-cholesterol and triglycerides, but not LDL-cholesterol, are associated with insulin secretion in non-diabetic subjects. Metabolism, 2017;69:33-42. Doi: 10.1016/j.metabol.2017.01.001

28. Ma M, Liu H, Yu J, He S, Li P, Ma C, et al. Triglyceride is independently correlated with insulin resistance and islet beta cell function: a study in population with different glucose and lipid metabolism states. Lipids Health Dis, 2020;19(1):121. Doi: 10.1186/s12944-020-01303-w

29. Riediger ND, Clark K, Lukianchuk V, Roulette J, Bruce S. Fasting triglycerides as a predictor of incident diabetes, insulin resistance and β-cell function in a Canadian First Nation. Int J Circumpolar Health, 2017;76(1):1310444. Doi: 10.1080/22423982.2017.1310444

30. Després JP, Lemieux I, Bergeron J, Pibarot P, Mathieu P, Larose E, et al. Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk. Arterioscler Thromb Vasc Biol, 2008;28(6):1039-1049. Doi: 10.1161/ATVBAHA.107.159228

31. DeFronzo RA. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. Diabetologia, 2010;53(7):1270-1287. Doi: 10.1007/s00125-010-1684-1

32. Labadzhyan A, Cui J, Péterfy M, Guo X, Chen YI, Hsueh WA, et al. Insulin clearance is associated with hepatic lipase activity and lipid and adiposity traits in Mexican Americans. PLoS One, 2016;11(11):e0166263. Doi: 10.1371/journal.pone.0166263

33. Petersen MC, Shulman GI. Roles of diacylglycerols and ceramides in hepatic insulin resistance. Trends Pharmacol Sci, 2017;38(7):649-665. Doi: 10.1016/j.tips.2017.04.004

34. Petersen MC, Madiraju AK, Gassaway BM, Marcel M, Nasiri AR, Butrico G, et al. Insulin receptor Thr1160 phosphorylation mediates lipid-induced hepatic insulin resistance. J Clin Invest, 2016;126(11):4361-4371. Doi: 10.1172/JCI86013

35. Xia C, Li R, Zhang S, Gong L, Ren W, Wang Z, et al. Lipid accumulation product is a powerful index for recognizing insulin resistance in non-diabetic individuals. Eur J Clin Nutr, 2012;66(9):1035-1038. Doi: 10.1038/ejcn.2012.83

36. Mazidi M, Kengne AP, Katsiki N, Mikhailidis DP, Banach M. Lipid accumulation product and triglycerides/glucose index are useful predictors of insulin resistance. J Diabetes Complications, 2018;32(3):266-270. Doi: 10.1016/j.jdiacomp.2017.10.007

37. Taverna MJ, Martínez-Larrad MT, Frechtel GD, Serrano-Ríos M. Lipid accumulation product: a powerful marker of metabolic syndrome in healthy population. Eur J Endocrinol, 2011;164(4):559-567. Doi: 10.1530/EJE-10-1039

38. Bermúdez V, Salazar J, Fuenmayor J, Nava M, Ortega Á, Duran P, et al. Lipid accumulation product is more related to insulin resistance than the visceral adiposity index in the Maracaibo city population, Venezuela. J Obes, 2021;2021:5514901. Doi: 10.1155/2021/5514901

39. Gong R, Luo G, Wang M, Ma L, Sun S, Wei X. Associations between TG/HDL ratio and insulin resistance in the US population: a cross-sectional study. Endocr Connect, 2021;10(11):1502-1512. Doi: 10.1530/EC-21-0414

40. Moriyama K. Associations between the triglyceride to high-density lipoprotein cholesterol ratio and metabolic syndrome, insulin resistance, and lifestyle habits in healthy Japanese. Metab Syndr Relat Disord, 2020;18(5):260-266. Doi: 10.1089/met.2019.0123

41. Rodríguez-Gutiérrez N, Vanoye Tamez M, Vázquez-Garza E, Villarreal-Calderón JR, Castillo EC, Laresgoiti-Servitje E, et al. Association of the triglyceride/high-density lipoprotein cholesterol index with insulin resistance in a pediatric population in northeast Mexico. Metab Syndr Relat Disord, 2020;18(7):333-340. Doi: 10.1089/met.2020.0046

42. Sumner AE, Finley KB, Genovese DJ, Criqui MH, Boston RC. Fasting triglyceride and the triglyceride-HDL cholesterol ratio are not markers of insulin resistance in African Americans. Arch Intern Med, 2005;165(12):1395-400. Doi: 10.1001/archinte.165.12.1395

43. Gasevic D, Frohlich J, Mancini GB, Lear SA. The association between triglyceride to high-density-lipoprotein cholesterol ratio and insulin resistance in a multiethnic primary prevention cohort. Metabolism, 2012;61(4):583-589. Doi: 10.1016/j.metabol.2011.09.009

44. Yeh WC, Tsao YC, Li WC, Tzeng IS, Chen LS, Chen JY. Elevated triglyceride-to-HDL cholesterol ratio is an indicator for insulin resistance in middle-aged and elderly Taiwanese population: a cross-sectional study. Lipids Health Dis, 2019;18(1):176. Doi: 10.1186/s12944-019-1123-3

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Effects of the tablets' organoleptic properties on patients' compliance

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ABSTRACT

This study aimed to investigate the effect of the organoleptic properties of tablets on patient compliance. A multidisciplinary approach is necessary to develop drugs that are suitable for each population and to ensure drug optimization. According to this, the patient's opinion should be sought. A personal interview (face-to-face) method was used for this study. Participants were asked seven questions about tablet shape, size, color, flavor, and coating. The target population for this study was chosen from five different age groups, and from each age group, fifty males and fifty females participated. As a result of this study, when all the responses were analyzed, the oval tablet shape, eight millimetres tablet size, white tablet color, tasteless tablet, and coated tablet were preferred. This study concluded which organoleptic properties of tablets were most preferred by different categories of patients.

