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

The historical development of bioanalytical method validation guidance

Editorial Article

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A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical and biological drug(s) and their metabolite(s). Bioanalytical method validation employed for the quantitative determination of drugs in biological fluids play a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic study data. The quality of these studies is directly related to the quality of the underlying bioanalytical data. It is therefore important that guiding principles for the validation of these analytical methods be established.

Prior to 1990, there were only regulations requiring the bioanalytical methods to be sensitive, specific, accurate and precise. There was a lack of uniformity in conducting validation of bioanalytical method, submission of data to the regulatory agencies and evaluation of the submitted data around the world. As reported in the articles published by Shah (2007) and by Shah and Bansal (2011), the first successful attempt at harmonizing the procedures and requirements for conducting bioanalysis was made in 1990 in a workshop dedicated to bioanalytical method validation. This workshop was co-sponsored by the American Association of Pharmaceutical Scientists (AAPS) and the Food and Drug Administration (FDA). The conference focused on requirements for bioanalytical methods validation, procedures to establish reliability of the analytical method, parameters to ensure acceptability of analytical method performance, method development and method application. The workshop defined essential parameters for bioanalytical method validation — accuracy, precision, selectivity, sensitivity, reproducibility, limit of quantification, and stability — and addressed "how to" evaluate and determine these parameters. In addition to defining various bioanalytical method validation parameters, the workshop discussed appropriate method validation procedures and defined the standard curve, recovery, and replicate analysis. One of the most important outcomes of the first workshop was that it defined "the acceptance criteria for a run". However, the workshop report was not an official document of the FDA. Therefore, the agency decided to develop and publish a draft guidance in January 1999.

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The second workshop was held in January 2000. The workshop focused on advancements in the field of mass spectrometery and discussed the ligand binding assays. Selectivity issues in ligand binding assays were discussed in detail. Two types of issues must be considered: interference from substances that are physicochemically similar to analyte and interference from matrix components (also termed "matrix effects") that are unrelated to the analyte. And also discussed different categories of validation; Partial Validation, Cross-Validation, and Full Validation. This workshop resulted in the report "A revisit with a decade of progress" and formed the basis for FDA-Guidance for Industry on Bioanalytical Method Validation" in 2001. This guidance was developed largely for the quantitation of small molecules using chromatographic techniques, but applied equally to large molecules and a variety of analytical techniques.

The third workshop discussed the requirements and procedures for bioanalysis using chromatographic or ligand-binding assay was held in May 2006. This workshop clarified the issues related to replacement of quality control samples, determination of matrix effect, use of internal standards and incurred samples reanalysis (ISR). The proceedings of the workshop were published in 2007 (Viswanathan, C. T. et al., Workshop/Conference Report — Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays). The following workshop was held in 2008 and discussed the strategies for conducting ISR. Recommendations concerning the basis for ISR, general operational principles, assessment timing and scope, sample selection, and acceptance criteria were offered. The workshop report was published in 2009 (Fast D, et al., Workshop Report and Follow-Up—AAPS Workshop on Current Topics in GLP Bioanalysis: Assay Reproducibility for Incurred Samples—Implications of Crystal City Recommendations).

In September 2013, the FDA released a draft revision of the Bioanalytical Method Validation Guidance, which included a number of changes to the expectations for bioanalysis, most notably the inclusion of biomarker assays and data. To inclusive discussion of the revised draft Bioanalytical Method Validation Guidance, the AAPS and FDA once again collaborated to convene a workshop during early December 2013. This Workshop included discussions on such science-driven topics such as the status of ISR after several years of application, new immunoassay technology, antibody-drug conjugates and the application of Liquid Chromatography Mass Spectrometry protein quantitation. Additionally, the Agency through the guidance and its presentations highlighted areas of additional focus for chromatographic and ligand-binding assays, such as stock solution expiry and most notably, the inclusion of biomarker assays within the Guidance. The workshop report was published in 2014 (Booth B, et al., Workshop Report: Crystal City V—Quantitative Bioanalytical Method Validation and Implementation: The 2013 Revised FDA Guidance) and formed the basis for FDA-Guidance for Industry on Bioanalytical Method Validation in 2018 provided recommendations for the development, validation, and in-study use of bioanalytical methods.

As a Founding Regulatory Member of ICH, FDA plays a major role in the development of each of the ICH guidelines. ICH Guidance "M10 Bioanalytical Method Validation and study sample analysis Guidance for Industry" finalised in 2022 addressed other industry concerns about bioanalytical assays, which are methods used to quantify the analyte(s) and/or their metabolite(s) in biological matrices. This guidance is intended to provide industry with harmonized regulatory expectations for bioanalytical method validation of assays used to support regulatory submissions. Namely to ensure that submissions advance toward the clinical trial phase, it would be necessary to adjust current clinical trial application approaches to the new ICH M10 requirements.

REVIEW ARTICLE

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Evaluation of the differences between acute and chronic asthma models with OVA/Alum exposure

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ABSTRACT

This review article discusses the differences between animal models using ovalbumin (OVA) and aluminum hydroxide (alum). OVA, derived from chicken eggs, is a widely used allergen due to its low cost and high purity. Environmental sensitization and/or using an adjuvant such as alum is required to induce an asthma-like response. Animal species and strain, as well as sex selection, influence the development of allergic airway inflammation and other asthma-related features *in vivo* models. Acute asthma models include OVA and alum to elicit airway inflammation, elevated IgE levels, and airway hyperresponsiveness. However, these models have limitations as they do not fully mimic the chronic inflammation and airway remodeling observed in human asthma. On the other hand, chronic asthma models involve prolonged exposure to low concentrations of allergens and have been shown to exhibit persistent airway hyperresponsiveness, airway remodeling, and other critical features of asthma. The aforementioned models have provided valuable insights into the pathophysiology of asthma and have been used to evaluate potential therapeutic agents. Overall, the use of OVA and alum in animal models has improved our understanding of asthma, and it is hoped that it has the potential to guide clinical therapies in the future.

Keywords: OVA, alum, asthma, *in vivo* models, mouse

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INTRODUCTION

Ovalbumin (OVA) from chicken eggs is the most frequently used model allergen due to its low cost and ability to be obtained in the highest purity[1]. In model formation, OVA administration induces immune tolerance. To generate a comprehensive asthma-like response in OVA-based models, environmental sensitization and/or the use of an adjuvant are necessary. Animal models may also favor proteins or extracts derived from human allergens. Ragweed, extract of house dust mites (HDM) extract, and fungi, including Alternaria alternata and Aspergillus fumigatus, are examples of such allergens[2]. When allergens such as OVA induce asthmatic responses in rodents, sensitization via a systemic route, particularly intraperitoneal, is frequently required. Additionally, to enhance the immunogenicity of the sensitizing allergen, alum is used to stimulate Th2 phenotype immune responses selectively. Most related research studies indicate that OVA and alum are commonly employed either sequentially or through repeated short/long-term applications. The exploration of various lung-related pathological conditions and the advancement of novel treatments are progressing rapidly, facilitated by *in vivo* models. This review compares and assesses three mouse models according to a selection of species/strain, acute, and chronic asthma models utilized thus far.

Determination of species and strain

Animal models of experimental allergic asthma permit the identification of disease development mechanisms, the examination of disease progression over time, and the evaluation of therapeutic agents' potential to reduce the severity of inflammation in allergic asthma[3]. Different animal species can be selected depending on the subject of the research. Mice, the most commonly used species in animal models, are preferred due to their short gestation period and large number of inbred strains.

Mice are used as animal models to study many diseases, such as asthma[2]. There are sensitization and challenge phases to mimic human asthma pathogenesis in animal models. OVA challenge models are valuable tools for understanding the effects and mechanisms of asthma disease, as it is a significant allergen. These *in vivo* models provide insights into the impact of OVA and the potential therapeutic agents for asthma. The choice of mouse strain directly affects the allergic inflammation response in OVA-induced asthma models[4]. A study by Kung et al. described an allergic pulmonary inflammation model using OVA[5]. Research with mice on asthma and the Th2 high asthma phenotype has led to an improvement in the pathophysiology of asthma.

Gender is among the most fundamental characteristics that differentiate individuals from one another. Gender disparities influence the incidence, intensity, and therapeutic response to asthma, among other conditions. Melgert et al. demonstrated that in mouse models of asthma, a chronic inflammatory disease of the airways, female mice are more likely to develop allergic airway inflammation and hyperresponsiveness than male mice[6]. The study demonstrated that the levels of total cells, eosinophils, and lymphocytes were notably elevated in female mice sensitized and challenged with OVA (OVA/OVA) compared to their male mice. However, hemotoxylin-eosin staining for histological analysis revealed a notable increase in peribronchial and perivascular inflammatory cells in female mice with OVA/OVA infection compared to their male mice. When cytokine levels in the bronchoalveolar lavage (BAL) fluid of mice were evaluated, another significant parameter was elevated levels of IL-13, IL-10, TGF-β cytokines, and IgE in female OVA/OVA mice compared to their male mice[7].

Asthma models

Asthma is a significant global health concern that is progressively worsening. The majority of knowledge about allergens is derived from research, particularly that which is conducted on animal models. The immunological pathways of asthma have been clarified, and the potential of candidate therapeutic agents has been evaluated as a result of these studies. Protocol variations within and between acute and chronic asthma models impact the physiological responses obtained, as shown in (Table 1), a summary of the OVA and alum models.

Table 1. Mouse models of acute and chronic asthma models

Model	Gender/ Strain	Allergen	Sensitization	Challenge	Response to Challenge	References
Acute	Balb/c	OVA	OVA+ alum (i.p) on days 1 and 14 (20mg OVA+2 mg alum in 200ml PBS)	OVA aerosol on days 21-23 (100mg in 20mL PBS)	Inflammatory cell infiltration in bronchiole, mucus hypersecretion, alveolar congestion, alveolar wall edema	8
Acute	Balb/c	OVA	OVA+ alum (i.p) on days 0-7 (20mg OVA+2 mg alum in 200ml PBS)	OVA (i.t) on days 14-20 (0,1% OVA in 30ml PBS)	Mucus hypersecretion, goblet cell hyperplasia, peribronchial, perivascular airway epithelial thickening	9
Chronic	Balb/c	OVA	OVA + alum aerosol on days 1-12 (0,01 mg/mouse in 200mL alum)	OVA aerosol 18-23 and 26-55 (aerosolized 5% OVA)	Airway hypersensitivity, increased airway smooth muscle mass, goblet cell hyperplasia, increased number of bronchial mucosal eosinophils, peribronchiolar inflammation	10
Chronic	Balb/c	OVA	OVA + alum (i.p) on days 0-14 (10 mg OVA +2 mg alum in 100ml PBS)	OVA aerosol on days 21-24 (aerosolized 1% OVA)	Goblet cell hyperplasia bronchitis and bronchovascular inflammation eosinophil infiltration in airway epithelium and lung tissue	11

Acute asthma

Asthma does not develop spontaneously in animals compared to humans; therefore, the animal species used to study the asthma model requires the administration of external allergens[12]. It has been reported that the OVA-induced mouse asthma model observed airway hyperresponsiveness, elevated IgE levels, goblet cell hyperplasia, and airway remodeling effects[12,13]. Although many different sensitization and challenge protocols have been applied, researchers consider the basic model more consistent. If an adjuvant is present in acute sensitization protocols multiple systemic allergen administration is required. Aluminum hydroxide (alum), one of the most commonly used adjuvants, is known to stimulate a Th2-directed immune response by the immune system when exposed to an antigen[12].

Airway inflammation, goblet cell hyperplasia, elevated IgE levels, and airway hyperresponsiveness to particular allergens are fundamental asthma charac-

teristics observed in acute asthma models. However, differences can also be observed in the models that are obtained. Allergens are exposed to elevated concentrations for short periods in the models. On the contrary, long-term exposure to low concentrations of allergens induces asthma in humans. Furthermore, many of the key features of asthma appear over a short period, with some model studies showing that airway inflammation and hyperresponsiveness patterns disappear within a few weeks of the last allergen administration[14]. Although acute and chronic airway wall inflammation is characteristic of human asthma, acute, perivascular, and peribronchial inflammation of the lung parenchyma was observed in a mouse model of acute asthma[15]. The research has demonstrated that acute asthma models do not undergo alterations in airway remodeling, including subepithelial fibrosis, epithelial proliferation, and chronic inflammation of the airway[16]. Despite these noticeable limits, models of acute asthma have been effectively employed to examine the correlation between inflammatory cells and the mediating mechanisms at play, as well as the effect of the stages occurring in the lungs. The understanding of asthma as a Th2-biased disease, the function of T cells and eosinophils in the allergic response, and their impact on airway processes have all been enhanced by research[12].

Chronic asthma

The research on specific issues encountered in acute asthma models leads to examining chronic asthma models. The purpose of chronic asthma models is to accurately replicate the medical symptoms of the condition, including persistent airway hyperresponsiveness (AHR) and airway remodeling, and evaluate potential therapeutic agents. Through up to 12 weeks, the airways are exposed to low concentrations of allergens to generate the models. Several studies have identified various allergens that are known to cause allergic reactions. These include OVA[17], Lipopolysaccharide (LPS)[18], house dust mites (HDM)[19], *Alternata alternatia*[20], cockroach extracts[21], and pollen[22]. Chronic exposure to allergens has been shown to cause allergen-induced sensitization, Th2-directed allergic inflammation owing to eosinophils in the mucosa of the airways, and AHR in mice. Furthermore, certain models have demonstrated the presence of goblet cell hyperplasia, subepithelial or peribronchial fibrosis, and airway remodeling, in addition to the mentioned asthma reaction patterns. These responses are followed by airway smooth muscle thickening[23]. These patterns lead to airway remodeling asthma observations in adult humans[24]. As a result of chronic allergen exposure, some of the key features of asthma have been to persist after model application[19,25].

Mouse models of chronic asthma induced by OVA exhibited peribronchial

inflammation, dysregulation of extracellular matrix proteins, increased tissue accumulation, and subepithelial collagen deposition due to prolonged allergen stimulation. These histopathologic data are known markers of airway remodeling in a chronic asthma model. A study demonstrated an increase in the cytokines IL-4, IL-5 and IL-13 in the lung samples of mice subjected to an ELISA assay[10]. In another study indicating the chronic asthma model, it has been observed that the levels of IL-4, IL-5 and IL-1β cytokines exhibit an elevation[11]. Previous research has indicated that IL-1β, a pro-inflammatory cytokine, attracts monocytes and macrophages during the OVA sensitization and challenge stages[26]. A reduction in the quantity of regulatory T cells (Treg cells) was observed during the analysis of lymphocytes from the spleen of mice[11]. Previous studies show that asthmatic inflammation leads to the suppression of Treg cells[27]. Chronic asthma models can be used to research the basic pathological mechanisms of asthma as a result of allergen exposure. Chronic asthma models provide a suitable system for the developing new therapeutic agents for asthma.

METHODOLOGY

The focus of the search was clarified by searching the PubMed database for current mouse models of asthma. Mouse strains and allergens were identified as common components of the search. The selection was based on the application, time intervals, frequency during the sensitization and provocation phases for the acute and chronic asthma models. The different concentrations and application times of allergens were highlighted in the selected articles.

RESULTS and DISCUSSION

Asthma is a heterogeneous disease for which much of the pathophysiology is still unknown. Animal models are used to map asthma's immunological pathways and to demonstrate the efficacy of many research components involved in critical regions of these pathways. Spontaneous resolution of the response in OVA-induced acute asthma models in a short period, such as a few days, is limited in its ability to reflect the clinical features of asthma[28]. Acute asthma models only allow us to focus on the mechanisms of asthma development. The limitations of acute models pave the way for developing chronic asthma models. Our review evaluated the results of selected acute and chronic models. A model example of acute asthma was presented in the study by Wu et al. which reported the presence of thickened lung tissues and alveolar walls and infiltration of inflammatory cells[8]. A similar result was obtained in a study of airway inflammation in an OVA-induced asthma model[29]. Mucus hypersecretion in the airway epithelium is one of the most important findings in allergic asth-

ma[30]. In addition, BAL fluid cell counts revealed high concentrations of eosinophils, which have been identified as contributing to allergic inflammation[31]. Lung tissue analysis revealed high levels of the cytokines IL-4, IL-5 and IL-13[25]. OVA and alum have been shown to stimulate Th2-directed immune pathways in acute asthma models[32]. Histopathological evaluation of the acute asthma model studied by Rajasekar et al. showed the presence of epithelial thickening, goblet cell hyperplasia, and mucus hypersecretion in the peribronchial and perivascular airways compared to the control group. The number of eosinophils, neutrophils and macrophages in the BAL fluid increased with OVA exposure. Increased levels of IgE, a known marker of airway inflammation, and IL-4, a member of the Th2 cytokine family, were observed. In addition, levels of TNF-α and IFN-γ, both pro-inflammatory cytokines, were elevated compared with the control group[9]. There were differences between the two models of acute asthma in the timing of OVA and alum administration during the sensitization phase and the days and route of OVA administration during the challenge phase. Despite these differences, histopathological markers of airway inflammation were obtained in both models, and Th2 cytokine-driven immunity was shown to occur with the cytokines analyzed from BAL fluid[8,9]. Acute asthma models are helpful for studying the pathophysiological patterns that occur in the acute phase of asthma. However, chronic asthma models are preferred to study the pathways involved in the chronic asthma process and the effect on airway remodeling. The two studies reviewed used comparable chronic asthma models[10,11]. The doses and duration of administration varied in the models. Despite the different doses and durations, common asthma phenotypes such as goblet hyperplasia, inflammation of BAL cells, and increase in Th2 cytokines, AHR, and eosinophil filtration were observed in mouse models in both studies[10,11]. In chronic asthma mouse models, the duration and dose of protocol modifications depend on the specific aspect of asthma being studied. Still, the common allergens used provide similar results in asthma pathogenesis. It has also been shown that repeated allergen challenges in cricket asthma models can mimic critical markers of human asthma.

Collectively, mouse models are often the preferred method to study asthma. The complex biochemical nature of the disease makes it difficult for mouse models to mimic human asthma. The phenotypes of mouse models of chronic asthma that differ from human asthma include:

late-phase bronchoconstriction,

a different distribution of lung inflammation than in humans,

tolerance after repeated allergen exposure[1].

Therefore, model studies have focused on specific patterns of asthma rather than a holistic approach to the disease. Since many primary phenotypes seen in asthma can be reproduced in OVA models, it was a suitable model choice. Although no mouse model completely mimics asthmatic patients' pathology and physiology, animal models using OVA and alum have accelerated the understanding of the biochemical mechanisms underlying asthma and the investigation of therapeutic targets. The timing of OVA and alum administration in animal models varies depending on the specific characteristics of asthma to be studied. Variations in model protocols lead to differences in the results obtained. In this way, asthma can be investigated in more detail through the specific mechanism of action established. This review will help researchers choose the more appropriate asthma models among differential asthma patterns for their studies. The review is expected to contribute to more detailed modeling of asthma disease by enabling essential science findings from model systems to help pre-clinical studies.

STATEMENT OF ETHICS

Ethical approval does not apply to this article.

CONFLICT OF INTEREST STATEMENT

The author declares that there are no conflicts of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

The author acknowledges sole responsibility for the following parts: conception, drafting, interpretation, and manuscript preparation.

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ORIGINAL ARTICLES

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Diabetes knowledge of pharmacy students: A cross-sectional study

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ABSTRACT

The knowledge level of diabetes mellitus (DM) is considered as an important factor for disease control and quality of life. The aim of this study is to determine the diabetes knowledge level of undergraduate students in a pharmacy faculty in Türkiye. A cross-sectional study using online questionnaire was conducted from 2-16 January 2023 among the 4th and 5th year pharmacy students. For scoring, responses were assigned a value of 1 point when answered is "true", while it is "false" or "I don't know" responses were designated as 0 points. The mean knowledge score was found to be as 34.07 ± 4.13 (maximum score is 40). The subdomain scores were found to be as follows; diabetes risk factor 2.88 ± 0.37 (maximum score is 3), diabetes symptoms 2.70 ± 0.53 (maximum score is 3), diabetes diagnosis 3.46 ± 0.91. The Cronbach's alpha value of the scale was found to be 0.767. Students were found to have sufficient knowledge of the diagnosis of DM, symptoms, and risk factors. Nonetheless, participants exhibited notable knowledge gaps, particularly in domains such as diet in diabetes and treatment in gestational diabetes have been identified among participants.