Keywords: tablet, organoleptic, patient compliance, patient preferences, survey

INTRODUCTION

Organoleptic properties have a significant impact on patient compliance, especially when the drug is administered orally[1]. By utilizing these properties, patients will perceive the medication as more pleasant, which improves the patient's quality of life[1–3]. The physical appearance of the drug needs to reflect the

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general preferences (shape, size, color, flavor, and coating) of the population in order to promote patient acceptance and compliance with drug treatment[4].

The patient's compliance with the drug changes according to the different organoleptic properties of tablets[3,5]. Many patients find swallowing drugs uncomfortable[6], and both physical and psychological factors, as well as the possibility of some tablets having an unpleasant taste or odor, can contribute to this discomfort[7,8] This could consequently lead the patient to be non-compliant with the drug[9]. An interdisciplinary approach is essential for the development of drugs that are tailored to specific populations and to ensure the optimization of drug efficacy[10,11] According to this, the patient's opinion should be sought. Evaluating the differences in the organoleptic properties of tablets and how they could impact patient compliance and drug acceptability is necessary[12,13].

This study aimed to investigate patient preferences for different organoleptic properties, which we determined in this study as the shape, size, color, flavor and surface properties (coating or uncoating) of the tablet. By conducting a survey, we were able to figure out which of the features mentioned above are mostly preferred by different categories of patients. Knowing the preferences of patients will increase the patient's compliance with the drug. This data is meant to be delivered to pharmaceutical industry companies and clinics to achieve the main goal of this study.

METHODOLOGY

This study is descriptive regarding research purpose and a quantitative survey method in terms of method type. For the research, a questionnaire with seven questions was prepared, and it included questions about various organoleptic property features.

Participants were asked to choose gender (male or female), and age group (twelve-seventeen, eighteen-thirty-four, thirty-five, fifty-four, fifty-five, seventy-four, or over seventy-five). Then, participants were prompted to indicate their preferences for tablets of various shapes (oval, circular, diamond, octagonal, or square), sizes (four mm, six mm, eight mm, ten mm, or twelve mm), colors (white, pink, blue, orange, red, and brown), flavors (tasteless, sugary, bitter, salty, or fruity) and surface properties (coated or uncoated surface).

The study setting

This study was conducted at multiple locations in the Republic of Türkiye. The decision to take part in the study was voluntary. All participants were informed of the research procedure and purpose. Participants who had trouble finishing the study were not included, and it was acceptable for participants to leave the study.

Sample strategy

The target population for this study was chosen from five different age groups. Twelve-seventeen, eighteen thirty-four, thirty-five, fifty-four, fifty-five, seventy-four, and over seventy-five were the age groups of the participants. From each age group, fifty males and fifty females made up a total of one hundred participants. The overall number of participants was five hundred. This deliberate choice ensured a balanced representation of both genders within each age category, allowing for a comprehensive analysis of potential variations in preferences. The study did not include visually impaired individuals because there were components of visual perception.

Data collection

This study was done by personal interview (face-to-face). The participants were given a survey consisting of structured questions. The data was collected and analyzed using the SPSS (IBM Statistical Product and Service Solutions Statistics, Version 26.0.) program.

Data analysis

The SPSS analysis program was utilized to analyze the data collected by the survey. The participants' preferences were analyzed and thoroughly examined, and percentage analysis was performed on the results using SPSS program applications.

RESULTS and DISCUSSION

Tablet shape results

The relation between age and tablet shape is demonstrated in Figure 1(a). It illustrates the preferred tablet shape in each age group. In the age groups of 12-17 and 35-54, the most preferable tablet shape was circular, while it was oval in the rest of the age groups. It was noticed that octagonal tablets are the least preferred in all groups.

The relation between tablet shape and gender is demonstrated in Figure 1(b). It shows the preferred tablet shape according to males and females. It is obvious that most males preferred circular and oval tablets, with a slightly increased preference for circular ones. On the other hand, most females preferred oval tablets. It is worth mentioning that a considerable percentage of females preferred circular tablets. Regarding the least preferred shape, both males and females considered the octagon as their least preferable shape.

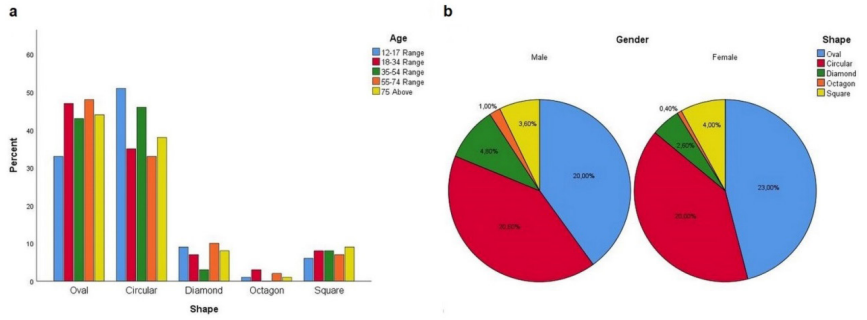

Figure 1. Percentage of preferred tablet shapes according to: (a) age; (b) gender

Tablet size results

The relation between age and tablet size is demonstrated in Figure 2(a). It demonstrates the preferred tablet size in each age group. According to the findings of this survey, for responders between the ages of 12-17, 18-34, and 35-54, eight millimeters was the most preferred size, while twelve millimeters was the least.

Moreover, four millimeters was the most preferred size in the age groups of 55-74, 75 and above. Regarding most minor preferable sizes, six and twelve millimeters were the least preferred among respondents aged 55-74, and both sizes were approximately equally preferred by this group. Finally, the least preferable size was ten millimeters in the 75 and above age ranges.

The relation between tablet size and gender is demonstrated in Figure 2(b). It illustrates the preferred tablet size among males and females. The results of this survey indicate that males tend to prefer sizes of eight millimeters the most, whereas, for females, it is four millimeters. Regarding the least preferred size, both males and females considered twelve millimeters their least preferable size.