Keywords: diabetes mellitus, knowledge level, pharmacy student, questionnaire, Türkiye

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INTRODUCTION

Diabetes mellitus (DM) is a global health problem[1]. According to the International Diabetes Federation (IDF), 537 million people had diabetes in 2021 and is predicted to be 784 million in 2045. In Türkiye, there are approximately 9 million diabetic patients between the ages of 20-79 in 2021 and its prevalence is 14.5%[2].

According to the American Diabetes Association (ADA), DM is "a chronic metabolic disease characterized by hyperglycemia caused by impairment in insulin secretion, insulin action, or both of these factors." Polyuria, polydipsia, weight loss, sometimes polyphagia and blurred vision are among the symptoms of hyperglycemia[3]. DM has microvascular (nephropathy, neuropathy, retinopathy) and macrovascular (atherosclerotic heart diseases, peripheral arterial disease, cerebrovascular diseases) complications[4]. As medication consultants and health advisors, pharmacists contribute to the management of many chronic diseases, including DM. With the pharmaceutical care education given by pharmacists providing primary health care services to individuals with diabetes, it is possible to prevent the development of these complications, increase adherence to treatment, increase quality of life and improve health outcomes[5-9]. Pharmacists are required to take on direct patient care roles through practice, provide comprehensive medication management and provide preventive care services. In this direction, the principles of pharmacy education have expanded from the traditional roles of preparing the prescription and presenting it to the patient, to the delivery of patient-oriented pharmacy services such as pharmaceutical care[10]. In our country, the concept of patient-oriented pharmacy education was introduced for the first time in the early 90s. Theoretical and practical training is given to students within the scope of clinical pharmacy courses in many pharmacy faculties. In this context, based on the concept of patient-centered pharmacy, students monitor patient treatment, evaluate patient-specific medical problems, drug therapy, therapeutic problems, and comprehensive drug treatment for these problems. Collaboration with patients, patient care providers and other health professionals is ensured[11].

There are studies in which the education given by pharmacy students to patients with diabetes significantly increases the patients' drug compliance, their knowledge about their diseases and complications is improved, and their problems such as coping with hypoglycemic crisis are improved[12]. In order to achieve these improvements, pharmacy students should have up-to-date and evidence-based information on diabetes. There are studies showing that pharmacy students need more knowledge to prevent diabetes and its compli-

cations[13,14]. It has been shown that the level of knowledge and confidence in presenting knowledge of pharmacy students who receive education on diabetes increases[15]. As far as we know, although there are studies investigating the knowledge and attitudes of students from different faculties (nursing, medicine, etc.) towards DM, more studies are needed regarding pharmacy students' knowledge and attitudes towards DM[16,17]. The primary objective of this research is to assess the diabetes knowledge level of undergraduate students in a pharmacy faculty in Türkiye.

METHODOLOGY

This cross-sectional study was conducted with 4th and 5th-grade pharmacy students at a university in Istanbul (Türkiye). Pharmacy education consists of 10 terms (5 years) in this pharmacy faculty. Fourth-grade students are introduced to pharmaceutical care and clinical pharmacy as follows; 2 hours theoretical and 2 hours practical per week- case studies. The clinic rotations of pharmacy students start in the 8th and 9th terms. At the time of the study, 4th-grade students had not yet started clinical rotation, while 5th-grader students completed their clinical rotation.

After obtaining the necessary permissions for the sample size for the questionnaire, 50% response distribution was predicted to give a 5% margin of error and 95% confidence interval, and it was determined that 139 pharmacy faculty students should be reached (http:/ /www.raosoft.com/samplesize.html, Accessed 12.12.2022). In this study, nonprobability sampling (purposive) was used.

After the participants were informed about the study, their informed consent was obtained via online. The sociodemographic characteristic of the students and the "Knowledge Level Questionnaire on Diabetes" questionnaire[16] were sent to the participants online via Google Forms. "Knowledge Level Questionnaire on Diabetes" questionnaire was developed by Al Sarayra and Khalidi[18] was performed. The validity process was not applied. After the translation from English to Turkish was made by 2 pharmacists, it was applied as a pretest to 10 pharmacy students and the adaptation was adjusted. The questionnaire consisted of 40 questions: General information about the definition of diabetes (8 items), information about the risk factor of diabetes (3 items), information about the symptoms of diabetes (3 items), information about the diagnosis of diabetes (4 items), information about the treatment of diabetes (10 items), information about the complications of diabetes (4 items) and information about the diabetes diet and exercise (5 items), about the control of

diabetes information (3 items). The answer choices were “true; false; or I do not know”. For scoring, responses were assigned a value of 1 point when answered correctly, while incorrect or “I don’t know” responses were designated as 0 points (“I don’t know” answer was evaluated as “false”).

The Statistical Package for Social Sciences (SPSS), Version 11.5 (SPSS Inc., Chicago, IL) was used for statistical analysis. The Kolmogorov-Smirnov test and Shapiro-Wilk test were used to test the normal distribution of data. The quantitative data of the binary groups were made with the Mann Whitney U test, and the comparison of the quantitative data of more than two groups was made with the Kruskal-Wallis H test. In case of significance in more than two groups, pairwise analyzes of the groups were made with the Mann Whitney U test. The median (interquartile range) values of the quantitative data that did not show normal distribution were calculated. A Chi-square test was applied to compare categorical data. Whether there is any relationship between the numerical data was analyzed with the Spearman correlation test. Data with $p<0.05$ at the 95% confidence interval were considered statistically significant.

RESULTS and DISCUSSION

One hundred and ninety-six students completed the questionnaires; 77% were female. The mean age and standard deviation were 23.57 ± 1.1 years. Regarding the study year of students, 49.5% of the students participating in the study were 5th grade students and 50.5% were 4th grade students. The demographic characteristics of students were summarized in Table 1.

Table 1. Demographic information of the students

Variable	4th year n (%)	5th year n (%)
Sex		
Male Female	26 (26.3) 73 (73.7)	19 (19.6) 78 (80.4)
Age (years) (IQR)	23.05 (22-24)	24.09 (22-24)
Rotation (day per year) (IQR)	0	7.35 (7-8)
Family history of diabetes Diabetic Not diabetic Unknown	 50 (50.5) 48 (48.5) 1 (1)	 35 (36.1) 61 (62.9) 1 (1)
Did you take part in the care of a patient diagnosed with diabetes? Yes No	 17 (17.2) 82 (82.8)	 64 (66) 33 (34)
Duration of community pharmacy internship (mean month for per student)	1.9	2.8
Duration of clinic rotation (mean month for per student)	0.4	0.9
Total	**99**	**97**

n: numbers of the students, IQR: Interquartile range

The diabetes information access sources used by the students are classified in Table 2.

Table 2. Information resources that the students used

Resources	4th year n (%)	5th year n (%)
Lecture notes	98 (99)	97 (100)
International and national guidelines	67 (67.7)	95 (97.9)
Internet	84 (84.8)	87 (89.7)
Brochure	6 (6.1)	97 (100)
Text book	21 (21.2)	78 (80.4)

n: numbers of the students

The answers given by the students to the Knowledge Level Questionnaire on Diabetes are classified in Table 3.

Table 3. Knowledge of diabetes mellitus among pharmacy students

Questions	True n (%)	False n (%)	I don't know n (%)
Definition of diabetes (8 items)			
1. Definition: DM is an increased blood sugar above acceptable level (True)	**174 (88.78)**	20 (10.2)	2 (1.02)
2. Diabetes Mellitus is a chronic disease (True)	**188 (95.92)**	7 (3.57)	1 (0.51)
3. Commonest type of DM is type 2 (True)	**191 (97.45)**	1 (0.51)	4 (2.04)
4. DM may be present in pregnant women (True)	**195 (99.49)**	0 (0)	1 (0.51)
5. Insulin deficiency is found in type 1 DM (True)	**171 (87.24)**	24 (12.24)	1 (0.51)
6. Insulin dysfunction is found in type 2 DM (True)	**177 (90.31)**	17 (8.67)	2 (1.02)
7. Insulin deficiency is found in type 2 DM (False)	45 (22.96)	**149 (76.02)**	2 (1.02)
8. Type 2 DM can be found in adolescent (True)	135 (68.88)	46 (23.47)	15(7.65)
Risk factor of diabetes (3 items)			
9. Risk factors for DM is obesity (True)	**193 (98.47)**	2 (1.02)	1 (0.51)
10. Risk factors for DM is family history (True)	**195 (99.49)**	0 (0)	1 (0.51)
11. Risk factors for DM is excessive sugar intake (True)	**176 (89.8)**	11 (5.61)	9 (4.59)
Symptoms of diabetes (3 items)			
12. One of the DM symptoms is excessive thirst (True)	**192 (97.96)**	1 (0.51)	3 (1.53)
13. One of the DM symptoms is weight loss (True)	**149 (76.02)**	35 (17.86)	12(6.12)
14. One of the DM symptoms is excessive urination (True)	**189 (96.43)**	2 (1.02)	5 (2.55)
Diagnosis of diabetes (4 items)			
15. Cut-off point for DM diagnosis is fasting blood sugar of 200mg/dl (False)	31 (15.82)	**163 (83.16)**	2 (1.02)
16. Cut-off point for DM diagnosis is fasting blood sugar of 126mg/dl (True)	**177 (90.31)**	14 (7.14)	5 (2.55)
17. Cut-off point for DM diagnosis is fasting blood sugar of 90mg/dl (False)	21 (10.71)	**168 (85.71)**	7 (3.57)
18. Urine sugar cannot be used to diagnose DM (False)	20 (10.2)	**171 (87.24)**	5 (2.55)
Treatment of diabetes (10 items)			
19. Mode of treatment in type 1 DM is diet therapy and insulin (True)	**186 (94.9)**	7 (3.57)	3 (1.53)
20. Mode of treatment in type 1 DM is diet therapy and hypoglycemic drugs (False)	21 (10.71)	**168 (85.71)**	7 (3.57)
21. Mode of treatment in type 1 DM is oral hypoglycemic drugs (False)	28 (14.29)	**155 (79.08)**	13(6.63)
22. Mode of treatment in type 1 DM is insulin alone (False)	105 (53.57)	88 (44.9)	3 (1.53)

23. Mode of treatment in type 2 DM is diet therapy and weight reduction (True)	**145 (73.98)**	48 (24.49)	3 (1.53)
24. Mode of treatment in type 2 DM is oral hypoglycemic drugs alone (False)	19 (9.69)	**171 (87.24)**	6 (3.06)
25. Mode of treatment in type 2 DM is insulin alone (False)	3 (1.53)	**190 (96.94)**	3 (1.53)
26. DM in pregnant can be treated by insulin and hypoglycemic drugs (False)	76 (38.78)	99 (50.51)	21(10.71)
27. DM in pregnant can be treated by insulin (True)	**163 (83.16)**	12 (6.12)	21(10.71)
28. DM in pregnant can be treated by oral hypoglycemic drugs alone (False)	41 (20.92)	133 (67.86)	22(11.22)
Complications of diabetes (4 items)			
29. Complications of DM may be seen in kidneys (True)	**191 (97.95)**	2 (1.03)	2 (1.03)
30. Complications of DM may be seen in eyes (True)	**189 (96.43)**	1 (0.51)	6 (3.06)
31. Complications of DM may be seen in nerves (True)	**184 (93.88)**	3 (1.53)	9 (4.59)
32. Complications of DM may be seen in lower limbs (as amputation) (True)	**182 (92.86)**	4 (2.04)	10 (5.1)
Diabetes diet and exercise (5 items)			
33. Diet therapy means 3 meals and 3 snacks (True)	115 (58.67)	31 (15.82)	50(25.51)
34. Diet therapy means 2 meals and 2 snacks (False)	34 (17.35)	108 (55.1)	54(27.55)
35. Diet therapy means not to eat carbohydrate (False)	12 (6.12)	**177 (90.31)**	7 (3.57)
36. Exercise in type 2 DM is recommended (True)	**193 (98.47)**	1 (0.51)	2 (1.02)
37. Exercise in type 1 DM is recommended (True)	**146 (74.49)**	26 (13.27)	24(12.24)
Control of diabetes information (3 items)			
38. Control of diabetes by measuring urine sugar (True)	**166 (84.69)**	19 (9.69)	11 (5.61)
39. Control of diabetes by measuring HbAc1 (True)	**185 (94.39)**	9 (4.59)	2 (1.02)
40. Control of diabetes by measuring daily blood sugar (True)	**189 (96.43)**	4 (2.04)	3 (1.53)

n: numbers of students DM: Diabetes Mellitus

The relationship between the education level and the answers given to the knowledge level questionnaire on diabetes is given in Table 4.

Table 4. Relationship between the education level and the answers given to the knowledge level questionnaire on DM

Subdomain	Questions	4th year n (%)		5th year n (%)		p value
		False	True	False	True	
Definition of diabetes	3. Commonest type of DM is type 2 (True)	0	99 (100)	5 (5.2)	92 (94.8)	0.022
Risk factor of diabetes	11. Risk factors for DM is excessive sugar intake (True)	4 (4)	95 (96)	16 (16.5)	81 (83.5)	0.004
Diagnosis of diabetes	18. Urine sugar cannot be used to diagnose DM (False)	7 (7.1)	92 (92.9)	18 (18.6)	79 (81.4)	0.016
Treatment of diabetes	21. Mode of treatment in type 1 DM is oral hypoglycemic drugs (False)	29 (29.3)	70 (70.7)	12 (12.4)	85 (87.6)	0.004
	22. Mode of treatment in type 1 DM is insulin alone (False)	46 (46.5)	53 (53.5)	62 (63.9)	35 (36.1)	0.014
	26. DM in pregnant can be treated by insulin and hypoglycemic drugs (False)	59 (59.6)	40 (40.4)	38 (39.2)	59 (60.8)	0.004
	27. DM in pregnant can be treated by insulin (True)	26 (26.3)	73 (73.7)	7 (7.2)	90 (92.8)	<0.001
	28. DM in pregnant can be treated by oral hypoglycemic drugs alone (False)	43 (43.4)	56 (56.6)	20 (20.6)	77 (79.4)	0.001
Complications of diabetes	31. Complications of DM may be seen in nerves (True)	2 (2)	97 (98)	10 (10.3)	87 (89.7)	0.016
Diabetes diet and exercise	33. Diet therapy means 3 meals and 3 snacks (True)	32 (32.3)	67 (67.7)	48 (49.5)	49 (50.5)	0.015
Control of diabetes	38. Control of diabetes by measuring urine sugar (True)	10 (10.1)	89 (89.9)	20 (20.6)	77 (79.4)	0.041

n: numbers of students DM: Diabetes Mellitus

There was not any significant relation between the sociodemographic characteristics of the participants, except for a family history of diabetes. 43.9% of those who answered the question 14 correctly had a family history of diabetes (p=0.002). Of those who answered question 15 correctly, 44.8% had a family history of diabetes (p=0.006). 66.7% of those who gave incorrect answers to questions 25 and 28 did not have a family history of diabetes (p<0.001), (p=0.007). Of those who answered the question 31 correctly, 45.7% had a family history of diabetes (p=0.002). Of those who answered the question 32 correctly, 44.5% had a family history of diabetes (p=0.039). 44.9% of those who answered the question correctly had a family history of diabetes (p=0.008).

The primary objective of this research is to assess the diabetes knowledge level of undergraduate students in a pharmacy faculty in Türkiye. In the literature, although there are many studies investigating the DM knowledge level of university students, there are not many studies investigating the DM knowledge level of pharmacy students. The percentage of female students in this study (77%) was found to be higher in contrast to similar studies (37%-64%)[17-20]. The percentage of the students with a family history of diabetes (43%) was found to be lower in contrast to other studies (67%)[17,19].

It was found that the students had sufficient knowledge about DM diagnosis with 3.46 points, symptoms with 2.7 points, and risk factors with 2.88 point. In a study conducted in Uganda, it was observed that 86% of university students had sufficient knowledge about the signs and symptoms of DM[21]. In a study conducted in Iraq, it was found that university students had an adequate level (a response of more than 50%)[22]. In a study conducted with university students studying in the field of health technicians in Türkiye, DM knowledge level of students was found to be limited[16]. However, in a study conducted in Poland, the knowledge level of medicine students was found to be insufficient[23].

Response rates of participants to questions regarding the knowledge level of DM ranged between 69% and 99%. This rate is quite high compared to studies conducted with other university students[24,25]. One of the reasons for this may be that it was conducted with pharmacy students. Because similar studies show that students studying in the field of health have a higher level of knowledge than students in other fields.

There are studies in which female students have a significantly better knowledge of DM general knowledge, risk factors, signs and symptoms, control and management, complications, and total knowledge scores[24-26]. Since the number of female students was much higher than male students in our study, it was not found statistically significant in our study ($p>0.05$).

Considering the participants with and without a family history of diabetes, there is a significant difference in the answers given to the following questions; One symptoms of DM is excessive urination, cut-off point for DM diagnosis is fasting blood sugar of 200 mg/dL, mode of treatment in type 2 DM is insulin alone, DM in pregnant can be treated by oral hypoglycemic drugs alone, complications of DM may be seen in nerves, complications of DM may be seen in lower limbs (as amputation) ($p<0.05$). In a similar study, the answers given to the questions such as risk factors for DM is family history and decreased physical activity, one symptom of DM is weight loss differed significantly depending on whether there was a family history of diabetes ($p<0.05$)[27]. In a study con-

ducted with university students, a significant relationship was found between the family history of DM and the knowledge and awareness of diabetes of those with no family history of diabetes. This may be due to caring for relatives of DM patients[24].

When the 4th and 5th-grade students were compared, it was found that the knowledge level of the 4th grade students was significantly higher in the correct answers to questions such as definition, risk factors, diagnosis, treatment, complications of DM ($p<0.05$). One of the reasons for this may be that the theoretical knowledge of 4th-grade students who have not yet started clinical rotation may be more up to date. Contrary to our study, in a study involving medical school students, clinical group had significantly better diabetes knowledge test results than preclinical group ($p<0.05$)[23].

All 4th and 5th grade students stated that they benefit from textbook notes as information sources about DM. In a study conducted with Saudi and Jordanian students, television was the highest source of information about DM, with 35% and 28%[28].

Students were found to have a sufficient foundational understanding regarding DM diagnosis, symptoms, and risk factors. Nonetheless, participants exhibited notable knowledge gaps, particularly in domains such as diet in diabetes and treatment in gestational diabetes have been identified among participants. This study will be helpful for future researchers who will investigate the knowledge level of DM among pharmacy students.

STATEMENT OF ETHICS

This study was granted ethical approval by the University of Health Sciences Hamidiye Scientific Research Ethics Committee, (Decision no: 28/9, Registration no: 22/634, Date: 30.12.2022), and was conducted in accordance with the ethical guidelines stipulated by the Declaration of Helsinki.

CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

The authors contributed equally.

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Effect of kaempferol, amygdalin and methylprednisolone alone and in combination in induced cytokine storm in mice

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ABSTRACT

Cytokine storm can cause organ failure and even death in severe cases. There is ongoing research into developing drugs that can target cytokines and modulate the immune response to prevent or treat cytokine storm for that reason the present study aimed to evaluate the effect of kaempferol, amygdalin and methylprednisolone alone and in combination for their potential effectiveness in managing cytokine storm in a study including the collection of blood samples from groups included an apparently healthy, negative control, positive control and ten other groups of prophylaxis and treatment with methylprednisolone, kaempferol, amygdalin either alone or in combination to measure interleukin -1β, 6, 8 (IL-1β, 6, 8) and tumor necrosis factor- α (TNF-α) which demonstrated that in comparison with controls, the blood levels of IL-1, IL-6, IL-8, and TNF-α were significantly higher in the Lipopolysaccharide (LPS) group which decreased by using the studied drugs either alone or in combination for one hour before or after LPS induction which demonstrated that natural compounds such as amygdalin, and kaempferol have shown promise in reducing the levels of pro-inflammatory cytokines and may have therapeutic potential for the treatment of chronic inflammatory diseases.