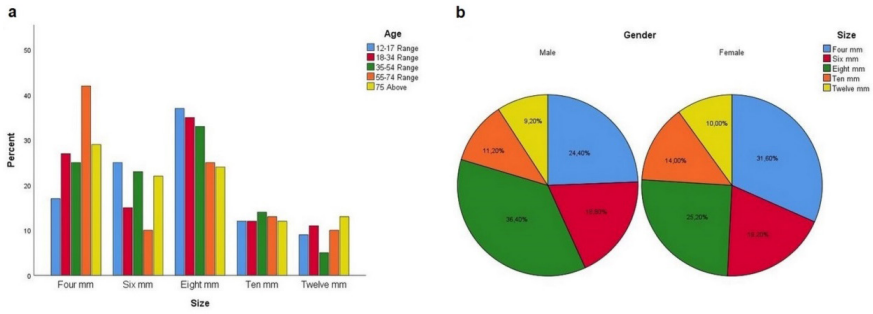

Figure 2. Percentage of preferred tablet sizes according to: (a) age; (b) gender

Tablet color results

The relation between age and tablet color is demonstrated in Figure 3(a). It demonstrates the preferred tablet color in each age group. Across all age ranges, white was the most preferred tablet color. When compared to other age groups, individuals aged 75 and above preferred the white color the most. The least preferable color was brown in the 12-17, 18-34, and 75 and above age ranges. Moreover, red was the least preferred color in the 35-54 and 55-74 age ranges.

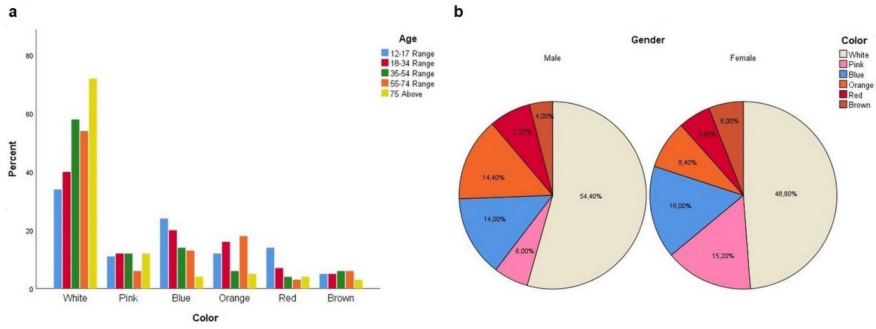

Figure 3. Percentage of preferred tablet colors according to: (a) age; (b) gender

Tablet flavor results

The relation between age and tablet flavor is demonstrated in Figure 4(a). It demonstrates the preferred tablet flavor in each age group. The most preferred tablet flavor across all age ranges was tasteless. Regarding least favorites, salty was the least preferred tablet flavor across all age ranges except 55-74. However, the flavors that people between the ages of 55-74 appreciated the least were salty and bitter.

The relation between tablet flavor and gender is demonstrated in Figure 4(b). It illustrates the preferred tablet flavor among males and females. According to the findings of this study, both males and females preferred the tasteless flavor the most. Figure 4(b). clearly concludes that males appreciated the tasteless flavor considerably more than females. In terms of the least preferable flavor, salty was the least preferred by both genders.

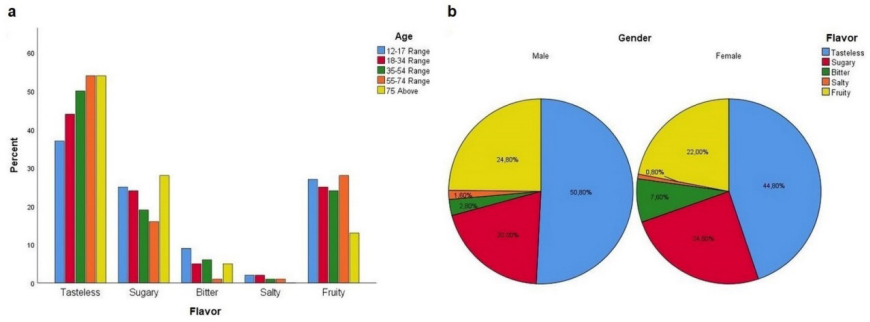

Figure 4. Percentage of preferred tablet flavors according to: (a) age; (b) gender

Tablet cover results

The relation between age and the tablet cover is demonstrated in Figure 5(a). It demonstrates the preferred tablet cover in each age group. Across all age ranges, coated tablet covers were the most preferred, while uncoated tablets were the least.

The relation between the tablet cover and gender is demonstrated in Figure 5(b). It illustrates the preferred tablet cover among males and females. According to the results of this survey, the most preferred cover for both males and females was coated. Though almost equally chosen by both genders, it is important to note that females preferred the coated cover slightly more than males.

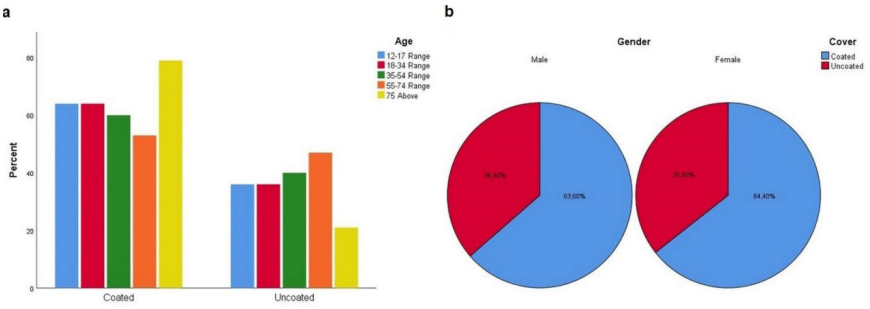

Figure 5. Percentage of preferred tablet covers according to: (a) age; (b) gender

Insights into patient preferences for tablets' organoleptic properties

The organoleptic properties of tablets enhance the drug's acceptability to patients and thereby positively influence their quality of life[2,14]; however, a paucity of knowledge persists regarding the organoleptic properties of tablets and their impact on acceptability. Recognizing these attributes is crucial for understanding patient preferences and, consequently, patient compliance[15,16]. Regarding the tablet's shape, multiple studies have demonstrated that rounder shapes are favored over angular shapes in diverse contexts, and according to the findings, patients chose rounder shapes, stating that they were easier to swallow[9,17,18]. Participants in our study reveal that rounder shapes are favored over angular shapes. Participants expressed that they made this choice because they perceived round tablets as easier to swallow. Our findings align with previous research, reinforcing the established preference for rounder tablet shapes. This consistent pattern highlights the practical significance of considering tablet shape in pharmaceutical design. Moreover, participants further emphasized the importance of tablet shape in differentiating and remembering the drug.