Keywords: amygdalin, cytokine storm, kaempferol, methylprednisolone

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INTRODUCTION

Cytokine storm is a situation in which cytokines are released in excess in reaction to an infection or damage, resulting in an overactive immune response[1]. It is most commonly associated with severe cases of infections, such as coronavirus disease-2019 (COVID-19), H1N1 influenza virus, and is also seen in autoimmune disorders and certain cancers[2].

Interleukin 1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α). are part of the cytokine network that has pro-inflammatory effects. Pro-inflammatory IL-1β is released in response to cell damage and lipopolysaccharide (LPS) metabolites from bacterial cell membranes[3]. An inflammatory cytokine with pleiotropic effects that promotes hematopoiesis, acute phase reactions, and particular immunological responses is IL-6. The greater death rate of COVID-19 has been related to IL-6[4]. One of the most important cytokines studied was TNF-α, which is involved in the cascade of inflammation and has a variety of intricate roles in the immune system[5,6].

There is currently no specific treatment for cytokine storm. Treatment is primarily supportive and may include measures to manage symptoms, such as oxygen therapy for respiratory distress, and medications to manage inflammation and fever[7,8]. However, certain drugs such as corticosteroids, tocilizumab, anakinra and baricitinib have been used to help regulate the immune response and reduce the risk of organ damage and other complications associated with cytokine storm but the use of these drugs is limited by their side effects[9-12]. In addition, some medications used to treat cytokine storm can increase the risk of blood clots, which can be a serious complication[13]. Immunosuppression drugs can increase the risk of infections and other complications which is a serious concern for immunocompromised patients[14].

For these reasons the scientific community need to development or find a new drug used for treatment of patient with cytokine storm with low or without side effect such as Kaempferol and Amygdalin. Kaempferol is one of the most commonly encountered aglycone flavonoids in the form of a glycoside which have been shown in numerous preclinical studies to have a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, anti-diabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic, and antiallergic activities[15].

Amygdalin is a plant glucoside from the Rosaceae family. The anti-fibrosis, anti-inflammatory, analgesic, auxiliary anticancer, immunoregulation, anti-atherosclerosis, anti-cardiac hypertrophy, anti-ulcer, and hypoglycemic prop-

erties of amygdalin have all received much research over the years. Additionally, it has the ability to cure neurological illnesses, stimulate ovulation, reduce endometriosis, and limit sperm hyaluronidase activity and motility[16].

The current work aimed to evaluate the efficacy of kaempferol, amygdalin, and methylprednisolone in treating cytokine storm by investigating their effect on the levels of IL-6, IL-18 and TNF-α in serum of Swiss Albino mice induced by lipopolysaccharide (LPS).

METHODOLOGY

A prospective control randomize experiment design was used in this study. Hangzhou Hyper Chemical Company (China) provided the amygdalin, methylprednisolone acetate, and kaempferol; Sigma Aldrich Chemical Company provided the LPS (USA). The dimethyl sulfoxide was given by Alpha Chemical (India) (DMSO). India's Edutek Co., Ltd. provided the formaldehyde. ELISA kits for IL-1β, IL-6, IL-8, and TNF were donated by SUN LONG Biological Technology Co. Ltd. in the USA.

Animals

In this investigation, 130 male Swiss albino mice weighing between 25 and 30g were used. They were purchased from the Center for Drug Control and Research. The Al-Nahrain University/College of Pharmacy animal ethics committee's guidance are used and care the experimental animals contains detailed instructions on how to handle and care for animals. Animals were kept in the Al-Nahrain University/College of Pharmacy's animal care facility for a 12-hour period in a light/dark cycle with frequent feedings of rodent food and water, the area was adequately ventilated and had fresh air.

Protocol for experimenting with cytokine storm models

To make the LPS solution, mix 10 mg of lyophilized LPS powder with 10 mL of normal saline in a sterile glass container, vortex mix it for 15 minutes, and then warm it in a water bath to thoroughly dissolve it before each use. The mice were randomly divided into thirteen groups of similar size (n=10).

- Group 1: (apparently healthy) ten male mice have not been received any treatment.
- Group 2: (negative control) ten males have been received 300 microliters of DMSO (<5%) intraperitoneally IP once daily for three days then received 0.13 ml IP equivalent to 5mg/kg of LPS and left for 48 hours.

- Group 3: (positive control) ten male mice have been received 0.13 ml single IP equivalent to 5mg/kg lipopolysaccharide (LPS) left for 48 hours.
- Group 4: (methylprednisolone prophylaxis group): ten male mice have been received methylprednisolone 1.25 ml IP from the stock solution equivalent to 50mg/kg once daily for three days then received 0.13 ml single IP equivalent to 5mg/kg of LPS left for 48 hours.
- Group 5: (kaempferol prophylaxis group): ten male mice receive 0.25 ml IP from the stock solution equivalent to 10mg/kg kaempferol once daily. for three days then received 0.13 ml single IP equivalent to 5mg/kg of LPS left for 48.
- Group 6: (amygdalin prophylaxis group) ten male mice have been received 0.08 ml equivalent to 3 mg/kg of amygdalin. IP once daily for three days then received 0.13 ml single IP equivalent to 5mg/kg of LPS left for 48 hours.
- Group 7: (kaempferol + methylprednisolone prophylaxis combination group); ten male mice receive 0.25 ml IP from the stock solution equivalent to 10 mg/kg kaempferol plus methylprednisolone 1.25 ml single IP from the stock solution equivalent to 50 mg/kg once daily for three days then received 0.13 ml single IP equivalent to 5 mg/kg of LPS after one hour left for 48 hours.
- Group 8: (amygdalin. + methylprednisolone prophylaxis combination group); ten male mice have been received 0.08 ml equivalent to 3 mg/kg of amygdalin plus, methylprednisolone 1.25 ml from the stock solution equivalent to 50 mg/kg IP once daily for three days then received 0.13 ml single IP equivalent to 5 mg/kg of LPS left for 48 hours.
- Group 9: (kaempferol treatment group); ten male mice have been received 0.13 ml microliters single IP equivalent to 5 mg/kg lipopolysaccharide (LPS) after one hour receive 0.25 ml IP from the stock solution equivalent to 10 mg/kg kaempferol. Twice daily for five days.
- Group 10: (amygdalin treatment group): ten male mice have been received 0.13 ml single IP equivalent to 5 mg/kg lipopolysaccharide (LPS) after one hour receive 0.075 ml IP from the stock solution equivalent to 3 mg/kg amygdalin. Twice daily for five days.
- Group 11: (kaempferol and methylprednisolone combination treatment group): ten male mice have been received 0.13 ml microliters single IP equivalent to 5 mg/kg lipopolysaccharide (LPS) after one hour receive 0.25

ml IP from the stock solution equivalent to 10 mg/kg kaempferol Plus methylprednisolone 1.25 ml single IP from the stock solution equivalent to 50 mg/kg Twice daily for five days.

- Group 12: (amygdalin + methylprednisolone combination treatment group): ten male mice have been received 0.13 ml single IP equivalent to 5mg/kg lipopolysaccharide (LPS) after one hour receive 0.075 ml IP from the stock solution equivalent to 3mg/kg amygdalin plus methylprednisolone 1.25 ml single IP from the stock solution equivalent to 50mg/kg twice daily for five days.
- Group 13: (methylprednisolone treatment group): ten male mice have been received 0.13 ml single IP equivalent to 5mg/kg lipopolysaccharide (LPS) after one hour receive methylprednisolone 1.25 ml IP from the stock solution equivalent to 50mg/kg. Twice daily for five days.
- Collect blood from all mice group from jugular vein under light anesthesia with chloroform after treatment period for each group, centrifuging for serum collection to quantitative measuring IL-1β, 6,8 and TNF-α by Elisa kit technique then scarified and collect liver, lung for histopathological examination.

Statistical analysis

The Social Sciences Software Statistics Package (SSPS) statistical software version (26) was used to collect, tabulate, and conduct all statistical analyses. The result was presented as Means SD. To compare across groups, a one-way analysis of variance (ANOVA) with post-hoc Tukey test. The level of significance was set at the p values, $p<0.01$ as significant, high significant and very high significant respectively[17].

RESULTS and DISCUSSION

The results showed that all of the tested compounds, including methylprednisolone, kaempferol, and amygdalin, were effective in preventing the elevation of IL-1β, IL-6, IL-8 and TNF-α levels when administered before or after LPS induction. Furthermore, the combined dosing of kaempferol, amygdalin, and methylprednisolone produced a synergistic effect in reducing the levels of IL-1β, IL-6, IL-8 and TNF-α levels (Figure 1-4, Table 1).

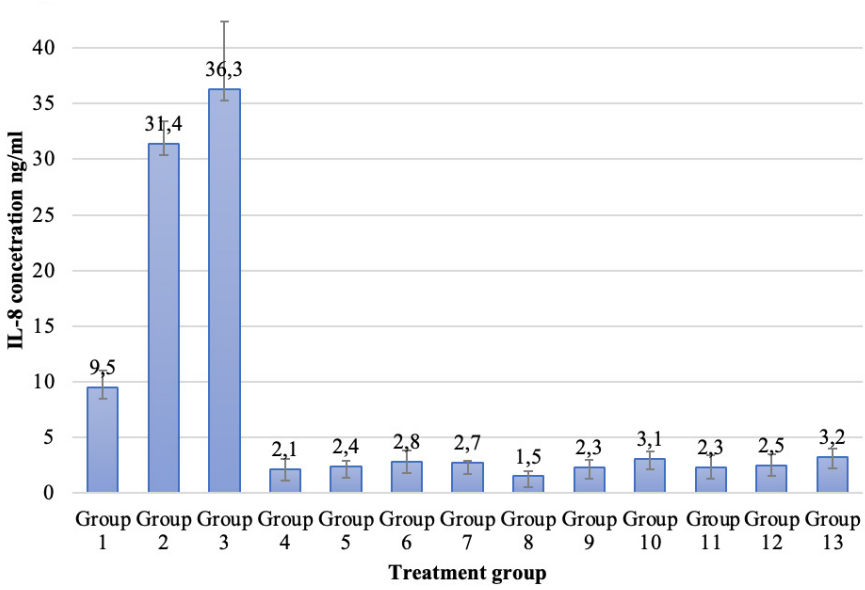

Figure 1. IL-8 level in all studied groups

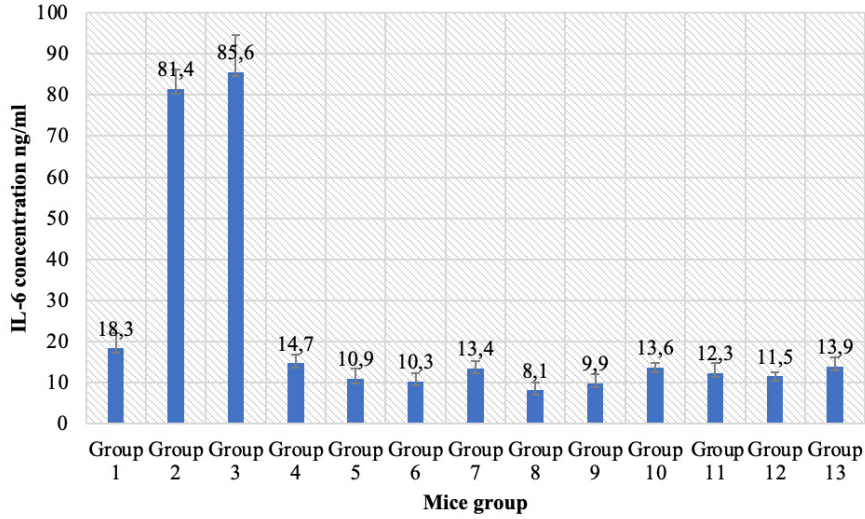

Figure 2. IL-6 level in all studied groups

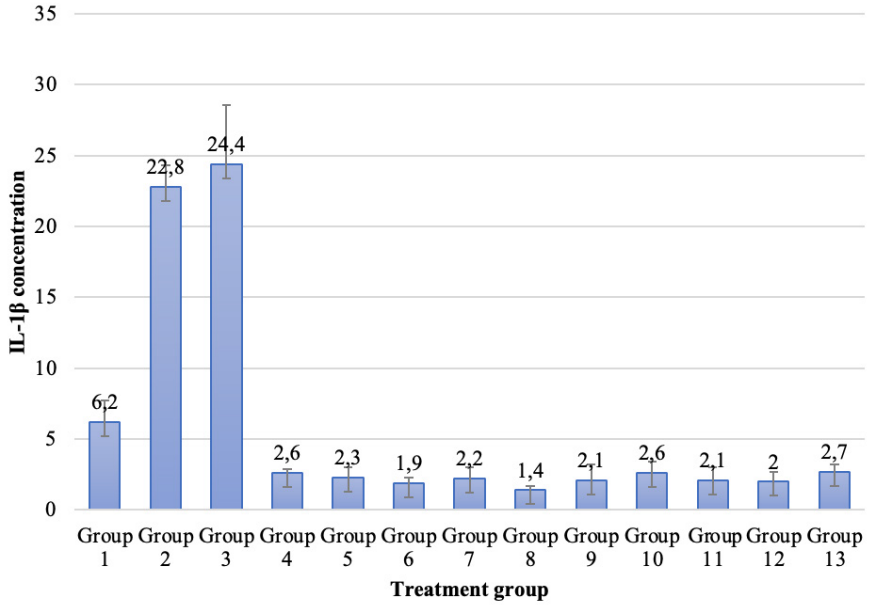

Figure 3. IL-1β level in studied groups

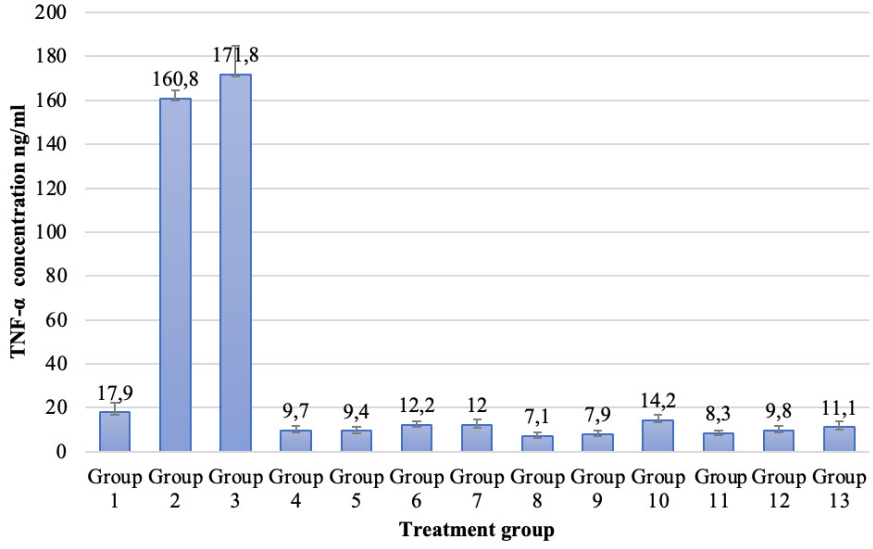

Figure 4. TNF-α level in studied groups

Table 1. Interleukin concentration in mice group after and before LPS induction

Group	IL-1β level as Mean ± SD in pg/ml	IL-6 level as Mean ± SD in ng/ml	IL-8 level as Mean ± SD in ng/ml	TNF-α level as Mean ± SD in ng/ml
Group 1 (Healthy)	6.2 ± 1.5ab	18.3 ± 3.8bb	9.5 ± 1.5ab	17.9 ± 4.4bb
Group 2 Negative Control (Dmso)	22.8 ± 1.5bb	81.4 ± 4.9a	31.4 ± 2.0bc	160.8 ± 3.9 dd
Group 3 Positive Control (Lps)	24.4 ± 4.2bb	85.6 ± 8.9a	36.3 ± 6.1bc	171.8 ± 13.1dd
Group 4 (Methylprednisolone Prophylaxis)	2.6 ± 0.3cc	14.7 ± 2.1bd	2.1 ± 1.0cc	9.7 ± 2.0ab
Group 5 (Kaempferol Prophylaxis)	2.3 ± 0.7cc	10.9 ± 2.5ab	2.4 ± 0.5cc	9.4 ± 1.8ab
Group 6 (Amygdalin Prophylaxis)	1.9 ± 0.4cc	10.3 ± 2.1ab	2.8 ± 1.0cc	12.2 ± 1.7ab
Group 7 (Kaempferol + Methylprednisolone Prophylaxis)	2.2 ± 0.8cc	13.4 ± 1.9bd	2.7 ± 0.2cc	12.0 ± 2.8ab
Group 8 (Amygdalin+ Methylprednisolone Prophylaxis)	1.4 ± 0.3cc	8.1 ± 1.9ab	1.5 ± 0.5cc	7.1 ± 1.5ab
Group 9 (Kaempferol Treatment)	2.1 ± 1.1cc	9.9 ± 2.1ab	2.3 ± 0.7cc	7.9 ± 1.6ab
Group 10 (Amygdalin Treatment)	2.6 ± 0.8cc	13.6 ± 1.1bd	3.1 ± 0.6cc	14.2 ± 2.4ab
Group 11 (Kaempferol+ Methylprednisolon Treatment)	2.1 ± 0.9cc	12.3 ± 2.4bd	2.3 ± 1.1cc	8.3 ± 1.3ab
Group 12 (Amygdalin + Methylprednisolon Treatment)	2.0 ± 0.7cc	11.5 ± 1.1bd	2.5 ± 1.0cc	9.8 ± 2.1ab
Group13 (Methylprednisolone Treatment)	2.7 ± 0.5cc	13.9 ± 2.3bd	3.2 ± 0.8ccc	11.1 ± 2.6Aa

Group 4-8 administration of compounds before receiving LPS, group 9-13 administration of treatment compounds after cytokine induction by LPS. similar later not have significant difference at $p<0.01$ by using LSD. different later have significant difference at $p<0.01$ by using LSD.

The body's immunological reaction to an infection or damage involves inflammation, which is a critical step. On the other hand, persistent inflammation has been linked to a number of illnesses, including as cancer, autoimmune disorders, and cardiovascular conditions. Pro-inflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α are important in the beginning and development of inflammation[18,19]. Thus, inhibition of these cytokines can be a therapeutic strategy for various inflammatory diseases. In recent years, natural compounds have gained attention as potential anti-inflammatory agents due to their safety profile and effectiveness[20].

The study aimed to assess the effect of various compounds on the levels of interleukin- IL-1β, IL-6, IL-8 and TNF-α levels in serum samples collected from different groups of mice. The serum samples were collected from healthy mice (Group 1), mice treated with DMSO (Group 2), and mice treated with LPS (Group 3). Groups 4 to 13 were pre-treated or treated with various compounds before or after LPS administration. The IL-1β, IL-6, IL-8 and TNF-α levels were quantitatively assessed using enzyme-linked immunosorbent assay (ELISA) kits, and the findings were statistically analyzed.

Kaempferol is a flavonoid found in various plant-based foods such as tea, broccoli, and berries. The production of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α has been shown to be inhibited by kaempferol, giving rise to its anti-inflammatory effects[21]. The findings shown that Kaempferol substantially and dose-dependently decreased the levels of IL-1β, IL-6, and TNF-α which is agreed with a study conducted by Chen et al. (2019) who demonstrated that kaempferol treatment significantly reduced the levels of IL-1β, IL-6, and TNF-α in LPS-induced RAW264.7 macrophages. Furthermore, kaempferol treatment also reported to suppress the activation of NF-κB and MAPK signaling pathways, which are critical pathways involved in the regulation of inflammatory responses[22].

According to reports, amygdalin has anti-inflammatory characteristics by preventing the formation of cytokines that promote inflammation, such as IL-1β, IL-6, and TNF-α[16,23] which is consistent with the results obtained in the current study. *In vivo* study conducted by Li et al. (2023) also demonstrated that amygdalin treatment group significantly reduced the expression of IL-1β, IL-6, and TNF-α after lipopolysaccharide (LPS) induction[24]. It was reported that Amygdalin significantly reduced the levels of IL-1β, IL-6, and TNF-α in a dose-dependent manner. Amygdalin significantly inhibited the production of IL-6 and TNF-α in LPS-stimulated macrophages. The authors suggested that the anti-inflammatory effect of amygdalin might be mediated through the inhibition of NF-κB and MAPK signaling pathways[25].