As for the tablet size, concerns emerged regarding the perceived smallness of four-millimeter tablets, with participants expressing worry about potential tracheal escape during swallowing. Older participants cited visibility issues with smaller tablets while acknowledging that twelve-millimeter tablets might be too large for comfortable swallowing. Interestingly, some older participants without swallowing difficulties preferred the larger tablet due to enhanced visibility.

Evidence suggests that the prevalence of swallowing issues and dysphagia is anticipated to rise among the aging population in the upcoming years, consequently posing challenges to the administration of oral medications[19,20]. Medication noncompliance is more prevalent among elderly patients, emphasizing the necessity for enhanced research and strategies for overcoming challenges[21,22]. In light of these projections, exploring innovative solutions and interventions to address this emerging issue is imperative.

Regarding tablet color, findings in numerous previous studies suggest that altering a drug's color can influence perceived efficacy[17,23,24]. Although there is a consensus on human perception of colors, it is crucial to acknowledge that factors including gender and culture can alter color perception[23]. The most preferred color for tablets, as indicated by various studies, was found to be white. Furthermore, participants believed that white tablets are perceived as more effective than tablets of other colors[9,17]. In our study, analysis of participant

feedback indicated favoring white tablets, perceived as more 'natural,' while other colors were noted to compromise the tablet's naturalness and potentially reduce drug efficacy.

Furthermore, the flavor is a major factor significantly impacting patients' compliance[25] and can be altered by different flavoring agents, affecting the drugs' acceptability[26,27]. Pharmaceutical formulations that lack palatability face challenges in achieving acceptability among patients[28,29] and, consequently, face obstacles in achieving desired therapeutic outcomes[29]. In evaluating tablet flavor within our study, participants preferred tasteless tablets, associating them with naturalness due to the absence of additives. Participants suggested that the flavor of tablets could influence or diminish the drug's effectiveness.

Finally, in the discussion on tablet cover, recent studies emphasize a substantial preference for coated tablets among patients, primarily because uncoated tablets pose greater difficulty in swallowing[9,18,30,31]. Reduced stickiness was observed in coated tablets, highlighting the advantageous impact of promoting a smooth passage through the esophagus[9,32,33]" Our study similarly concluded that participants favored the coated tablet due to its ease of passage through the esophagus. Notably, within the entire tested population, the uncoated tablet emerged as the least preferred option.

The outcomes emphasize the importance of considering diverse patient needs in developing and optimizing pharmaceuticals. Preferences can exhibit variability among various societies and individuals. Therefore, considering patients' needs and preferences can make treatment options more personalized and patient-centric. As the study unveils specific preferences across demographics, it highlights a pathway for enhancing patient compliance, ultimately promoting improved health outcomes.

When the survey's findings were analyzed, it was revealed that the oval, eight millimeters, white, tasteless, and coated tablets were the most preferred choices, while the octagonal, twelve millimeters, brown, salty, and uncoated were the least preferred among the overall tested population.

STATEMENT OF ETHICS

Research participants voluntarily agreed to take part after receiving information about the research procedure and purpose. They were informed that they could withdraw from the study if they experienced any difficulties or wished to leave at any point. Furthermore, prior to commencing the study, ethical approval was obtained from the relevant ethics committee. This approval ensured that the research protocol adhered to ethical guidelines and standards set forth by the Declaration of Helsinki.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interests.

AUTHOR CONTRIBUTIONS

Study concept and design: M.S., N.S.Ü.; Acquisition of data: F.B.; Data analysis/interpretation: M.S., F.B., N.S.Ü.; Preparation of figures: G.Ç.; Drafting/writing of manuscript: N.S.Ü.; Critical revision: M.S., N.S.Ü.

REFERENCES

1. Guhmann M, Preis M, Gerber F, Pöllinger N, Breitkreutz J, Weitschies W. Development of oral taste masked diclofenac formulations using a taste sensing system. Int J Pharm, 2012;438(1-2):81-90. Doi: 10.1016/j.ijpharm.2012.08.047

2. Diamond R. Drugs and the quality of life: the patient's point of view. J Clin Psychiatry, 1985;46(5 Pt 2):29-35.

3. Kardas P, Muras M. A blinded comparison of palatability of 13 common pediatric antibiotic suspensions. Wiadomosci Lek Wars Pol 1960, 2005;58(1-2):15-20.

4. Hasamnis AA, Patil SS, Kumar A, Thu K, Mohanty BK. Preferences of medical students regarding physical characteristics of oral solid dosage formulations in Malaysia. J Pharmacol Pharmacother, 2011;2(2):119-120. Doi: 10.4103/0976-500X.81909

5. Tanaka Y, Fukahori S, Ishii S, Saikusa N, Koga Y, Higashidate N, et al. Adherences to oral nutritional supplementation among hospital outpatients: an online cross-sectional survey in Japan. PLoS One, 2019;14(9):e0222972. Doi: 10.1371/journal.pone.0222972

6. Dhone PG, Jitendra M, Watsala S, Tanaji S, Lalan MS. Preparation and characterization of hydralazine mouth dissolving tablet by using super-disintegrates. Int J Health Sci, 2022;6(S5):3622-3629. Doi: doi.org/10.53730/ijhs.v6nS5.9566

7. Soares N, Mitchell R, McGoff T, Bailey T, Wellman GS. Taste perceptions of common pediatric antibiotic suspensions and associated prescribing patterns in medical residents. J Pediatr Pharmacol Ther, 2022;27(4):316-323. Doi: 10.5863/1551-6776-27.4.316

8. Schiffman SS. Influence of medications on taste and smell. World J Otorhinolaryngol - Head Neck Surg, 2018;4(1):84-91. Doi: 10.1016/j.wjorl.2018.02.005

9. Overgaard ABA, Møller-Sonnergaard J, Christrup LL, Højsted J, Hansen R. Patients' evaluation of shape, size and colour of solid dosage forms. Pharm World Sci, 2001;23(5):185-188. Doi: 10.1023/a:1012050931018

10. Sander O, Magnusson B, Ludwig I, Jullion A, Meille C, Lorand D, et al. A framework to guide dose & regimen strategy for clinical drug development. CPT Pharmacomet Syst Pharmacol, 2021;10(11):1276-1280. Doi: 10.1002/psp4.12701

11. Stegemann S, Ecker F, Maio M, Kraahs P, Wohlfart R, Breitkreutz J, et al. Geriatric drug therapy: neglecting the inevitable majority. Ageing Res Rev, 2010;9(4):384-398. Doi: 10.1016/j.arr.2010.04.005

12. Navarro V. Improving medication compliance in patients with depression: use of orodispersible tablets. Adv Ther, 2010;27(11):785-795. Doi: 10.1007/s12325-010-0073-y

13. Urquhart J. Patient non-compliance with drug regimens: Measurement, clinical correlates, economic impact. Eur Heart J, 1996;17(suppl A):8-15. Doi:10.1093/eurheartj/17.suppl_a.8

14. Liu F, Ranmal S, Batchelor HK, Gul M, Ernest TB, Thomas IW, et al. Patient-centered pharmaceutical design to improve acceptability of medicines: similarities and differences in paediatric and geriatric populations. Drugs, 2014;74(16):1871-1889. Doi: 10.1007/s40265-014-0297-2

15. Hofmanová JK, Mason J, Batchelor HK. Sensory attributes of coated tablets: developing a formal lexicon and sensory wheel. Int J Pharm, 2020;590:119883. Doi: 10.1016/j.ijpharm.2020.119883

16. Hofmanová JK, Mason J, Batchelor HK. Sensory aspects of acceptability of bitter-flavoured 7.5 mm film-coated tablets in adults, preschool and school children. Int J Pharm, 2020;585:119511. Doi: 10.1016/j.ijpharm.2020.119511

17. Wan X, Woods AT, Salgado-Montejo A, Velasco C, Spence C. Assessing the expectations associated with pharmaceutical pill colour and shape. Food Qual Prefer, 2015;45:171-182. Doi: 10.1016/j.foodqual.2015.06.009

18. Hofmanová JK, Rajabi-Siahboomi A, Haque S, Mason J, Teckoe J, To D, et al. Developing methodology to evaluate the oral sensory features of pharmaceutical tablet coatings. Int J Pharm, 2019;562:212-217. Doi: 10.1016/j.ijpharm.2019.03.046

19. Stegemann S, Gosch M, Breitkreutz J. Swallowing dysfunction and dysphagia is an unrecognized challenge for oral drug therapy. Int J Pharm, 2012;430(1-2):197-206. Doi: 10.1016/j.ijpharm.2012.04.022

20. Radhakrishnan C, Sefidani Forough A, Cichero JA, Smyth HE, Raidhn A, Nissen LM, Steadman KJ, et al. A difficult pill to swallow: an investigation of the factors associated with medication swallowing difficulties. Patient Prefer Adherence, 2021;15:29-40. Doi:10.2147/PPA.S277238

21. Shah AK. Compliance with medication among elderly people. BMJ, 1995;311(6999):259-259. Doi:10.1136/bmj.311.6999.259b

22. Boczkowski JA, Zeichner A. Medication compliance and the elderly. Clin Gerontol, 1985;4(1):3-15. Doi:10.1300/J018v04n01_02

23. Tao D, Wang T, Wang T, Qu X. Influence of drug colour on perceived drug effects and efficacy. Ergonomics, 2018;61(2):284-294. Doi:10.1080/00140139.2017.1349935

24. De Craen AJM, Roos PJ, De Vries AL, Kleijnen J. Effect of colour of drugs: systematic review of perceived effect of drugs and of their effectiveness. BMJ, 1996;313(7072):1624-1626. Doi:10.1136/bmj.313.7072.1624

25. Sohi H, Sultana Y, Khar RK. Taste masking technologies in oral pharmaceuticals: recent developments and approaches. Drug Dev Ind Pharm, 2004;30(5):429-448. Doi:10.1081/ddc-120037477

26. Uhari M, Eskelinen L, Jokisalo J. Flavoring agents and acceptance of antibiotic mixtures. Pediatr Res, 1985;19(10):1124-1124. Doi: 10.1203/00006450-198510000-00306

27. Alyami H, Koner J, Huynh C, Terry D, Mohammed AR. Current opinions and recommendations of paediatric healthcare professionals – the importance of tablets: emerging orally disintegrating versus traditional tablets. PLoS One, 2018;13(2):e0193292. Doi: 10.1371/journal.pone.0193292

28. Ruiz F, Keeley A, Léglise P, Tuleu , Lachuer C, Rwabihama JP, et al. Sex differences in medicine acceptability: a new factor to be considered in medicine formulation. Pharmaceutics, 2019;11(8):368. Doi: 10.3390/pharmaceutics11080368

29. Anurova M, Bakhrushina E, Moiseyeva A, Krasnyuk I. Principles of masking organoleptic properties of bitter tasting medicines. Pharmaceutical Management and Pharmaceutical Technology, 2020;1:25-32. Doi: 10.33920/med-13-2001-02

30. Shailesh K, Vaishali L. Review on: alternatives to large dosage forms for ease of swallowing. J Drug Deliv Sci Technol, 2020;57:101712. Doi: 10.1016/j.jddst.2020.101712