Synthetic glucocorticoids like methylprednisolone are frequently used to treat inflammation and inhibit the immune system. By inhibiting the synthesis of pro-inflammatory cytokines such IL-1β, IL-6, IL-8, and TNF-α, methylprednisolone reduces inflammation[26]. The methylprednisolone treatment significantly reduced the levels of IL-1β, IL-6, and TNF-α in a rat model of acute lung injury. Methylprednisolone treatment also attenuated the histological damage and improved the respiratory function in the rat model[27].

The results of the present study are consistent with previous research that has demonstrated the anti-inflammatory properties of kaempferol, amygdalin, and methylprednisolone. Kaempferol has been shown to inhibit the production of pro-inflammatory cytokines such as IL-1β and TNF-α in various cell lines[28]. Similarly, amygdalin has been found to suppress the production of inflammatory mediators in macrophages[24], while methylprednisolone is a well-known anti-inflammatory agent that is frequently used to treat inflammatory conditions such as asthma and rheumatoid arthritis[29].

Interestingly, the present study also suggests that the combination of these compounds produces a synergistic effect in reducing IL-1β levels. This finding is consistent with previous studies that have demonstrated the benefits of combining natural compounds with synthetic drugs to enhance their therapeutic effects[30]. For example, mixing between dexamethasone and quercetin may have a synergistic impact on cytokine storm inhibition. Dexamethasone suppresses the immune system largely, but quercetin can target particular inflammatory pathways and alter cytokine production in covid 19 patient[31]. Moreover, the present study highlights the potential use of these compounds as a combination therapy in the treatment of inflammatory disorders.

Natural substances including amygdalin and kaempferol have been shown to have anti-inflammatory characteristics by lowering the levels of pro-inflammatory cytokines like IL-1β, IL-6, IL-8, and TNF-. These substances could be able to cure inflammatory illnesses that are chronically present. To assess these substances' effectiveness and safety in people, more research is required.

STATEMENT OF ETHICS

The study received approval from the "Institute Review Board (IRB) of Al-Nahrain University/College of Medicine" in January 2022 (58/2022).

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

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

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The effect of a combination therapy of spironolactone and metformin on women with polycystic ovary syndrome

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ABSTRACT

Many women of childbearing age are affected by polycystic ovary syndrome (PCOS), which is a complex endocrine disorder that has no one ideal treatment. This research aimed to assess the effect of the combination therapy of metformin and spironolactone on the levels of hormones and some PCOS symptoms. This study is a prospective study that conducted on 60 females with PCOS recruited from an Obstetrics and Gynecology private clinic in Baghdad, Iraq, between December 2022 and May 2023 who received a combination therapy of metformin and spironolactone for at least 2 months. Blood samples were taken for the assessment of LH, FSH, and prolactin levels, as well as other PCOS symptoms before and after receiving the treatment for ≥ 2 months. Results showed that LH and prolactin levels were significantly reduced with a significant decrease in duration of the menstrual cycle absence to approximately 30 days, and a significant reduction in the number of cases that suffer from hirsutism after receiving treatment in comparison with the prevalence of hirsutism before treatment. In conclusion, spironolactone in combination with metformin showed to improve hirsutism and the menstrual cycle frequency as it caused an improvement in the levels of LH, FSH, and prolactin in PCOS patients.

Keywords: metformin, polycystic ovary syndrome, spironolactone

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INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a complex endocrine disorder that affects a sizable fraction of reproductive-aged women around the world[1]. Ovarian enlargement and dysfunction, increased androgen levels, insulin resistance, and other factors are often seen in patients with this syndrome[2]. About 10% of women have polycystic ovary syndrome (PCOS) before menopause[3] and many of these women have to deal with the difficulties associated with this condition. Although it is known that an increased ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) and increased frequency of gonadotropin-releasing hormone (GnRH) are contributing factors in polycystic ovary syndrome (PCOS)[4,5], the etiology and pathophysiology of PCOS remain poorly understood. Insulin resistance (IR), hyperandrogenism (HA), environmental factors, genetic factors, and epigenetic mechanisms are just a few examples of what we know to play a role in determining outcomes. Importantly, polycystic ovary syndrome (PCOS) has been linked to an increased risk of other health problems, including metabolic syndrome, type 2 diabetes mellitus, cardiovascular disease[5, 6] as well as melancholy and anxiety[7].

Considering the increasing prevalence of polycystic ovary syndrome (PCOS) and its associated problems, as well as the limited efficacy of current treatments and drugs, it is imperative to thoroughly explore the underlying mechanisms of PCOS and identify novel pharmacological targets[3]. The primary pharmacological treatments utilized in the management of polycystic ovary syndrome (PCOS) encompass various drug classes. These include oral contraceptive pills, insulin sensitizers such as metformin and pioglitazone, anti-androgens like spironolactone, cyproterone acetate, finasteride, and flutamide, as well as ovulation inductors such as gonadotropin-releasing hormone analogues and clomiphene. Those drugs have been used for different expectations of the patients with PCOS[8,9]. Since insulin resistance and hyperandrogenemia are among the main interacting causes, as well as overproduction of luteinizing hormone (LH) and androgenic hormones, in PCOS[10], a combined approach utilizing insulin sensitizers and anti-androgens may offer synergistic benefits in the management of PCOS.

Researches have demonstrated that metformin has the ability to reduce insulin resistance, augment the number of cycles in individuals diagnosed with polycystic ovary syndrome (PCOS), and maybe ameliorate hirsutism to a limited extent in certain people[11-13]. The administration of spironolactone has been shown to improve hyperandrogenism through its dual mechanism of action, which involves the inhibition of both androgen production and receptor-bind-

ing[14,15]. Therefore, in theory, the combination of metformin and spironolactone therapy has promise as a potentially successful and safe treatment option for polycystic ovary syndrome (PCOS). One could postulate that the concurrent administration of metformin and spironolactone may yield superior efficacy compared to individual medication usage, owing to their mutually reinforcing modes of action.

A recent study conducted in India demonstrated that the combination of metformin and spironolactone yielded superior results compared to either drug used individually. Specifically, this combination exhibited improvements in various factors including the number of menstrual cycles, Ferriman-Gallwey score (FGS), serum total testosterone (T) levels, and area under curve (AUC) measurements for glucose and insulin. However, it did not show significant effects on body mass index (BMI), homeostasis model assessment of insulin resistance (HOMA-IR) index, and blood pressure[10]. The objective of our study was to examine the effects of this combination therapy on some hormonal levels and several features of PCOS.

METHODOLOGY

Study protocol

A prospective study was done on 60 female patients with polycystic ovarian syndrome (PCOS) who were recruited from Obstetrics and Gynecology private clinic, Baghdad, Iraq, between December 2022 and May 2022. Ages of the patients ranged between 17 and 35 years (mean ± SD 24.83 ± 4.27 years). The practical part of the study was conducted at private laboratory in Baghdad, Iraq. The diagnosis of PCOS was based on Rotterdam Revised criteria (2003)[3,16]. Those individuals who meet at least two of the following criteria were included in this study: oligo- or anovulation, such as amenorrhea (absence of menstruation >180 days) or oligomenorrhea (menstrual periods occur at intervals of >35 days), and clinical and/or biochemical signs of hyperandrogenism. Polycystic ovaries on ultrasound are defined as an ovary containing 12 or more follicles measuring 2–9 mm in diameter or an ovary that has a volume of greater than 10 ml. Patients received metformin (1700 mg/day), spironolactone (100 mg/day) and a hypocaloric diet and the levels of studied markers were measured before and after receiving the treatment.

Exclusion criteria

Pregnancy was ruled out by human-chorionic gonadotropin measurement[17]. Normal thyroid function was established by hormonal evaluation because hypothyroidism cause an increase in the prolactin levels which in turn inhibits

gonadotrophins that lead to a reduction in the levels of serum FSH and serum LH[18]. Late-onset nonclassical congenital hyperplasia was excluded by values of basal 17-hydroxy progesterone less than 2 ng/ml. Cushing syndrome was also ruled out by hormonal evaluation. Patients who administered drugs included oral contraceptives, antihypertensive agents, anti-diabetic drugs, and agents for weight loss were also ruled out from this study as they interfere with the treatment used.

Anthropometric tests

The only anthropometric parameter specified in the study was BMI calculated as:

BMI = weight (kg) / height square (m^2)

All subjects were weighed on the same scale, barefoot. Height was measured using the same measuring tape.

Methods

Patients who had fasted for 12 hours had five milliliters of blood drawn for the initial estimation of the studied markers with a clinical examination that included the presence of acne and hirsutism were recorded in addition to the age, BMI, and marital status that were obtained from all patients subjected to the current study. The collection of blood samples was repeated after 2-5 months of receiving the combination therapy, in addition to examining the patients for acne and hirsutism in addition to their BMI. After letting the samples clot for 30 minutes at room temperature, they were centrifuged at 4000rpm (1252 x g) for 10 minutes to separate the serum. The sera were then aliquoted and stored at -20°C until they could be evaluated for LH, FSH, and Prolactin using the enzyme-linked immunosorbent assay (ELISA).

Limitation

We faced a problem in convincing the patients to subject to the Ferriman-Gallwey scoring system for hirsutism, therefore, we replaced it by asking patients about their hirsutism condition.

Statistical analysis

The research data were entered into a Microsoft Excel spreadsheet, and then analyzed using SPSS 20 and Excel (2016). All numerical variables were presented as means and standard deviations. Statistical significance was set at a P value of ≤0.05 for all t-test and ANOVA comparisons. Cross tabulation was used to determine the frequency and percentage of each categorical variable

across the different groups. All relationships between parameters were analyzed using either the Pearson correlation test for numerical parameters[19] or the Chi-square test for categorical variables.

RESULTS and DISCUSSION

Some demographic characteristics of the studied groups were summarized in Table 1 and Figures 1 and 2 which showed non-significant differences in the age and the body mass index (BMI) between single and married patients. Results also revealed that the majority of cases were overweight to obese.

Table 1. Demographic characteristics of PCOS patients: a comparison between single and married patients

	Total patients	Single	Married	p-value
n	60	32	28	-
Age (year) (mean ± standard deviation)	26.3 ± 5.27	25.75 ± 5.11	27.21 ± 3.83	0.3
BMI (kg/m^2) (mean ± standard deviation)	30.79 ± 6.18	30.49 ± 7.44	31.13 ± 4.6	0.78

Table 2 and Figure 3 revealed that the level of LH and Prolactin were reduced significantly after treatment with metformin and spironolactone for at least 2 months. It was also noticed that the duration of the absent of menstrual cycle reduced dramatically from about 80 days to approximately 30 days. On the other hand, a non-significant reduction in BMI and the levels of FSH were obtained while the duration of menstrual bleeding where a non-significantly increased from average of 5.5 days to about 6 days after receiving the treatment.

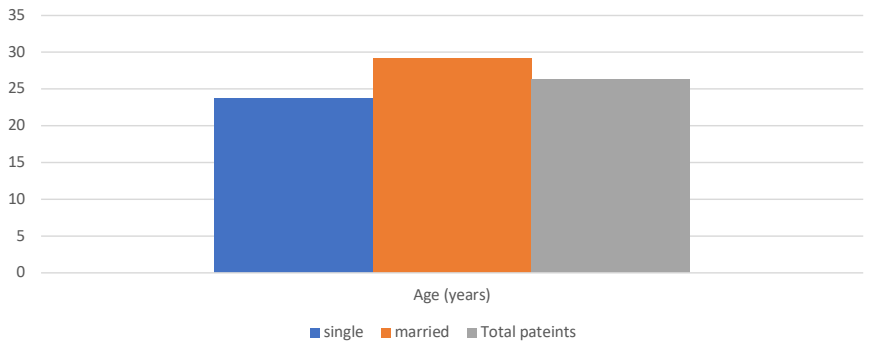

Figure 1. Averages of age in all studied groups

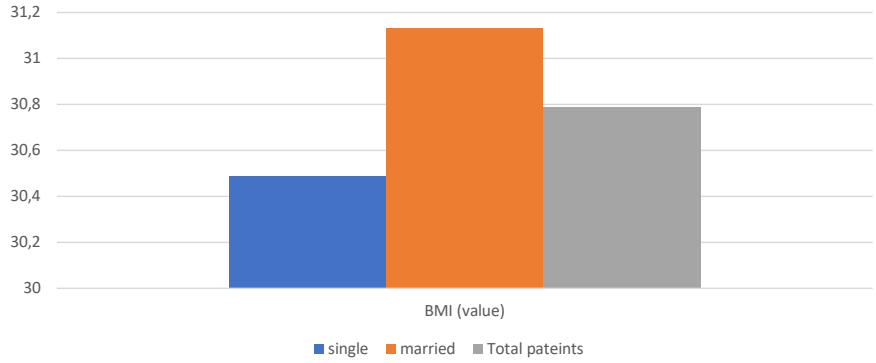

Figure 2. Averages of BMI in all studied groups

Table 2. Levels of LH, FSH, Prolactin and BMI with the duration (in days) since the last menstrual period and the duration of menstrual bleeding in patients before and after receiving the treatment

	Before treatment	After treatment	P value
LH (mIU/mL) (Mean ± SD)	11.7 ± 3.79	3.8 ± 0.46	<0.001
FSH (mIU/mL) (Mean ± SD)	6.06 ± 1.67	5.72 ± 0.68	0.3
Prolactin (ng/mL) (Mean ± SD)	21.07 ± 10.61	13.62 ± 2.28	<0.001
Duration since the last menstrual period (days) (Mean ± SD)	80.13 ± 44.01	30.6 ± 2.34	<0.001
Duration of bleeding in the last period (days) (Mean ± SD)	5.5 ± 1.91	6.07 ± 0.789	0.14
BMI (kg/m2) (Mean ± SD)	30.79 ± 6.18	29.2 ± 5.48	0.3

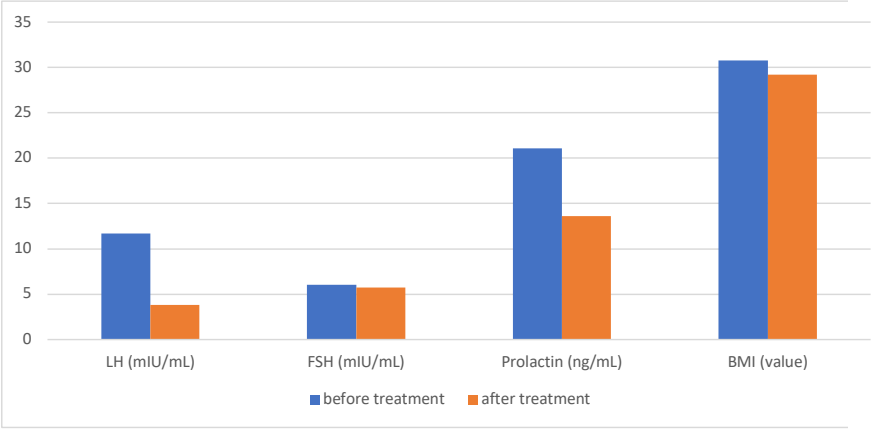

Figure 3. Averages of LH, FSH, Prolactin and BMI levels in all studied groups

Results illustrated in Table 3 and Figures 4 and 5 showed that the prevalence of hirsutism was significantly reduced in patients who received the combination therapy in comparison with the prevalence of hirsutism before treatment in that about 80% of patients were suffered from hirsutism before treatment which reduced to only 3.3% of patients after receiving the treatment for at least two months. On the other hand, the prevalence of acne was reduced non-significantly which may be owned to that the number of cases which suffer from acne were only four cases who recovered after receiving treatments.

Table 3. Prevalence of hirsutism and acne in PCOS patients before and after receiving treatments

Yes No			Hirsutism		Acne	
			Yes	No	Yes	No
Groups	Before treatment	Count	24	6	4	26
		% within Groups	80.0%	20.0%	13.3%	86.7%
	After treatment	Count	1	29	0	30
		% within Groups	3.3%	96.7%	0.0%	100.0%
Chi^2 value			<0.001		0.056	

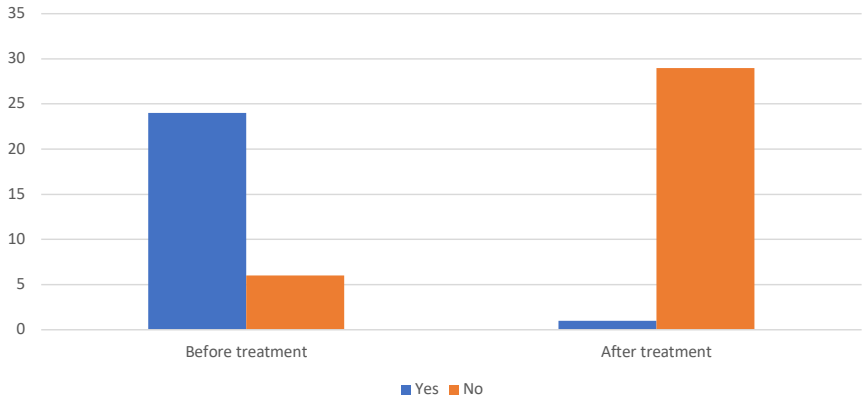

Figure 4. Prevalence of hirsutism in PCOS patients before and after receiving treatments

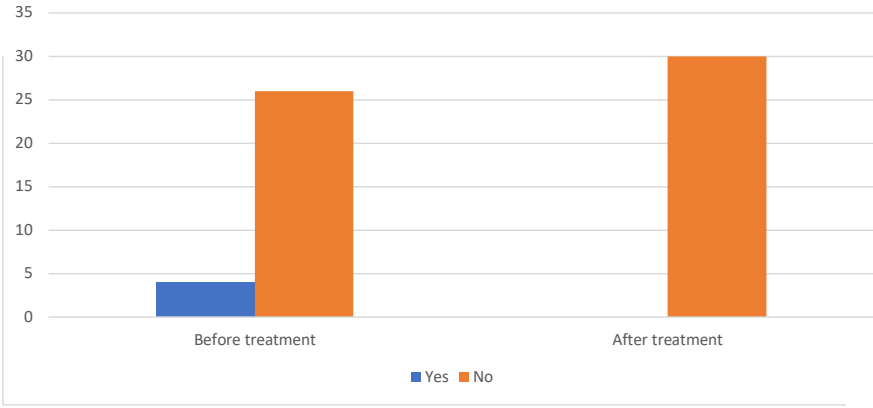

Figure 5. Prevalence of acne in PCOS patients before and after receiving treatments

Table 4 revealed that there were significant positive (direct) correlations between FSH and the levels of both LH (r=0.669, p<0.001) and prolactin (r=0.405, p=0.026) in PCOS patients before receiving the treatment. Moreover, levels of LH also showed a positive significant correlation with the duration of time since the last menstrual period (r=0.381, p=0.038). In contrast, all other parameters showed non-significant correlations with each other in these patients before receiving the treatment.

Table 4. Correlations between all studied parameters in PCOS patients before treatment

		FSH	LH	Prolactin	Duration since the last menstrual period	Duration of bleeding in the last period	BMI
Age	r	- 0.082	0.081	0.092	- 0.012	- 0.070	- 0.228
	p	0.667	0.669	0.629	0.948	0.712	0.226
FSH	r		0.650**	0.405*	0.119	- 0.285	0.033
	p		<0.001	0.026	0.530	0.126	0.862
LH	r			0.312	0.381*	- 0.069	- 0.214
	p			0.093	0.038	0.719	0.257
Prolactin	r				- 0.018	- 0.154	0.189
	p				0.925	0.415	0.318
Duration since the last menstrual period	r					0.279	0.079
	p					0.135	0.677
Duration of bleeding in the last period	r						- 0.110
	p						0.563

As demonstrated in Table 5, the levels of LH were positively and significantly correlated with duration of receiving treatment (r=0.513, p=0.004) whereas all other parameters showed non-significant correlations with the duration of receiving combined therapy. Moreover, FSH showed a positive and significant correlation with LH (r=0.388, p=0.034) in PCOS patients receiving treatment whereas Prolactin levels were negatively (inversely) significantly correlated with the duration of menstrual bleeding in the last menstrual period (r=-0.463, p=0.01). It was also demonstrated that the duration of treatment non-significantly correlated with levels of FSH and prolactin and the results also showed that in spite of the improvement of menstrual frequency it didn't correlate to the levels of FSH, LH and Prolactin. The bleeding duration in each menstrual period showed a non-significant correlation with the levels of LH and FSH that assessed in this study and also showed a non-significant correlation with the duration of receiving the combined treatment. All other parameters were non-significantly correlated with each other.

Table 5. Correlations between all studied parameters in PCOS patients after treatment

		Duration of treatment (days)	FSH	LH	Prolactin	Duration since the last menstrual period	Duration of bleeding in the last period	BMI
Age	**r**	- 0.110	- 0.003	0.115	0.209	0.111	- 0.113	- 0.234
	p	0.564	0.988	0.544	0.268	0.561	0.551	0.213
Duration of treatment (days)	**r**		0.029	0.513	- 0.192	- 0.213	- 0.101	0.025
	p		0.878	0.004	0.310	0.258	0.597	0.896
FSH	**r**			0.388	0.091	0.118	- 0.314	0.169
	p			0.034	0.632	0.534	0.091	0.373
LH	**r**				- 0.155	- 0.006	- 0.037	0.211
	p				0.413	0.975	0.845	0.264
Prolactin	**r**					0.199	- 0.463	0.191
	p					0.292	0.010	0.313
Duration since the last menstrual period	**r**						- 0.154	0.187
	p						0.417	0.322
Duration of bleeding in the last period	**r**							- 0.140
	p							0.460

Polycystic ovary syndrome (PCOS) represents the prevailing manifestation of World Health Organization (WHO) type II anovulatory infertility, commonly accompanied by hyperandrogenemia. Additionally, it is worth noting that this particular endocrine anomaly is prevalent among women of reproductive age. The use of the new Rotterdam diagnostic criteria[3], has revealed a greater prevalence of polycystic ovary syndrome (PCOS) (11.9 ± 2.4%) compared to the prior estimates provided by the National Institute of Health (10.2 ± 2.2%) and the Androgen Excess Society (8.7 ± 2.0%). Insulin resistance and hyperandrogenemia are recognized as prominent contributing factors along with excessive production of luteinizing hormone (LH) and androgenic hormones in the development of polycystic ovary syndrome (PCOS), Consequently, a therapeutic approach combining insulin sensitizers and antiandrogens may offer synergistic benefits in the management of PCOS[17].

Research has demonstrated that metformin has the capacity to enhance insulin resistance, augment the frequency of menstrual periods in individuals diagnosed with polycystic ovary syndrome (PCOS). Previous literatures also proposed that metformin maybe ameliorate hirsutism in certain people. A multitude of studies have been conducted to investigate the clinical effectiveness and safety of various medication combinations for the treatment of polycystic ovary syndrome (PCOS). Nevertheless, the number of studies evaluating the clinical and laboratory effectiveness of the combined use of spironolactone and metformin is somewhat limited[17]. Our hypothesis posits that the combination of metformin and spironolactone would yield improved results compared to using either drug alone. This is based on the understanding that both medications are commonly employed to address certain components of the condition in question[20].

In the current study, the levels of LH and prolactin were reduced significantly ($p<0.001$ for both) in patients receiving the combined therapy of metformin and spironolactone, as illustrated in Table 2. Given that patients subjected to the current study received the treatment for a variable duration ranging from 2–10 months with an average of 101 days, this explains the heterogeneity of the results obtained. Regarding LH levels, results demonstrated in the present study were consistent with previous studies conducted by Abd Elaal et al. in 2020[17], who investigated the effect of combined therapy on 20 PCOS patients for 6 months, whereas Ganie et al. (2013)[10] and Diri et al. (2016)[20] demonstrated a non-significant change in the levels of LH in patients subjected to their studies.

The significant reduction in prolactin levels was in agreement with several previous studies[21,22] and evidence from these studies suggests that metformin has a

significant impact on pituitary function in PCOS women, as evidenced by changes in LH and, in ovulatory responders, prolactin PRL secretion dynamics after intervention[21]. As a consequence, it increases the number of cycles in PCOS patients, as indicated by the dramatic reduction in the period ($p<0.001$) since the last cycle that is presented in Table 2, which is consistent with previous literature[17,20].

In accordance with previous literature, Table 3 revealed that patients subjected to the current study showed a significant ($p<0.001$) improvement in hirsutism as a consequence of restoring the levels of LH, FSH, and prolactin. In the current study, about all subjects experienced a reduction in hirsutism, given that in our study the patients were asked about hirsutism and not as in previous studies that conducted a hirsutism score. Ganie and his co-workers reported in 2013 reported that the combination of metformin (1000 mg/d) and spironolactone (50 mg/d) was better than either drug alone for improving the number of menstrual cycles and hirsutism score, which is completely compatible with the current research findings[10].

Correlation studies revealed that in patients with PCOS, the levels of FSH were directly and significantly correlated to the levels of LH ($p<0.001$) and prolactin ($p=0.026$) before receiving treatments, as illustrated in Tables 4, which is a classical finding that has been approved since 1978[23,24]. Before receiving treatments, patients showed a significant positive correlation between LH levels and the duration of the last menstrual period ($p=0.038$), as shown in Tables 4. Increasing the durations of the menstrual cycle indicates a decrease in the number of cycles, so, the high ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) and the increased frequency of gonadotropin-releasing hormone (GnRH) which are known as the underlying causes of PCOS involved in the elongation of the menstrual cycle period that reduce its frequency[3].

It was demonstrated in Table 5 that after at least two months of treatment, the correlation studies showed that the LH-FSH positive significant ($p=0.034$) association persists, which may indicate that the reduction in the levels of both hormones run in parallel to each other. However, it was noticed that the correlation before receiving treatment was more significant ($r=0.65$; $p<0.001$) whereas the correlation after receiving treatment was less significant ($r=0.388$; $p=0.034$). The reduction in the significancy of the LH-FSH correlation might be caused by the fact that the reduction in LH levels were more pronounced than the reduction in FSH levels, which also led to a lower LH/FSH ratio, which is mentioned above as one of the underlying causes of PCOS, i.e., using this combination therapy affect the levels of LH to higher extent than FSH which may be contributed to that reduction in the significancy of their correlation[3].

Additionally, results illustrated in Table 5 showed that a significant positive correlation (p=0.004) was obtained between the levels of LH and the duration of treatment. It was also noticed that patients treated with this therapy for more than 3 months showed a slight increase in LH levels that may be caused by the poor compliance of patients to the therapeutic regime after a long term of treatment and the observed improvement while patients receiving treatment for less than three months showed a higher compliance rate. Furthermore, results presented in Tables 5 revealed that a significant negative (p=0.01) correlation was obtained between prolactin and the duration of menstrual bleeding, which is also compatible with the previous literature, which reported that high levels of prolactin cause menstrual abnormalities[25].

In conclusion, spironolactone in combination with metformin was effective on hirsutism and also caused an increase in the menstrual cycle frequency as it caused an improvement in the levels of LH, FSH, and prolactin in our patients with PCOS. The most significant result of the study was that when spironolactone was combined with metformin, both drugs were more effective and more likely to be taken as prescribed. One possible explanation for these oligoammenorrhoic women's improved adherence is the combination's enhanced efficacy in restoring regular menstrual cyclicity. The relatively small sample sizes of the treatment groups in the present study are a significant limitation that necessitates additional research using a larger patient population.

STATEMENT OF ETHICS

The study has approved by the Institutional Review Board (IRB) of the Department of Pharmacy, Al-Yarmouk University College, Diyala, Iraq (YUC3982-2022).

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Design – Khadir FK, Ajeed AM; Acquisition of data – AlSahaf DM; Analysis of data – Ajeed AM; Drafting of the manuscript – Khadir FK, AlSahaf DM; Critical revision of the manuscript– Ajeed AM; Statistical analysis– AlSahaf DM; Technical or financial support– Khadir FK, Ajeed AM; supervision – Khadir FK.

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Preparation and evaluation of topical hydrogel containing ketoconazole-loaded bilosomes

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ABSTRACT

Ketoconazole (KET) is an antifungal drug that was used for the first time as loaded bilosome that incorporated into Carbopol hydrogels to enhance topical skin application and improve therapeutic efficacy. KET-bilosome which contained span 60: cholesterol: SDC at milligrams weight (350:60:15) was prepared using ultra-sonication method. Then, the bilosome formulation was incorporated into the hydrogel of Carbopol 934 and 940 as a gelling agent. All hydrogels were evaluated for their physical appearance, pH, KET content, viscosity and *in-vitro* release. The optimum KET-bilosomal hydrogel was further evaluated for skin irritation test and antifungal activity. The optimized KET-bilosome loaded hydrogel (FKC934-1%) showed good viscosity, highest spreadability (11.5 ± 0.5 g.cm/sec), and acceptable pH (5.9 ± 0.1) and drug content (98.4 ± 0.5%). Ketoconazole-bilosomes loaded hydrogel was found to be safe and non-irritating when tested on a rat skin. It also exhibited the sustained release profile (82.16% at 8 hours) with a Korsmeyer–Peppas kinetic release model (R^2 = 0.9967). FTIR study shows no important interaction between ketoconazole and the polymers used. The antifungal study revealed a significant ($p<0.05$) enhancement of antifungal activity against *C. albicans* compared to marketed ketoconazole cream.

Keywords: ketoconazole, bilosome, hydrogel, antifungal activity, spreadability

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INTRODUCTION

Ketoconazole (KET) is a broad-spectrum antifungal medication effective against various fungi and yeasts[1]. KET is classified as a Class II biopharmaceutical, which means it has poor aqueous solubility with log P value equal 4.74 which considered that it has good permeability[2]. The creation of a solubilized KET form is difficult, and hence it is required to overcome this limitation to prevent a formulation issue, minimize side effects, and improve the drug's antifungal activity[3].

Antifungal medications treat and prevent the progression of cutaneous fungal disorders. These medications found over the years have little effect on drug resistance, inability to reach target areas, limited residence duration, low bioavailability, lack of penetration, and other issues[4]. One of strategies to overcome these constraints in topical application is the preparation of drug-loaded lipid nanovesicular systems[5]. One of the novel nanocarriers that have been first emerged by Conacher in 2001 is bilosomes, which are defined as bile salt-loaded bilayer vesicles, structurally similar to liposomes and niosomes and differ in composition, stability and storage conditions. Bilosomes are usually made from phospholipid or non-ionic surfactant, cholesterol, bile acid, or salts. They are more stable and flexible compared to conventional liposomes. Bile salts have become widely utilized because of their stability, safety and biological compatibility[6,7]. Also, the bile salts in nanovesicles play an important role as edge activators to impart vesicular elasticity and as charge inducers to supply vesicular stability against aggregation. Due to its biological membrane fluidizing impact, sodium deoxycholate is the most typically used bile salt. It leads to enhanced drug permeation and its ability to enhance the dissolution of insoluble drugs[8,9]. Furthermore, bilosomes have been used as permeation enhancers in topical dosage forms such as buccal, ocular, nasal, and transdermal routes of administration, as well as for increasing the hydrophilicity of water-insoluble active pharmaceutical ingredients[10].

Topical drug delivery is the most basic and easy technique of delivering localized medications to any portion of the body via routes such as ophthalmic, rectal, vaginal, and skin[11].Topical ketoconazole formulations that have been granted approval by the FDA include 2% shampoo, 1% shampoo, 2% cream, 2% gel, and 2% aerosol foam[12]. The bulk of topical dermatological formulations such as creams and ointments, have the drawbacks of having a lower spreading coefficient, being sticky, and requiring rubbing during application. These restrictions can be overcome by using a hydrogel composition[13]. Gels, particularly hydrogels, have attracted much interest due to their appealing look and

nice cold feeling. They are simple to apply and remove[14]. They also provide regulated release characteristics for a variety of drugs[15]. Carbopol vesicular gel with miconazole improved drug permeability, residency time, and medicinal efficacy. A carbopol-gelling substance was additionally identified to extend the liberation time of the drug-loaded nanovesicle system[16]. Since up-to-date there is no research on ketoconazole-loaded bilosomes prepared by ultrasonication and then incorporated into carbopol gel base for topical application. So the goal of this study was the preparation of ketoconazole bilosomal-loaded hydrogel to enhance its solubility, antifungal activity and patient compliance.

METHODOLOGY

Materials

Ketoconazole, sodium deoxycholate, cholesterol, span 60 and span 40 were purchased from Baoji Guokang Bio-technology Co., China. Methanol was supplied from Thomas Baker, India. Phosphate buffer 5.5 from Himedia laboratories, Carbopol 934, Carbopol 940 (Hi Media lab., Ltd, Mumbai, India), glycerin and triethanolamine (Thomas baker/ India).

Preparation of ketoconazole bilosomes

The ultra-sonication method was carried out for the preparation of different ketoconazole bilosomes[17]. Briefly, this method involves mixing 350 mg of span 60 and 50 mg of KET with 60 mg of cholesterol and 15 mg of sodium deoxycholate. Then, 20 mL of distilled water was added to the prepared mixture and the resultant dispersion was homogenized with a homogenizer (Homogenizer HG-150, Witeg Labortechink, Germany) operating for 5 minutes at 5000 rpm. After that, the resultant dispersion was subjected to probe sonication (QSONICA Sonicator, Qsonica, USA) for 5 minutes (50 seconds on and 10 seconds off with 30% amplitude). Finally, the resultant milky dispersion was stored in the refrigerator overnight to allow vesicles to mature and remained there until further evaluation of the bilosomal formula's as previously prescribed[2,18].

Preparation of ketoconazole bilosomal hydrogel / ketoconazole plain gel

Bilosomal hydrogels containing KET-loaded biolosomes were formulated with varying percentages (1% and 2% w/w) of Carbopol 934 and Carbopol 940. The calculated amounts of Carbopol 934 and Carbopol 940 were soaked in distilled water overnight. A defined quantity of KET bilosomal suspension underwent centrifugation at 4 °C and 30,000 rpm for 90 minutes in a cooling centrifuge. The resulting semisolid bilosomal mass, equivalent to 1% w/w, was separated

from the supernatant and incorporated into the Carbopol 934 and Carbopol 940 hydrogel base using an electric homogenizer (1000 rpm) for 30 minutes. Triethanolamine (TEA) was added dropwise for neutralization until the desired pH range of 5.5-7 was achieved. The plain hydrogel was prepared by following above mention method but replacing bilosomal dispersion by pure KET levigated with propylene glycol.

The prepared hydrogels were then stored in the refrigerator for subsequent hydrogel evaluation[19]. Table 1 illustrated the composition of the prepared KET-bilosomal hydrogel and plain ketoconazole hydrogel.

Table 1. Formulas composition of ketoconazole plain hydrogel and ketoconazole bilosomal hydrogel

Formula Code	Carbopol 934 (% w/w)	Carbopol 940 (% w/w)	Ketoconazole bilosomes (% w/w)	TEA	Distilled water (g)
FKC934 1%	1%		1%	q.s	Up to 100
FKC934 2%	2%		1%	q.s	Up to 100
FKC940 1%		1%	1%	q.s	Up to 100
FKC940 2%		2%	1%	q.s	Up to 100

Ketoconazole plain hydrogel (1% w/w) was made by dissolving a measured quantity of Carbopol 934 1% in distilled water to form an aqueous dispersion. A known amount of ketoconazole (equivalent to 1% w/w) was levigated with 5% w/w propylene glycol and added to the hydrogel base, where it was thoroughly dispersed with continuous homogenization until a homogeneous hydrogel was formed. Triethanolamine drops were added until the desired pH was achieved.

Evaluation of ketoconazole bilosomal hydrogel

Physical appearance

All preparation formulas were visually evaluated for color, uniformity, homogeneity, aggregation, presence of grittiness, and separation. Once the hydrogels had been set in their receptacles[20].

pH measurement

The pH of KET-bilosomal hydrogels were measured utilizing a digital pH meter. The pH of topical formulations is meaningful for their association with the skin pH to avert any irritation[21].

Viscosity measurement

A digital viscometer with spindle number R7 (Myr Rotational Viscometer, Spain) was used for investigating the rheological properties of prepared KET-bilosomal hydrogel. Viscosity measurements were taken at room temperature. The spindle was immersed in the tube, and the speed was rotating at 6, 10, 12, 20, 30, 50, 60, 100 and 200 rpm. The observed viscosity values had been expressed in centipoise[22].

Spreadability test

The spreadability of the prepared formulations was studied. Briefly, 0.5 g of hydrogel were placed in the center of a glass slide (14x14 cm) after that, another glass slide had to be placed on top of the first one and then 500 grams weight was placed on the upper glass slide[13]. Following the removal of the weight, the final diameter of the spread hydrogel was measured.

Drug content determination

The drug content in the hydrogel was determined by utilizing a UV spectrophotometer. One gram of the hydrogel was first dissolved in 50 milliliters of methanol. Following that, the solution was sonicated to guarantee full drug solubility in the methanol. Approximately 1 milliliter of this solution was extracted and diluted to a final volume of 10 milliliters. The absorbance was then measured at 243 nanometers after appropriate dilution depending on previous constructed calibration curve which gave straight line equation ($y= 0.029x-0.0007$) and revealed high correlation coefficient ($R^2 = 0.9993$). Finally ,the content of the drug was calculated by using a linear regression of the drug in methanol[23].

Extrudability

The typical method for determining the force required to push material out of a tube is to conduct an extrudability test. In this test, 30 grams of hydrogel were put within a closed collapsible tube, and the plunger was adjusted to keep the tubes in place. A load of 1 kg was used for 30 seconds, and the poured hydrogel was precisely weighed. This procedure was done three times throughout the tube at regular intervals[13].

In-vitro drug release from a hydrogel loaded with ketoconazole bilosomes

In-vitro drug release was performed for the KET-bilosomal hydrogel formulations utilizing a Paddle type II dissolution apparatus[24]. A 0.5 gram hydrogel containing 5 mg of ketoconazole was uniformly put over a dialysis membrane

that soaked overnight in the dissolution medium, about 150 mL of 30% ethanolic phosphate buffer solution is used as dissolution medium to achieve sink condition. With the aid of a rubber band, the dialysis membrane was affixed to the circular open end of a tube. The tubes were then inverted and secured to the lower part of a paddle with rubber bands, ensuring that the gel's lower portion was just submerged beneath the surface of a 150 mL solution of phosphate buffer (pH 5.5) as the receiving medium. The apparatus temperature was maintained at 37 ± 0.5 °C, and the paddle rotation speed was 100 rpm. At predetermined time (1, 2, 3, 4, 5, 6, 7 and 8 hours), five milliliters' samples were withdrawn and replaced by fresh ethanolic phosphate buffer solution. The withdrawal samples were tested for KET amount spectrophotometrically by measuring the absorbance at the maximum wavelength (λ_{max}) of KET in ethanolic phosphate buffer at 235 nm, after appropriate dilution depending on previous constructed calibration curve which gave straight line equation (y= 0.0327x-0.0006) and revealed high correlation coefficient (R^2 = 0.9994).

Kinetic model study

The release mechanism of KET from the prepared formulas was investigated by fitting the release data into the Zero, First, Higuchi, and Korsmeyer Peppas equations. Using a DDSolver Excel Microsoft Add-in application, k and R^2 values were calculated for each equation, as well as the n value for the Korsmeyer Peppas equation at 60% release[25].

FTIR spectrum for the optimum KET bilosomes loaded-hydrogel

Fourier-transform infrared spectroscopy (FTIR) analysis was used to ensure purity, compatibility and the absence of drug-excipient interaction; it was performed for the pure KET, physical mixture of KET with utilized formulation excipients and the optimum bilosome formula[26].

Skin irritation test

This test was carried out by applying sufficient hydrogel to a small region of rat skin under supervision (at the time of application, after one hour and 24 hours). The skin was observed for any visible change and checked for skin hypersensitivity (redness, irritation and edema) or any visible necrosis that could be happened[27].