31. Yamamoto S, Taniguchi H, Hayashi H, Hori K, Tsujimura T, Nakamura Y, Sato H, et al. How do tablet properties influence swallowing behaviours? J Pharm Pharmacol, 2013;66(1):32-39. Doi: 10.1111/jphp.12155

32. Ershad AL, Rajabi-Siahboomi A, Missaghi S, Kirby D, Mohammed AR. Multi-analytical framework to assess the *in vitro* swallowability of solid oral dosage forms targeting patient acceptability and adherence. Pharmaceutics, 2021;13(3):411. Doi: 10.3390/pharmaceutics13030411

33. Drumond N, Stegemann S. Better medicines for older patients: considerations between patient characteristics and solid oral dosage form designs to improve swallowing experience. Pharmaceutics, 2020;13(1):32. Doi: 10.3390/pharmaceutics13010032

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Naproxen-phospholipid complex, a new vesicular drug delivery system: Design, development, and evaluation

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ABSTRACT

This study was aimed at preparing and assessing the Naproxen-phospholipid complex (NP-PL) as a vesicular drug delivery system prepared by solvent evaporation method. Attenuated total reflection (ATR) spectroscopy was used to confirm the interaction, while a dissolution study was used to compare the dissolution profiles for the NP drug, the NP-PL complex, and the marketed NP suspension. Results revealed that the ATR spectra showed some changes that confirm the complexation between the PL and NP, and better dissolution behavior was shown by the NP-PL pharmacosomes compared to the NP suspension and the pure NP, which indicates that the potential of making a phospholipid complex with an improved dissolution profile of Naproxen was investigated and a successful NP-PL pharmacosomes was prepared. ATR data confirmed NP-PL complex formation. *In-vitro* dissolution profiles of NP pharmacosomal dispersion showed improvement over marketed NP and pure NP.

Keywords: naproxen, naproxen-phospholipid complex, phospholipid, solvent evaporation method

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INTRODUCTION

Phospholipids (PL) are one of the most common amphiphilic molecules that have both hydrophobic and hydrophilic regions, In the presence of water, phospholipids arrange themselves into one or more concentric bilayers (vesicles)[1]. Complexes of drugs with lipids that have a functional group capable of interacting with the functional group in the drug, for example, phosphatidylcholine, have a phosphate functional group that can interact with Naproxen (NP) to form pharmacosomes (vesicles) that show several advantages, such as enhancing orally administered drug bioavailability and reducing the toxicity of drugs[2,3]. Naproxen (NP) is from the profen family (2-arylpropionic acid) of the non-steroidal anti-inflammatory drugs. Naproxen has good lipid solubility which make it insoluble in water and causes some problems in dissolution[4]. The oral administration of Naproxen is available as suspension or tablet (immediate and extended release) dosage form[5]. From the primary adverse effects for naproxen is gastro-intestinal ulcers[6,7]. Phospholipids can interact through hydrogen bond and/or van der Waals forces with drug molecules that have an active hydrogen atom[8]. The aim of this study is to prepare and assess of Naproxen-phospholipid complex (NP-PL) as vesicular drug delivery system.

MATERIALS AND METHODS

Materials

Lipoid S 100, which is a soybean phosphatidylcholine with a purity of 94%, was obtained from Lipoid GmbH, Germany. The pure Naproxen provided by Sigma-Aldrich, Germany, and the purity of NP were determined and assessed by UV lambda max and melting point methods. Reagents and solvents used are commercially available in analytical grade.

Preparation of Naproxen-phospholipid complex

Naproxen-phospholipid complex (NP-PL) was prepared by using the solvent evaporation method. Highly purified (94%) soybean phosphatidylcholine and Naproxen (NP) were dissolved in 30 mL of dichloromethane, which is used as a solvent due to its good solubility for both naproxen and lipoid S100 and also because it is considered a good option for the solvent evaporation method due to its low boiling point. The components were placed in a round bottom flask in a ratio of 1:1 mole (50 mg Naproxen with 157 mg PL), which has been proven in previous research to be the best ratio for complexation.

Three hours of stirring at 35°C were done to the mixture by a magnetic stirrer, then a rotary evaporator with low pressure used for solvent removal. The obtained lipid film retained in a desiccator for a day[9].

Attenuated total reflection (ATR)

The ATR analysis of pure NP, PL, NP-PL were carried out with a Bruker Alpha-P ATR FTIR, Germany.

Preparation of Naproxen vesicular systems

The hydration of the obtained lipid film that contain NP-PL by a solution of phosphate buffer saline (PBS, pH 7.4) at 55±2°C resulted in pharmacosomal dispersion[10].

Investigation by transmission electron microscopy (TEM)

A TEM instrument (Philips, Netherland) was used to investigate the NP-PL Pharmacosomal dispersion. 1% phosphotungstic was used to stain a little amount from the dispersion that placed on carbon grids that coated with copper[11].

In-vitro dissolution study

A rotating paddle dissolution apparatus type II containing phosphate buffer (pH 7.4) with a volume of 900 mL at speed 100 rpm was used to compare the dissolution behavior of NP pharmacosomal dispersion, NP suspension, and the pure NP powder. Each formula containing 125 mg of Naproxen[12] the concentration of the marketed NP used is 25mg/ml and a 5 ml of the product were used to obtaind the 125mg of NP. The used media was preconditioned and maintained at a 37 °C. At a proper time interval, 5 mL sample were drawn then replaced with a new dissolution medium. The filtration of the samples was done through a 0.45 μm filter syringe. A UV-Visible spectrophotometer was used to determine the drug content of the solution.