Antifungal activity of optimum KET bilosome-loaded hydrogel

The antifungal activities of the optimum KET-bilosomal hydrogel formula, plain hydrogel as a control and local marketed cream were evaluated using the agar diffusion method on *C. albicans* ATCC 10231, fungal strains. The auto-

claved aqueous solution of the required quantity of Muller-Hinton agar with 2% glucose for support fungal growth was prepared and poured into sterilized Petri plates. Each plate was then planted with fungi, and the holes were made in the agricultural media after solidification. The samples to be examined were added into the holes, and then plates were incubated at 28°C for 48 h[28]. The zones of inhibition (ZOI) were measured to compare the results.

Statistical analysis

All evaluation tests were carried out in triplicate, and the findings were presented as mean ± SD. Microsoft Excel2010 utilized one-way ANOVA for statistical analysis. P values of 0.05 or less were considered statistically significant, whereas values of 0.05 or higher were considered statistically insignificant.

RESULTS and DISCUSSION

Preparation of ketoconazole bilosomes

Span60, sorbitan monoester, was selected as membrane forming nonionic surfactant own to its lipophilic saturated alkyl chain, high transition temperature and optimum HLB value (4-8) that can create stable single and/or multilamellar nanovesicles layers. The appearance of the prepared bilosomal formula was a homogenous milky white liquid dispersion. Furthermore, it had optimum vesicles nanosize (229.63 ± 6.22 nm) and PDI about 0.376 ± 0.029 that indicate homogeneity and approximately can be considered monodispersion as well as good % EE which was 83.13 ± 1.21%. similar result were attained by previous study[29].

Evaluation of ketoconazole bilosomal hydrogel

Physical appearance

All prepared KET-bilosomal hydrogel formulas had an off-white appearance and were smooth in texture with no grittiness or evidence of phase separation. This result agrees with the previous study, which stated that all prepared gelling systems were evaluated for visual appearance, clarity, and pH. The pH of the formulation decreases as the carbopol concentration increases. The pH of the formulation becomes acidic due to the polymer's acidic nature[30].

pH measurement

The developed KET-bilosomal hydrogel formulations had pH values ranging from 5.9 ± 0.1 to 5.5 ± 0.1 as shown in Table 2, which in agreement with the regulations required for topical treatment to avoid any skin irritation[31].

Table 2. The pH, spreadability, extrudability and % drug content evaluation of the different KET-bilosomal hydrogel

Formula	pH	Spreadability (cm)	% Drug content	Extrudability
FK C934 1%	5.9 ± 0.1	11.5 ± 0.5	98.4 ± 0.5	Excellent 93%
FK C934 2%	5.8 ± 0.1	9 ± 1	98.3 ± 1.05	Good 85%
FK C940 1%	5.6 ± 0.05	7 ± 0.5	98.7 ± 0.6	Fair 76%
FK C940 2%	5.5 ± 0.1	5 ± 0.5	98.76 ± 0.25	Fair 66%

Spreadability

Spreadability is an important issue in topical drug delivery systems since the efficacy of therapy is dependent on patients evenly applying the drug formulation to give a standardized dosage. It was revealed that increasing the polymeric content has a significant impact on spreadability since increasing the viscosity of the gel diminishes the formulation's spreadability. It is clear from the Table 2 that the spreadability of hydrogel formulas of Carbopol 940 is less than Carbopol 934. This is because the spreadability of any semisolid preparation decreases as the viscosity of the polymer increases. Similar results were attained by previous study which stated that the ejected gel was gathered and measured. The results were explained by difference in the concentration and nature of crosslinking of polymers[32].

Extrudability

The amount of extruded gel was measured and collected. The extrudability of the product exhibited decreasing as the gelling polymer percent raised[13], and the result shown in Table 2 that Carbopol 934 1% has excellent outcome. Similar results were gained by prior study[33].

Drug content

The ketoconazole content in the hydrogel formulations was ranged from 98.3 ± 1.05 to 98.76 ± 0.25% which indicated uniform distribution of KET in all hydrogel formulations and to be in good agreement with the theoretical drug content.

Viscosity

Viscosity plays a meaningful role in determining drug content and release from prepared KET-bilosomal hydrogel formulations. The viscosity experiment was carried out to determine the impact of Carbopol base type and concentration

on hydrogel viscosity. Hydrogel viscosity was measured at various shear rates was shown in Figure 1. It was revealed that as the shear rate raised, the viscosity of the hydrogel decreased, Likewise, it was found that increasing in the viscosity of the prepared hydrogel is caused by an increase in the amount of polymer. The results agreed with the earlier study, which stated that viscosity greatly affected the drug's content and release. The viscosity had done to evaluate the base's type and concentration impact on gel viscosity. The study showed that the gel's viscosity a decrease as the shear rate increases[34].

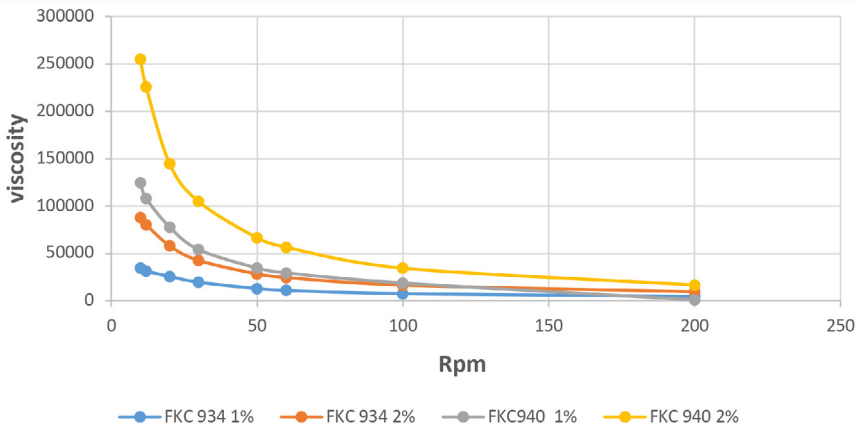

Figure 1. Viscosity versus share rate of the KET-bilosomal hydrogel

In-vitro drug release from ketoconazole –bilosome-loaded-hydrogel

The study of ketoconazole's *in-vitro* release patterns from several bilosome hydrogel compositions was performed and Figure 2 showed the results which revealed that the formulations containing 1% Carbopol 934 exhibited the highest drug release, whereas those containing 2% Carbopol 934, 1% Carbopol 940, and 2% Carbopol 940 exhibited slower drug release rates. This indicated the importance of Carbopol grade and concentration as gelling agent on drug release kinetics[35].

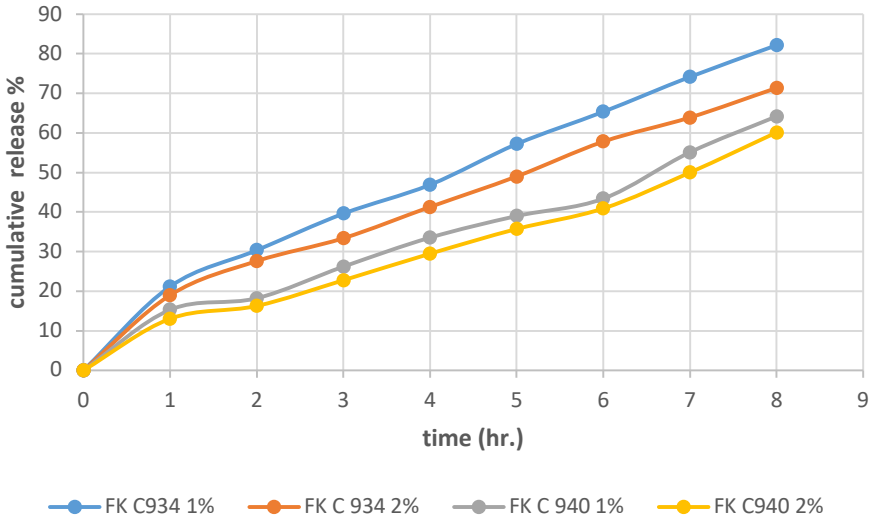

Figure 2. *In-vitro* drug release from ketoconazole -bilosome-loaded-hydrogel

Kinetics of release

The values of the release rate constant and regression coefficient (R^2) of the KET released profile obtained by applying different mathematical models are listed in Table 3. KET releasing profile for all tested bilosomal formulas best fit the Korsmeyer-Peppas model since it exhibited the highest R^2. The release exponent (n value) was less than 0.85 and larger than 0.43, which indicated that non-fickian diffusion (anomalous) is a drug transport mechanism[29].

Table 3. Mathematical model data of in-vitro ketoconazole bilosome releasing from hydrogel

Formula	Zero order		First order		Higuchi model		Korsmeyer Peppas model		
	k_0	R^2	k_1	R^2	k_H	R^2	k_{KP}	n	R^2
FKC934 1%	10.993	0.9514	0.181	0.9836	26.194	0.9599	19.269	0.671	0.9967
FKC934 2%	9.556	0.9453	0.145	0.9854	22.792	0.9619	17.064	0.662	0.9939
FKC940 1%	7.936	0.9732	0.108	0.9689	18.731	0.9067	12.809	0.682	0.9904
FKC940 2%	7.288	0.9828	0.096	0.9706	17.135	0.8905	10.486	0.753	0.9912

So, 1% Carbopol 934 (FKC934 1% formula) was selected as the optimum formula for KET-bilosomal hydrogel with good and acceptable physical properties include its in-vitro release, viscosity, spreadability and extrudability.

FTIR spectrum for the optimum KET bilosomes loaded-hydrogel

Any interaction or incompatibility of KET with the hydrogel formulation excipient mixture was investigated by comparing their FTIR spectra, as illustrated in Figure 3. The primary ketoconazole characteristic bands have been identified at 1645.28, 1583.58, 1510.26, 1285, and 814 cm^{-1}, which correspond to C=O stretching, C=C aromatic symmetrical stretching, C=C aromatic asymmetrical stretching, tertiary amine, and -C-Cl stretching, respectively. The FTIR spectrum of the physical combination revealed all of the principal KET peaks with no alterations in position. That suggests no interaction exists between ketoconazole and the components employed in crucial hydrogel production[36].

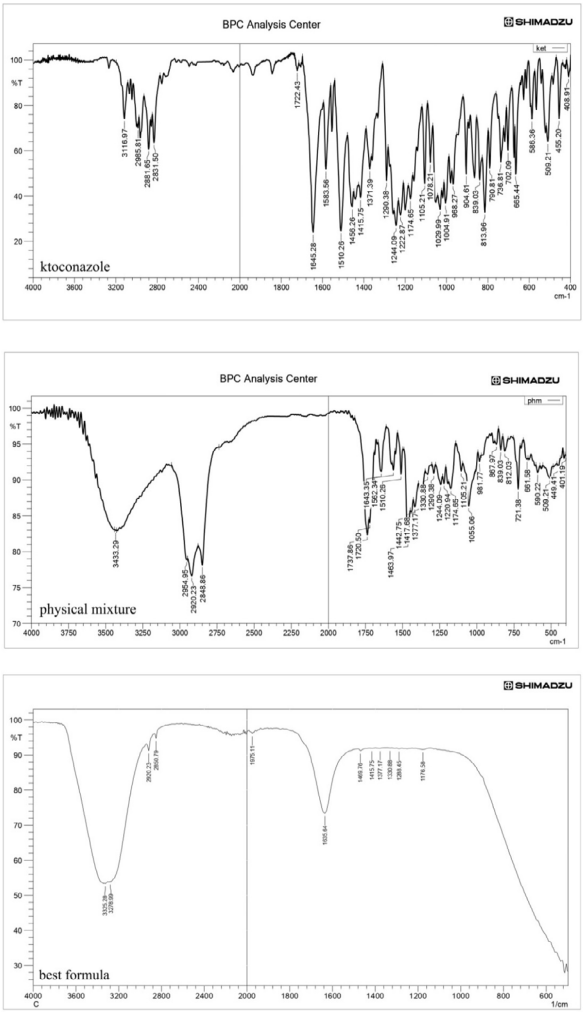

Figure 3. The FTIR spectra of ketoconazole, physical mixture and the best selected formula

Skin irritation test

To achieve the safety of the optimal hydrogel formula (FKC934 1%), a skin irritation test was done after topical hydrogel application on skin rats. Application of the hydrogel on the skin was found to have no irritation and no redness as shown in Figure 4, and this result agrees with a previous study[37].

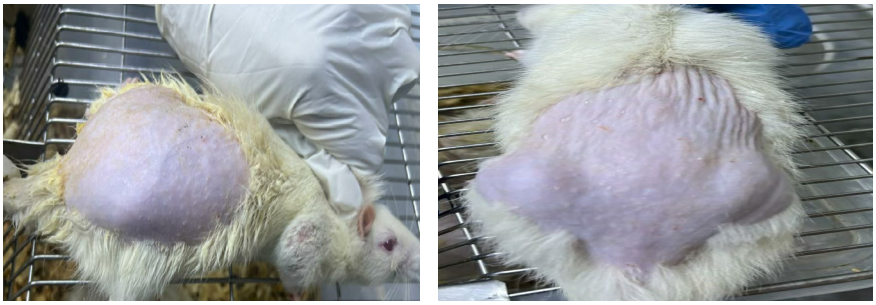

Figure 4. Rats' skin at time of application (left) and after one hour (right) after application KET bilosomal hydrogel

Antifungal activity of optimum hydrogel loaded with ketoconazole bilosomes

The antifungal activity assessment of KET-bilosomal hydrogel, plain hydrogel, ethanolic ketoconazole solution, and marketed ketoconazole cream was carried out against *Candida albicans,* the most common human pathogenic fungus. As shown in Figure 5, the plain hydrogel showed no growth inhibition. Meanwhile, KET-bilosomal hydrogel (FKC934 1%) gave a zone of inhibition diameter of about 18.5 ± 0.5 mm, ethanolic KET-solution 1% (as positive control used) showed growth inhibition 19.16 ± 0.28 mm and marketed ketoconazole cream zone inhibition 1.06 ± 0.11 mm. The highest inhibition zone of ketoconazole compared to plain hydrogel could be due to the highest release of the ketoconazole from hydrogel bilosomal formulation. This result confirmed with the earlier study[38].

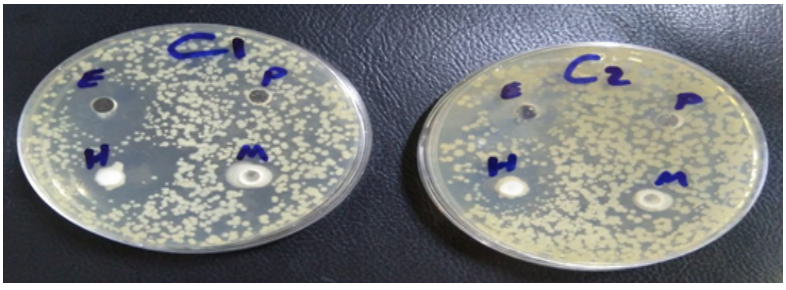

Figure 5. Photo illustrated; H: KET-bilosomal hydrogel, E: ethanolic ketoconazole solution, P: plain hydrogel, M: marketed ketoconazole cream

The characterization approaches and *in-vitro* release studies of the prepared KET-bilosomal hydrogel using Carbopol exhibited acceptable pH, good viscosity, spreadability, excellent extrudability, and good release profile. The optimum formula that containing KET bilosomes loaded in 1% Carbopol 934 showed 18- and 17-fold enhancement in antifungal activity against *C. albicaes* compared to plain gel and local marketed cream, respectively which provide a promising result for further studies.

STATEMENT OF ETHICS

All the necessary ethical rules were followed while performing research. Research ethical approval form number RECAUBcD151020BA have assigned by Research Ethics Committee at College of Pharmacy/University of Baghdad.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the study as follows: study conception and design: Lubna Abdalkarim Sabri (L.A.S.); methodology and data collection: Amer Sajjed (A.S.); analysis and interpretation of results: L.A.S and A.S.; draft manuscript preparation: A.S.; reviewed the results and approved the final version of the manuscript: L.A.S.

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The comparison between the results of turbidimetric method for C-Reactive Protein measurement using different instruments

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ABSTRACT

C-reactive protein (CRP) is considered a marker of chronic inflammation and also a mediator of the atherosclerotic process. The purpose of this study is to compare the results of three quantitative immunoturbidimetric method used to measure CRP amounts in patient serum samples in a clinical laboratory. Freshly collected patient serums (n=100) were analyzed with 2 different analyzer (Siemens Advia 1800 and Abbott Architect C 8000) and 3 different reagents (Siemens, Sentinel, Archem). In order to determine whether they provide equivalent results in terms of traceability, the EP09-A3 guideline was used, and comparisons were evaluated within the scope of this standard. Limit of quantification (LoQ), Inter27 assay, Intra-assay, precision studies have been done. The relationship between these three reagents was determined by regression analysis and Bland Altman method. Regression coefficiencies between these three methods were found: Archem-Sentinel r^2=0,9987, Archem – Siemens r^2=0,9986 and Sentinel – Siemens r^2=0,9984. Regression equations between Archem-Sentinel y= - 0,1359+1.0035x, between Archem- Siemens y= -0,02646+1,002x and between Sentinel – Siemens y=0,1326+0,9978x were found. For the first time in the literature, our results indicated that these three immunoturbidimetric methods were compatible.

Keywords: Siemens, sentinel, archem, CRP, method comparison

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INTRODUCTION

C-reactive protein (CRP) is an acute phase protein that increases in the blood in case of infection and inflammation. It is a pentraxin protein consisting of 5 subunits of 206 amino acids each, synthesized mainly in liver and fat cells. Its molecular mass is approximately 106 kilodaltons (Kd)[1]. Acute phase proteins include various proteins secreted mostly from the liver under the influence of cytokines, mainly interleukin 6 (IL-6), which increase as a result of acute or chronic inflammatory events. CRP can increase more than 10,000-fold in inflammatory conditions. Although CRP is not a disease-specific test, it is recognized as a very important parameter in the diagnosis, risk assessment and monitoring of some diseases. Chronically high levels of CRP, even mildly elevated, are more important risk factor for coronary artery disease (CAD) than elevated LDL (Low Density Lipoproteins). Chronically high levels of CRP, which is considered a systemic marker of tissue damage, are inversely correlated with life expectancy[2,3].

In bacterial infections, systemic fungal infections, systemic viral infections, erythema nodosum, acute rheumatic fever, rheumatoid arthritis, juvenile chronic arthritis, spondyloarthropathies, systemic vasculitis, polymyalgia rheumatica, familial mediterranean fever, Crohn's disease, tumor necrosis, acute pancreatitis, surgery, burn, fracture, lymphoma, carcinoma, sarcoma, CRP level is significantly increased. Chronic conditions that decrease CRP levels include alcohol intake, exercise and statin use[4-6].

CRP starts to be synthesized approximately 6 hours after infection. Therefore, even detection of low levels of CRP may provide early identification of infection[4].

Many methods are available to determine CRP levels. Turbidimetric and nephelometric methods are the most preferred methods for quantitative analysis of CRP. In addition, complex latex technology, enzyme-linked immunosorbent assay (ELISA), particle enhanced turbidimetric immunoassay (PETIA), particle enhanced nephelometric immunoassay (PENIA), etc. are also available for CRP mesurement. Although nephelometry theoretically provides an advantage in sensitive measurement of antigen-antibody reactions at low concentrations, stable and high-resolution photometric systems have become as sensitive as nephelometric methods in immunologic measurements of serum proteins[7,8].

In Turkey, the same analyte can be measured in the same laboratory using different devices and kits. This situation causes problems in terms of traceability of patient results. In this study, it was aimed to evaluate the compatibility and

correlation of the results of three different kits (Siemens Advia CRP, Sentinel CRP, Archem CRP) using turbidimetric method on two commonly used devices. In order to determine whether they provide equivalent results in terms of traceability, the EP09-A3[9] guideline developed by the Clinical Laboratory Standards Institute (CLCI) was used and comparisons were evaluated within the scope of this standard.

METHODOLOGY

The laboratory experiments of the study were carried out in Archem Diagnostics Quality Control and Analysis Laboratory. The study was conducted with the permission of Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee dated 19.12.2018 and numbered 773. Statistical analyses were performed with MedCalc (Mariakerke, Belgium) program.

Serum samples

Serum samples of patients who gave blood to Medipol University Mega Medipol Hospital Biochemistry Laboratory for routine analyses, which were to be discarded after the desired tests were run, were used. Samples were collected in a sitting position from the antecubital vein into clot activator-containing, anticoagulant-free gel tubes (Beckton Dickonson, New Jersey, USA). Within 30 minutes after blood collection, each sample was centrifuged at 2000xg for 15 minutes and serum was separated. The age range of the patients was 5-56 years, and the mean age was 24.5 years. 48 of the patients are women and 52 are men.

CRP measurements

Sentinel (Ref No: 6K26-10) and Archem reagents (Ref No: TA101S-4) were performed on an Abbott Architect C 8000 (Abbott Laboratories 100 Abbott Park Road Abbott Park, Illinois 60064-3500, USA) at different times to inhibit the carryover effect between reagents. USA). Then, the same samples were measured on the Siemens Advia 1800 (Siemens Medical Solutions USA, Malvern, PA, USA) autoanalyzer with the Siemens kit (Ref No: 00337402; B03-4815-01).

All autoanalyzers were calibrated before measurements were performed. Control standard deviation (SD) values were within ± 1SD. Suitable conditions were ensured for all measurement methods before experiments.

Siemens CRP method

ADVIA Chemistry wr CRP is a latex immunoassay that developed to measure blood CRP levels in serum and plasma using the turbidimetric/immunoturbidimetric method. It results in agglutination due to an antigen-antibody re-

action between CRP in the sample and anti-CRP antibody adsorbed on latex particles. The change in absorbance proportional to the CRP level is detected at 571 nm. Measurements were run as a double reading. All samples were run randomly on the autoanalyzer.

Sentinel CRP method

Multigent CRP Vario is a latex immunoassay developed to measure blood CRP levels in serum and plasma using the turbidimetric/immunoturbidimetric method. It results in agglutination due to an antigen-antibody reaction between CRP in the sample and anti-CRP antibody adsorbed on latex particles. The change in absorbance proportional to the CRP level is detected at 572 nm.

Archem CRP Method

Archem CRP reagent is a latex immunoassay developed to measure blood CRP levels in serum and plasma using the Turbidimetric/Immunoturbidimetric method. It results in agglutination due to an antigen-antibody reaction between CRP in the sample and a specific anti-CRP antibody adsorbed on latex particles. The change in absorbance proportional to the CRP level is detected at 572 nm.

Comparison of method performance

To assess suitability for use in the clinical laboratory, methods were compared using EP09-A3 guidelines developed by the Clinical Laboratory Standards Institute (CLCI)[9,10].

Precision and accuracy

Precision and error calculations were performed using data from a single study. For both studies, as quality control material Archem Specific Protein Control Level 1 (14,0mg/L) (Ref:04R42-01) and Level 2 (68,2mg/L) (Ref:04R43-01) were used. For the intra-day control study, the two levels of control sera were run in a single run on the same day with 20 replicates. For inter-day reproducibility, CRP values were obtained by repeated measurements of the same level controls stored at -20 ^{o}C for ten consecutive days, 5 times each day. The obtained data were used for precision and accuracy assessment[11]. Arithmetic mean (AM), SD and repeatability (intra-measurement and inter-measurement percentage coefficient of variation/coefficient of variation- percentage distribution coefficient CV%) values were calculated with the data obtained to evaluate precision and accuracy.

Analytical sensitivity (detection ability)

The term analytical sensitivity describes tests used to evaluate the precision performance of a measurement method at low analyte concentration. The tests are listed as "Limit of the Blank" (LoB), "Limit of Detection" (LoD), "Limit of Quantitation" (LoQ). In this study, LoQ is defined as the lowest concentration of analyte that can be measured with acceptable precision and accuracy. "Functional sensitivity" is the analyte concentration at which CV:20% can be achieved.

Comparison Analyses (Regression Analysis, Bland Altman): In our study, 100 serum samples were measured with two different autoanalyzers. Scatter plots and difference plots (Bland-Altman) were used to examine the distribution of the data obtained[12,13]. Pearson correlation and regression analyses were performed to compare the methods.

Hemolysis, icterus and lipemia were considered as sample rejection criteria. Samples covering the entire measurement range were selected instead of only clinical decision points and sample results within reference ranges.

Sera with values below the LoQ value were not included in the study. Stable and appropriate conditions were ensured during transportation and storage for the samples used during the study period.

Samples for the comparison study were kept under stable and appropriate conditions and the studies were performed on the same day.

Since bias deviations due to calibration, lots and instrumentation may be observed in the measurement results, biases were eliminated using the calibration procedure of the Ideal In Vitro Diagnostic (IVD) measurement procedure.

Scatter plots

For the scatter plots comparing methods, the plots of the Bland Altman and Passing Bablok methods were used. Intercept confidence interval, slope confidence interval, relative standard deviation interval are found with the help of the graph. Bias calculations were made according to the readings and Passing Bablok Regression graphs were drawn.

Difference plots

The Bland-Altman plot was considered appropriate when plotting difference plots for the methods compared.

RESULTS and DISCUSSION

Routine Internal Quality Control (Internal QC) results given to the autoanalyzers after the calibration results were found to be within ±1 SD as in the tables below (Table 1, Table 2, Table 3).

Table 1. Archem CRP control result

QC (Quality Control)	Mean (mg/L)	±1 SD	The Result (mg/L)
Specific Protein Control Level I	14,0	1,4	14,2
Specific Protein Control Level II	68,2	6,82	68,0

Table 2. Abbott (Sentinel) CRP control result

QC (Quality Control)	Mean (mg/L)	±1 SD	The result (mg/L)
Specific Protein Control Level I	14,0	1,4	13,8
Specific Protein Control Level II	68,2	6,82	68,9

Table 3. Siemens Advia 1800 CRP control result

QC (Quality Control)	Mean (mg/L)	±1 SD	The result (mg/L)
Specific Protein Control Level I	14,0	1,4	14,2
Specific Protein Control Level II	68,2	6,82	67,7

Precision and accuracy study

Intra-assay and Inter-assay studies were studied.

Intra-assay study (n:20) for control level 1 and 2 respectively for Siemens Advia (CV:1,62; CV:1,14), Sentinel (CV:1,3; CV:0,9) and Archem (CV:2,23; CV:1,02) were found below 5%.

Inter-assay study (n:10) for control level 1 and 2 respectively for Siemens Advia (CV:2.34; CV:2.32), Sentinel (CV:2.53; CV:1.98) and Archem (CV:3,32; CV:2.51) were found below 5%.

Analytical sensitivity (detection ability)

The LoQ was found by calculating the analyte concentration at which the CV was 20% (Table 4).

Table 4. LoQ (Limit of Quantitation) comparison chart

Kit Brand	LoQ Value (mg/L)
Archem CRP	0,5
Sentinel CRP	0,23
Siemens CRP	0,12

Archem – Sentinel comparison

Regression coefficiencies between Archem-Sentinel is r^2=0,9987, regression equations between Archem-Sentinel y=-0,1359+1.0035x were found (Figure 1 and Figure 2).

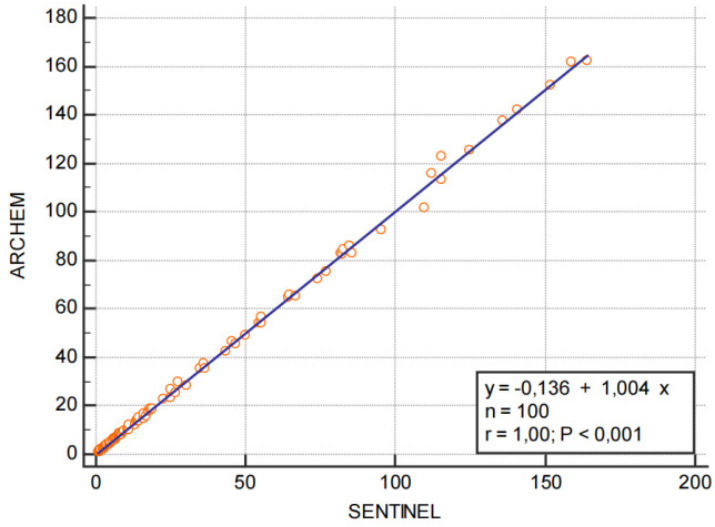

Figure 1. Archem – Sentinel Regression scatter plot

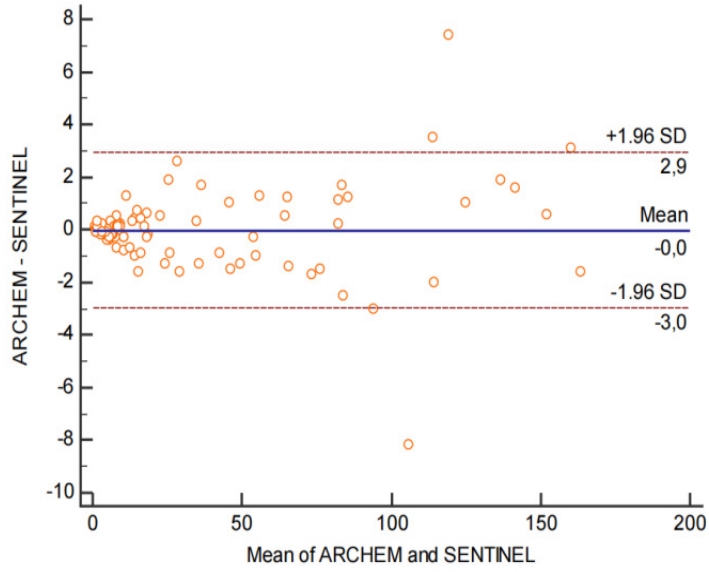

Figure 2. Archem – Sentinel Bland-Altman plot

Archem – Siemens comparison

Regression coefficiencies between Archem- Siemens is r^2=0,9986, regression equations between Archem-Sentinel y=-0,02646+1,002x were found (Figure 3 and Figure 4).

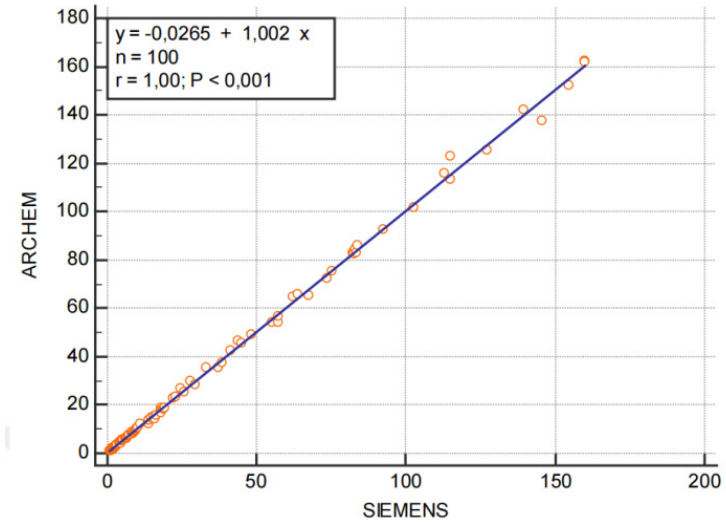

Figure 3. Archem – Siemens Regression scatter plot

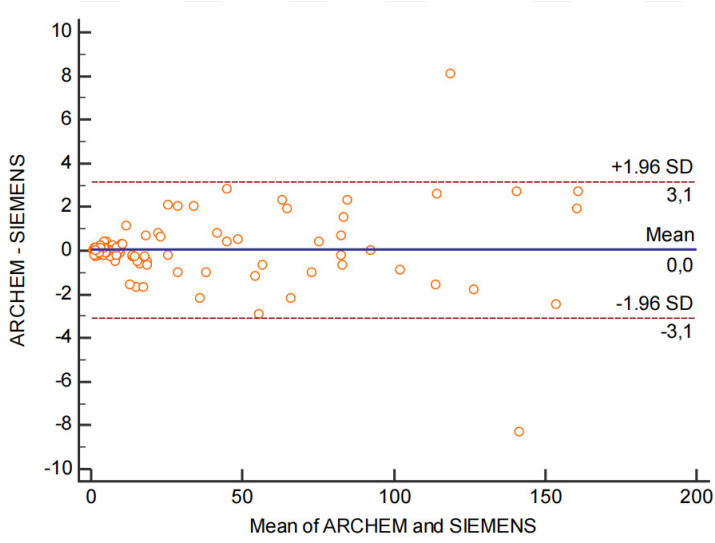

Figure 4. Archem – Siemens Passing and Bablok chart

Sentinel – Siemens comparison

Regression coefficiencies between Sentinel – Siemens is r^2=0,9984, regression equations between Sentinel – Siemens y=0,1326+0,9978x were found (Figure 5 and Figure 6).

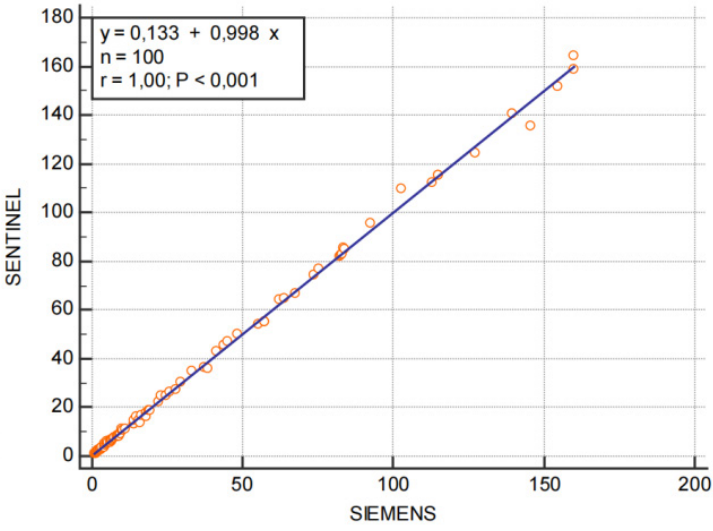

Figure 5. Sentinel – Siemens Regression scatter plot

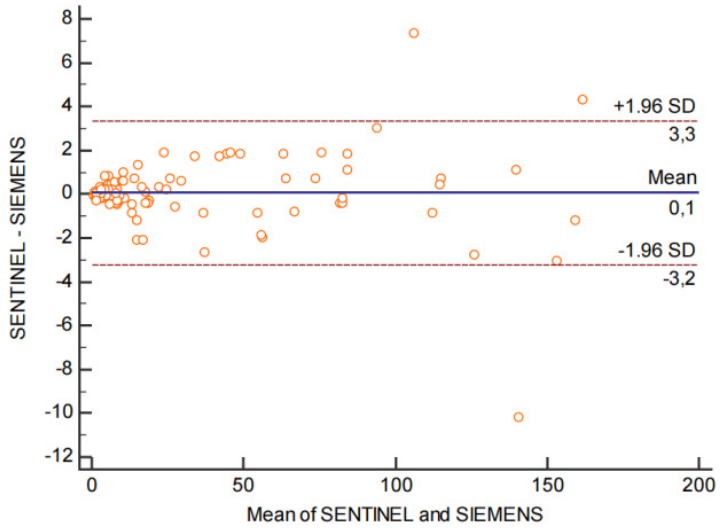

Figure 6. Sentinel – Siemens Passing and Bablok chart

In the 1990s, as CRP could be measured more precisely, its clinical use increased significantly. Although CRP measurement is a non-specific test, it is a very important parameter in determining the risk and monitoring of many diseases[2].

Immunoturbidimetric assay (ITA) and Immunonephelometric assay (INA) methods are the most frequently preferred methods in CRP quantitative analysis. In addition, CRP analysis can be performed using methods such as complex latex, ELISA, PETIA[7].

The main aim of clinical laboratories is to provide patients with the most accurate and reliable results in the shortest time, every time, by minimizing analytical errors. Nephelometry theoretically provides more sensitive measurement of low concentration antigen antibody reactions[14].

In a study comparing the Dade-Behring RCRP method with the Behring nephelometer (BN) method, they found that the correlation between the two methods was RCRP= (0.984 + BNx0.033 (mg/L) [15]. Due to the compatibility between INA and ITA methods, serum protein measurements with ITA, which is a faster, easier and cheaper method, have gained importance today.

In Turkey, the same analyte can be measured in the same laboratory using different analyzer and kits. This situation causes serious deficiencies in terms of traceability. Frequent device changes within the scope of tender, especially

in public hospitals and universities, make it necessary to evaluate the compatibility and correlation of test results.

In this study, it is aimed to compare the results of Siemens CRP reagent, Archem CRP reagent and Sentinel CRP reagent on Siemens Advia 1800 and Architect C8000 which are the most commonly used autoanalyzers in the clinical determination of CRP test.

There are comparison studies made with autoanalyzers, ITA and immunoluminometric assay (ILA) methods, using INA as a reference.

In the study conducted by Shiesh SC et al. comparing the ILA method and the INA method; Inter-assay CV; for mean CRP levels of 0.02 mg/L, 1.44 mg/L, and 11.04 mg/L, they were 7.0%, 5.2%, and 4.1%, respectively. Intra-assay CV: for mean CRP levels of 0.02 mg/L, 1.49 mg/L, and 10.90 mg/L, they were 9.2%, 7.0%, and 6.0%, respectively[16].

In current study, Intra-assay study (n:20) for control level 1(14,0 mg/L) and control level 2(68,2 mg/L) respectively for Siemens Advia (CV:1,62%; CV:1,14%), Sentinel (CV:1,3%; CV:0,9%) and Archem (CV:2,23%; CV:1,02%) were found below 5%. Inter-assay study (n:10) for control level 1 and control level 2 respectively for Siemens Advia (CV:2.34%; CV:2.32%), Sentinel (CV:2.53%; CV:1.98%) and Archem (CV:3,32%; CV:2.51%) were found below 5%.

In both studies, it was observed that CV% values increased as CRP levels decreased, as expected.

According to the study conducted by Shiesh SC et al., the lower CV% values that we found may be due to the higher CRP concentration in the control level 1 and 2 we used. In this study, in the intra-day and inter-day study conducted with two levels of control for all three kits, it was observed that the CV% remained below 5% and was suitable for clinical use, and there was no significant statistical difference between them.

In current study, LoQ values has been found for Siemens Advia (0,12 mg/L), Sentinel (0,23 mg/L) and Archem (0,5 mg/L). Patients with CRP levels below 1,0 mg/L are considered to be at low relative risk, patients with CRP levels between 1 and 3 mg/L are considered to be at medium risk, and patients with CRP levels above mg/L are considered to be at high risk for cardiovascular diseases[17]. The fact that every 3 reagents measure 1,0 mg/L can be considered an advantage.

In the study conducted by Buğdaycı et al.; serum samples were studied on the same day on Siemens Dade Behring BN ProSpec and Abbott Architect C8000 systems using the INA method. Serum samples with CRP values between 3.02-

170.00 mg/L were used. In the accuracy study, the regression coefficient was found to be r^2=0.997 and the regression equation was y=1.171x-1.084. The coefficients of variation of controls at three different levels (13.2 mg/L ± 1.33; 28.5 mg/L ± 2.85; 49.3 mg/L ± 4.93) were determined to be below 5%[18].

The following methods were compared in the study of Maggiore et al. in 2009. Immunoturbidimetry (AU2700 biochemistry analyzer; Olympus, Rungis, France) laser nephelometry (Behring Diagnostics, Marburg, Germany), and nephelometry (Beckman Instruments, Fullerton, Calif).

The Beckman method yielded with intraassay CVs ranging from 1 to 2 and interassay CVs ranging from 1 to 4. The Olympus method with intraassay CVs ranging from 1 to 3 and interassay CVs ranging from 1 to 10. The least precise assay was the Behring method, for which intraassay CVs ranged from 12 to 15 and interassay CVs ranged from 7 to 16[18].

In current study, when patient results measured using three different kits in the range of 0.8 mg/L to 159.8 mg/L were compared, it was determined that there was a linear correlation between the methods and no significant fixed or proportional error.

Regression coefficiencies between these three methods were found: Archem-Sentinel r^2=0,9987, Archem – Siemens r^2=0,9986 and Sentinel – Siemens r^2=0,9984. Regression equations between Archem-Sentinel y= -0,1359+1.0035x, between Archem- Siemens y= -0,02646+1,002x and between Sentinel – Siemens y=0,1326+0,9978x were found. The slope coefficient values of the study were very close to 1.0.