RESULTS and DISCUSSION

ATR of the prepared Naproxen-phospholipid complex

The prepared phospholipid complex of Naproxen by the solvent evaporation method were characterized by ATR to confirm the interaction between the two molecules. The ATR spectra of NP, PL, Naproxen-phopholipid complex (NP-PL) are displayed in Figure 1. Significant changes detected in the spectrum of NP-PL, the Naproxen OH stretching band absorption peak which occur at (3145 cm^{-1}) was shifted to lower wavenumber (3008 cm^{-1}), the stretching band of the C=O (1680 cm^{-1}) of NP was moved to lower wavenumber (1633 cm^{-1})[13]. Additionally, the P=O stretching band absorption peak of the of PL (1251 cm^{-1}) have been moved to higher wavenumber (1262 cm^{-1}). An interaction between the polar part of the PL (phosphate) and the carboxyl group of NP might be suggested by the obtained results[14]. The chemical structure of NP and PL were illustrated in Figure 2.

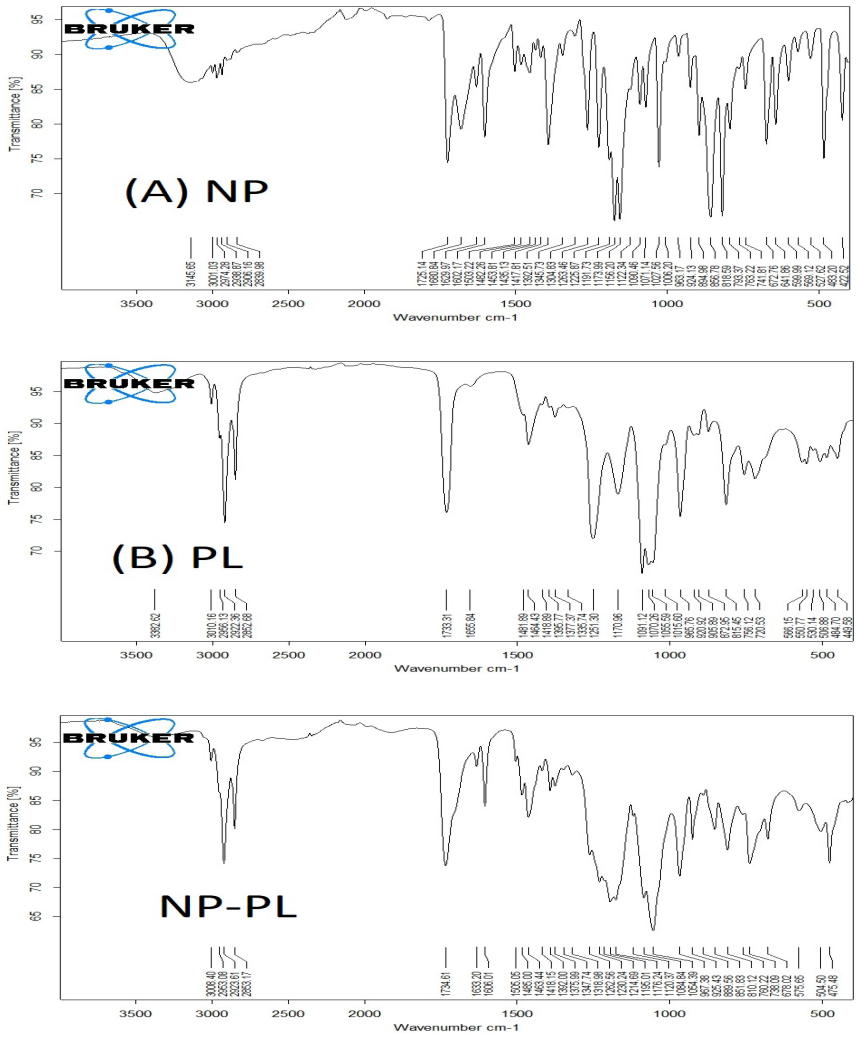
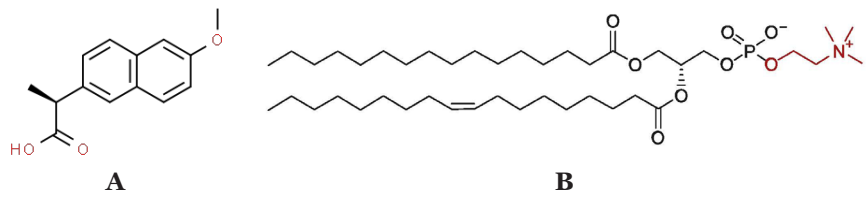


Figure 1. ATR spectra of (A) NP (B) PL (C) NP-PL



A **B**

Figure 2. Chemical structures of (A) Naproxen, (B) phospholipid

Examination using transmission electron microscopy (TEM)

The Naproxen pharmacosomal dispersion was examined by TEM. Figure 3 show the TEM photographs that revealed spherical structures, these structures indicating vesicle formation.

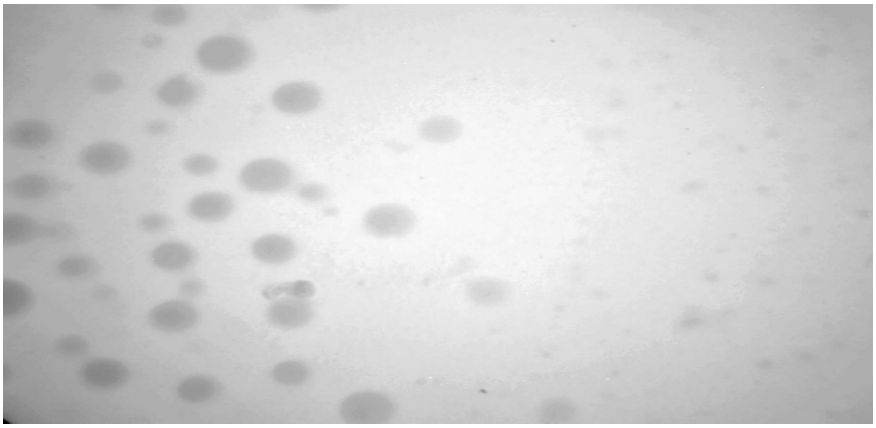


Figure 3. TEM photographs of Naproxen pharmacosomal dispersion

In-vitro dissolution study

The dissolution behavior of Naproxen pure powder (NP), NP pharmacosomal dispersion in comparison to marketed NP suspension in phosphate buffer (PB) pH 7.4 are shown in Figure 4. NP-PL dispersion show higher dissolution profile than pure NP and marketed NP suspension, one of the factors that the dissolution study depend on is the solubility of compounds. So, it will give an estimation for the enhancement in solubility and the rapid and enhanced dissolution rate of pharmacosomal dispersion in the dissolution behavior could be attributed to the role of lipid vesicles by decreasing the surface interfacial tension[15].

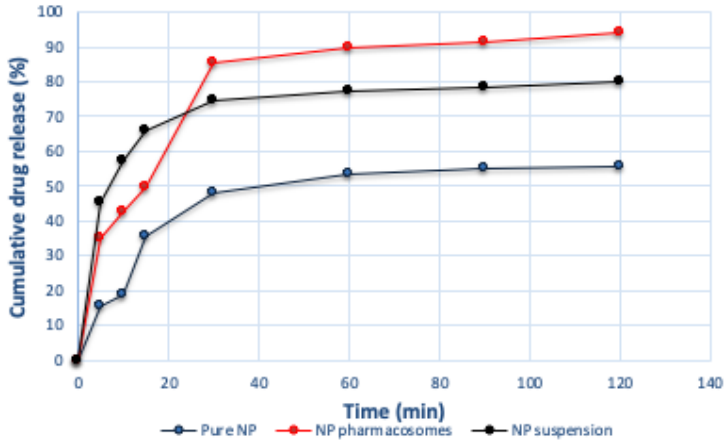


Figure 4. Dissolution profile of pure NP, NP pharmacosomal dispersion, and marketed NP suspension in phosphate buffer 7.4

A successful Naproxen-phospholipid complex (pharmacosomes) was prepared using solvent evaporation method and the ATR data confirmed NP-PL complex formation and *in-vitro* dissolution profile of NP pharmacosomal dispersion showed an improvement over marketed NP and pure NP.

STATEMENT OF ETHICS

Ethical approval was not required to perform this study.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed equally for this work.

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REFERENCES

1. Jain S, Jain V, Mahajan S. Lipid based vesicular drug delivery systems. Adv in Pharma, 2014;1-12. Doi: 10.1155/2014/574673

2. Date T, Paul K, Singh N, Jain S. Drug-lipid conjugates for enhanced oral drug delivery. AAPS Pharm Sci Tech, 2019;20:41. Doi: 10.1208/s12249-018-1272-0

3. Alexander RL, Greene BT, Torti SV, Kucera GL. A novel phospholipid gemcitabine conjugate is able to bypass three drug-resistance mechanisms. Cancer Chemo and Pharma, 2005;56:15-21. Doi: 10.1007/s00280-004-0949-0

4. Mouelhi M, Ruelius HW, Fenselau C, Dulik DM. Species-dependent enantioselective glucuronidation of three 2-arylpropionic acids. Naproxen, ibuprofen, and benoxaprofen. Drug Metabolism and Dispos, 1987;15(6):767-772.

5. Gargallo CJ, Sostres C, Lanas A. Prevention and treatment of NSAID gastropathy. Curr Treat Options Gastroenterol, 2014;12(4):398-413. Doi: 10.1007/s11938-014-0029-4

6. Pellicano R. Gastrointestinal damage by non-steroidal anti-inflammatory drugs: updated clinical considerations. Minerva Gastroente Dietol, 2014;60(4):255-261.

7. Gudis K, Sakamoto C. The role of cyclooxygenase in gastric mucosal protection. Dig Dis Sci, 2005;501:16-23. Doi: 10.1007/s10620-005-2802-7

8. Khudhur AQ, Maraie NK, Raauf AM. Highlight on lipids and its use for covalent and non-covalent conjugations. Al Mustansir J Pharm Sci, 2020;20(3):1-13. Doi: 10.32947/ajps.v20i3.754

9. Gnananath K, Nataraj KS, Rao BG. Phospholipid complex technique for superior bioavailability of phytoconstituents. Advan Pharm Bull, 2017;*7*(1):35. Doi: 10.15171/apb.2017.005

10. Khudhur AQ, Maraie NK, Raauf AM. Identification and quantitation of phospholipid binding sites in lipid drug conjugate pharmacosomes using model drug. System Rev Pharm, 2020;11(5):458-463. Doi: 10.31838/srp.2020.5.63

11. Gokce EH, Korkmaz E, Tuncay Tanrıverdi S, Dellera E, Sandri G, Bonferoni MC, Ozer O. A comparative evaluation of coenzyme Q10-loaded liposomes and solid lipid nanoparticles as dermal antioxidant carriers. Inter J Nanomed, 2012;*7*:5109. Doi: 10.2147/IJN.S34921

12. Jena SK, Singh C, Dora CP, Suresh S. Development of tamoxifen-phospholipid complex: novel approach for improving solubility and bioavailability. Inter J Pharm, 2014;473(1-2):1-9 Doi: 10.1016/j.ijpharm.2014.06.056

13. Akhter S, Paul S, Hasan I, Ayon NJ, Haider SS, Reza MS. Preparation, characterization and compatibility studies of Naproxen loaded microspheres of cellulosic and polymethacrylic polymeric blend. Dhak Uni J Pharm Sci, 2013;12(1):11-21. Doi: 10.3329/dujps.v12i1.16295

14. Zhang K, Gu L, Chen J, Zhang Y, Jiang Y, Zhao L, et al. Preparation and evaluation of kaempferol–phospholipid complex for pharmacokinetics and bioavailability in SD rats. J Pharm and Biomed Ana, 2015;114:168-175. Doi: 10.1016/j.jpba.2015.05.017

15. Semalty M, Badoni P, Singh D, Semalty A. Modulation of solubility and dissolution of furosemide by preparation of phospholipid complex. Drug Develop and Therapeut, 2014;5:172. Doi: 10.4103/WKMP-0090.139641