Bias values were calculated as-0.1359 / -0.02646 and 0.1326, which are quite low between Archem-Sentinel / Archem – Siemens, / Sentinel – Siemens, respectively. All of the values found are too small for the decision level (<5 mg/L). Compared to the studies carried out in previous years, it is seen that the new generation kits produced by commercial companies have achieved much more sensitive measurement success[15-19].

When Bland–Altman plots are applied for method comparison purposes; (In this method, (μD ± 1.96SD) is called «limits of agreement»), it was found that 95% of the differences between the measurement values obtained by the three methods were within the limits of agreement. According to the Bland-Altman method, it was observed that the average of the differences spread around zero and the results were within the limits of fit.

In conclusion, ITA systems offer easy, fast and economically advantageous solutions for CRP measurement. Our results have shown for the first time in the literature that these three ITA methods (Sentinel, Archem, Siemens) are compatible and can be alternatives to each other in terms of traceability.

STATEMENT OF ETHICS

Our study was approved by Medipol University local ethics committee (Ethical approval no: 773, Date: 19.12.2018).

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

These authors contributed equally.

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Liposome encapsulated curcumin in lysine-collagen hydrogel embedded with valsartan for treatment of diabetic wounds

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ABSTRACT

Impaired wound healing occurs due to factors such as diabetes, resulting in slow healing. The aim of this study is to develop and evaluate the wound healing potential of curcumin encapsulated liposomes in a lysine-collagen-hydrogel matrix embedded with valsartan for diabetic wounds. Formulations, CF1 (curcumin encapsulated liposomes in lysine–collagen hydrogel), CF2 (curcumin encapsulated liposomes in lysine-collagen hydrogel embedded with valsartan), and CF3 (valsartan loaded lysine-collagen hydrogel) were prepared and evaluated for physicochemical, histological, histomorphometric and wound healing properties. Formulation CF2 had the highest swelling ratio which was 89.2 ± 1.95%, while CF3 had the highest viscosity of 60000.00 ± 2.07 m Pas. Formulation CF2 showed the best wound closure, which was 100% by day seven, followed by CF1, CF3, Control and then diabetic wounds that were not treated. Formulation CF2 was found to be the most effective in promoting re-epithelization and angiogenesis. It can serve as an effective formulation for the treatment of diabetic wounds.

Keywords: liposome, hydrogel, wound healing, formulation, collagen

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INTRODUCTION

Diabetes is a condition that occurs when the pancreas does not produce enough insulin, or the body lacks the ability to utilize the insulin produced by the pancreas effectively[1]. This condition may lead to a consistent state of hyperglycaemia and, if poorly managed, may cause damage to the blood vessels and nerves[2]. Wounds that occur in people with poorly managed diabetes have a low chance of healing normally. The process of healing is impaired due to the hyperglycaemic state and the wound becomes chronic[3]. Chronic wounds emanating from poorly managed diabetes are an important primary public health issue. More than 430 million adults are likely to be affected by diabetic wounds within the next decade[4]. A diabetic wound can be described as an impaired wound characterized by hypoxia, impaired neovascularization, neuropathy and fibroblast abnormalities[4].

Diabetes has been identified as a leading cause of impaired wound healing[5]. Diabetic wounds are difficult to treat due to a variety of factors, which result in slow wound healing. The adverse microenvironment of a diabetic wound includes factors such as degenerative enzymes, alkaline pH and a complicated array of biochemical cues and processes that lead to a lack of progression through the primary phases of wound healing[6]. The impaired or chronic state of the wound is also caused by the high blood sugar levels, making it very unlikely that its entire process of angiogenesis will pass through all four structured phases of wound healing. Systemic factors that affect wound healing due to diabetes include sustained hyperglycaemia, peripheral neuropathy, and inflammation at the wound site[7].

The complicated process of tissue repair relies on the combined effect of cells, cytokines, enzymes and growth factors. In a diabetic wound, there is a lack of proper regulation necessary for wound healing, which leads to the wound into a chronic state[8]. Chronic diabetic wounds need an effective ultra-modern delivery system that will serve as an ideal wound treatment. This novel formulation should prevent infection, control the moisture level at the wound bed, facilitate the principal mechanisms involved in angiogenesis while stimulating wound closure, and finally, minimise scar formation[7].

Curcuma longa is a plant from which curcumin is gotten. It is a turmeric plant which belongs to a group of rhizomes[9]. The pathway through which curcumin enhances wound healing is through the localization of transforming growth factor β1 in the wound's microenvironment, coordination of collagen and reduction of reactive oxidative species. Curcumin is lipophilic, hence its de-

creased bioavailability and wound healing activity. Liposomes may be used for the encapsulation of hydrophobic drugs like curcumin to enhance their bioavailability and therapeutic activity[9].

The need for advancement of the curcumin-loaded liposome formulation may lead to the infusion of the loaded liposomes into a polymer hydrogel to improve the overall stability, efficacy, and dermal contact time of the formulation with the wound area. The development of a liposome-in-hydrogel delivery system will demonstrate improved hydrophilicity and controlled release of the loaded drug molecules curcumin, lysine and valsartan[10]. In this liposome-in-hydrogel delivery system, the surrounding polymer hydrogel provides a solid framework which serves as a mechanical cushion for the liposome bi-lipid membrane, enhancing its stability[11]. The delivery system provides an attenuated burst release effect via the liposome due to the mechanical cushioning effect of the hydrogel. It provides better muco-adhesion to the wound, higher tissue localisation, as well as efficient muco-penetration of loaded drug molecules into the wound microenvironment[12]. Research also indicates that the presence of lysine at the wound microenvironment may enhance wound healing, collagen will improve the tensile strength of the newly formed cells in the wound's microenvironment, while valsartan may be responsible for accelerated wound contraction, increased tensile strength, regulation of immune responses, as well as molecular and cellular processes. This makes this group of drug molecules an ideal choice for potentiating a synergistic wound healing effect[13,14,15]. The synergistic effect between these bioactive compounds presents a novel approach to diabetic wound management[16]. The choice of polymer used for the development of the hydrogel was based on its characteristic features that aid drug delivery at the wound site. Carbopol delivers maximum drug in an alkaline environment due to its greater swelling index at higher pH.[17]. The pH of a diabetic wound is slightly alkaline (6.95)[18]. Carbopol, an acrylic polymer and gelling agent, is safe for dermal applications and non-toxic[19].

Based on existing literature, little research has been carried out regarding synergistic interactions between bioactive molecules such as curcumin, lysine, valsartan, and collagen. In this study, curcumin-loaded liposomes in a lysine collagen hydrogel embedded with valsartan is formulated. This liposome-in-hydrogel based formulation stands out as a pioneer in its research space, as it provides a stable environment for the synergistic interaction between the therapeutic agents (curcumin, lysine, collagen and valsartan), while also providing sustained release of these agents. The formulation developed in this study could be used to enhance the healing of chronic diabetic wounds[20].

METHODOLOGY

Materials

The materials utilized for this study include are Phosphatidylcholine (Sigma-Aldrich Co., St. Louis®, MO, USA), Carbopol Ultrez (Surfachem, U.K), Valsartan (Merck, Germany), Curcumin (Sigma-Aldrich Co., St. Louis®, MO, USA), Phosphate buffer (Loba Chemie, Colaba Mumbai, India), Urethane (Sigma-Aldrich Co., St. Louis®, MO, USA), Methanol (Merck, Darmstadt, Germany), Cremophor (RH 40) (Macklin Biochemical, Shangai, China), Alloxan (Merck, Germany), Deionised water, Lysine (Sigma-Aldrich Co., St. Louis®, MO, USA), Collagen (**N**eocell, U.S.A), and Triethanolamine (Merck, New Jersey®, USA).

Development of curcumin-loaded liposomes

Liposomes were prepared according to 'thin film hydration method'[5]. Methanol (25 mL) was measured using a volumetric cylinder and poured into a round bottom flask. Curcumin and phosphatidylcholine (435 mg and 100 mg, respectively) were weighed using an analytical balance. The curcumin and phosphatidylcholine were dissolved in methanol in the flask, which was then attached to a rotary evaporator at 45°C. After 20 min, a lipid film is observed on the inner surface of a spherical flask. Phosphate buffer (pH 7.4) was prepared, and 25 mL of buffer was used to rehydrate the thin lipid film. The mixture was then sonicated for 15 min. Finally, the solution was vortexed for 10 min, and transferred into a 100 cm^3 transparent bottle. It was labelled and stored at 4°C. Liposomes were analysed in terms of shape, size, and surface morphology using Scanning electron microscopy[21].

Curcumin *in-vitro* drug release profile (Flow rate using Franz cell)

In vitro drug release study was performed with Franz diffusion cell. The diffusion fluid was a mixture of phosphate buffer (pH 7.4) and Cremophor RH40 (pH 7.0). The receptor compartment was filled with the diffusion fluid. A membrane filter was soaked in the diffusion fluid for 45 min, and then blotted on both sides. The membrane was fixed on the lower side of the donor compartment and fitted tightly with a ring. The Franz diffusion cell was fixed on a magnetic mixer and allowed to be stable at 37°C by warming. The donor compartment was filled with the curcumin-loaded liposomes. A stopwatch was started, and exactly 1 mL of sample was withdrawn from the diffusion fluid at intervals of 5, 10, 30, 60, 120, and 180 min. Then, 1 mL of fresh diffusion fluid was used to replace the fluid withdrawn from the receptor compartment of the Franz diffusion cell. Samples from the different time points were taken for UV analysis, and the experiment was performed in triplicates[17, 22].

Determination of encapsulation efficacy

Exactly 5 mL of the liposomal suspension was poured into a centrifuge tube and loaded onto a centrifuge, then allowed to centrifuge for 10 min at 400 rpm. The centrifuge tube was then removed, and the supernatant was discarded, while the loaded liposomes which had settled at the bottom were taken for UV analysis. Encapsulation efficacy was calculated using Equation (1). The experiment was performed in triplicate[23,24].

$$\text{Encapsulation Efficiency} = \frac{\text{TAC} - \text{NEAC}}{\text{TAC}} \; x \; 100 \quad \text{(Equation 1)}$$

TAC: Total amount of curcumin

NEAC: Non encapsulated amount of curcumin

Preparation of hydrogel formulations

Hydrogels were formulated by dissolving 8 g of Carbopol Ultrez in 0.4 L of distilled water, then left to soak overnight. Hydrogel cross-linking was carried out by adding three drops of triethanolamine, and the pH of the hydrogel was adjusted to pH 5.8 using sodium hydroxide or hydrochloric acid. The compositions of the different formulations are shown in Table 1. Curcumin-loaded liposomal formulation, 0.005 L, was incorporated into 0.4 L of hydrogel. Then, 0.005 g of lysine and 1g of collagen were added to the hydrogel to obtain formulation CF1. Formulation CF2 was prepared by adding 0.005 L of curcumin-loaded liposomal formulation into 0.4 L of hydrogel, then incorporating 0.004 g of valsartan, 5 mg of lysine and 1000 mg of collagen. Finally, formulation CF3 was prepared by adding 0.004 g of valsartan and 1000 mg of collagen into 400 cm^3 of polymer hydrogel. They were stored at 4°C[17,19].

Table 1. Formulations CF1-CF3 and their varying constituents

Ingredients	CF1	CF2	CF3
(2% w/v Carbopol) Polymer Hydrogel	0.4 L	0.4 L	0.4 L
Curcumin loaded liposomes	0.005 L	0.005 L	-
Lysine	0.005 g	0.005 g	-
Collagen	1 g	1 g	1 g
Valsartan	-	0.004 g	0.004 g

Physicochemical characteristics of hydrogel formulations CF1-CF3

Characterisation and pH evaluation of the hydrogel formulations CF1-CF3

Physical evaluation and pH determination of the formulations were carried out after preparation. The formulations were optically examined for consistency, homogeneity, and colour. The pH of the hydrogel formulations was measured using a pH meter (Mettler Toledo, Columbus USA in triplicate[25].

Rheology test

The rheological behaviour of the hydrogel formulations was determined my measuring their viscosity at 24 °C, at 20–100 rpm using Spindles 6.0 and 7.0 cone and plate viscometer (Brookfield Engineering Laboratories, Middleboro, USA). All measurements were performed in triplicate[25,26].

Swelling test

The extent of water absorbed by the hydrogel formulations was determined by incubating 0.1 g of dry thick hydrogel film in 50 cm^3 of phosphate-buffer saline (pH 7.4) at 37 °C. The dry weights of the formulations were denoted as Sa and equilibrium swelling weight as Sb. All measurements were carried out in triplicate[25].

The swelling ratio was expressed as:

$$\% \text{ Swelling ratio } = \frac{(Sb - Sa)}{Sa} x\ 100 \quad \text{(Equation 2)}$$

Stability studies

Stability tests were performed after the formulations CF1-CF3 were stored for one month at room temperature (23–24 °C). The appearance, texture properties and bio adhesiveness of the hydrogel were determined one day after preparation and during storage on days 3, 7, 14, 15, 30 and 60[25].

In-vivo wound healing studies

Twenty-four male Wistar rats, each weighing 380–420 g, were acquired at the beginning of the study. The rats were allowed to adapt to their new environment for one week and were housed individually in polypropylene cages. Ethical approval was obtained for this investigation with the approval number, CMUL/ACUREC/08/21/923. Twenty-four randomly selected rats were divided into two sets: diabetes-induced rats (n=20) and healthy rats (n=4). Both sets of animals were made to fast overnight. Diabetes was induced by

the intraperitoneal injection of a freshly prepared solution of alloxan (50 mg/kg) in 0.9% normal saline (pH 5.5). After seven days, blood sugar levels were tested using a glucometer (Accu-Check, Roche Diabetes Care Limited, U.S.A). Rats with blood sugar levels equal to or greater than 250 mg/dL were considered diabetic. All rats were anaesthetized intraperitoneally with urethane (0.03 cm^3/kg). The dorsal area of each Wistar rat was carefully shaved and cleaned with 70% ethanol. One excision wound was created on the upper back of each animal using a scalpel. Bioactive dressing containing formulations CF1, CF2 and CF3 were used to dress the diabetic wounds starting from day three, while untreated non-diabetic wounds were designated "control" and diabetic wounds not treated were designated "DNT". Photographs of the wound surface were taken, and wound closure was measured on days 1, 3, 7, and 14 post-treatment. The wound dressing was changed on days 3, 7 and 14, and wound size was measured using a calliper (Mitutoyo 500-196-30, Europe). Data were reported as percentage wound closure against time. The percentage of wound contraction was calculated using Equation 3[27].

$$\% \text{ wound closure} = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{(Equation 3)}$$

A_0 = Wound size on day 0

A_1 = Wound size by day 3, 7, 14 and 21 after-treatment

Skin patch test

The hydrogel formulations (0.4 g) were applied to a shaved dorsal surface (3.0 cm^2) of three male Wistar rats. The skin appearance was visually examined for redness and swelling 1h after application[25].

Histological and histomorphometric examination

After the completion of the *in-vivo* wound studies, Wistar rats were euthanized. The healed wound sites were excised and fixed in 10% neutral buffered formalin. The tissues were immersed in alcohol then xylene. Embedding was carried out using paraffin wax. The tissues were stained with haematoxylin and eosin (H&E) and viewed under a microscope (Leica Microsystems microscope, Mannheim, Germany)[25].

Statistical analysis

The level of significant difference was considered if $p<0.05$. Statistical analyses were performed using Graph Pad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA)[28].

RESULTS and DISCUSSION

Size and morphology of curcumin-loaded liposomes

Liposomes were viewed under a scanning electron microscope, as seen in Figure 1. The liposomes were within the size range of 5 μm-10 μm in diameter. They were spherical with smooth surfaces, and also appeared stable. The large size of the liposomes enabled the encapsulation of a higher quantity of curcumin, which allowed for the release of more of curcumin locally at the wound area[29].

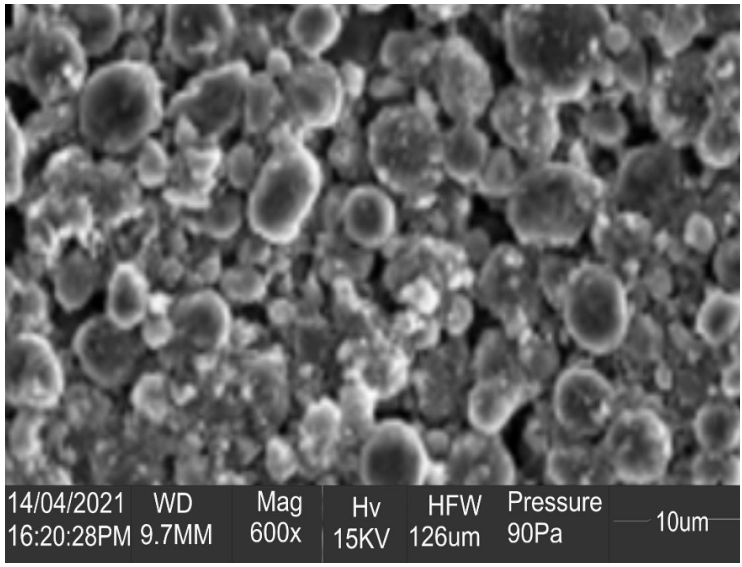

Figure 1. Scanning electron microscopic image of stable curcumin loaded liposomes

Encapsulation efficiency

The encapsulation efficiency of the curcumin-loaded liposomes was 99.934%, which is above 90%, indicating that the liposome loading of curcumin was optimal. This also implies that the liposomes remained stable with minimal leakage of curcumin. The stability of liposomes can be due to a couple of factors, such as the technique employed in its preparation, the type of phospholipid used, their size (which is influenced by the sonication time and frequency), and the storage conditions. Curcumin-loaded liposomes were prepared using a well-established technique (thin lipid film hydration), liposomes were also sonicated at a high frequency and an ideal time lapse. Also, liposomes were stored at 4°C immediately after preparation to prevent bilayer membrane disruption due to possible lipid hydrolysis[29].

In-vitro permeation rate (flux)

Flux illustrates a phenomenon in which a substance, in this case, drug molecule curcumin, appears to pass through a membrane selectively. Flux is also affected by the size, morphology, and encapsulation efficiency of the liposome formulation. A larger liposome size provides a larger of bi-lipid area to house the lipophilic drug curcumin. More efficient encapsulation of curcumin is associated with higher concentrations of curcumin per liposome and better flux, as more curcumin will be available to sip through the membrane. *In-vitro* permeability, also known as flux, as seen in Figure 2 was optimal (51.229 μg/cm²/h)[30].

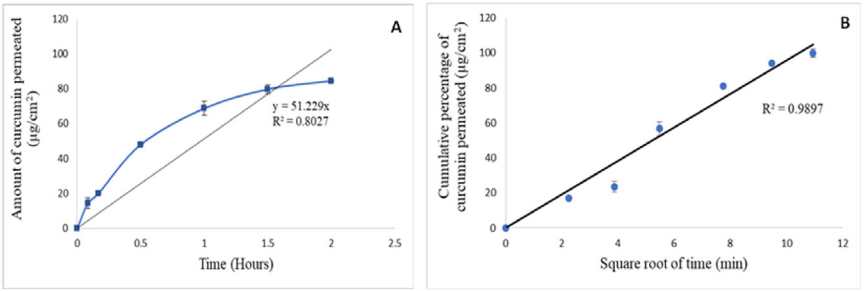

Figure 2. The flux of the optimized curcumin loaded liposomal formulation (A). The Higuchi plot of percentage cumulative of curcumin released from the optimized formulation against square root of time (B). Data are shown as mean ± standard deviation.

Figure 2B shows a plot of percentage cumulative drug release against the square root of time. This plot is that of the Higuchi release model. This model can help to understand the basic drug release system of the curcumin-loaded liposomes. Analysis of the release behaviour of the liposome formulations shows a controlled release of curcumin over a definite period. The release of curcumin from the liposome involved both dissolution and diffusion mechanisms. As a result, Higuchi release model can be used to fit the release of curcumin from liposomes[30].

Rheological evaluation

Rheology can be described as the science of flow and deformation of a material. It addresses the relationship between a given deformation and the stress response for a material such as hydrogel. Rheological techniques are commonly used to evaluate a material's viscosity and viscoelastic properties in relation to time, temperature, and shear.

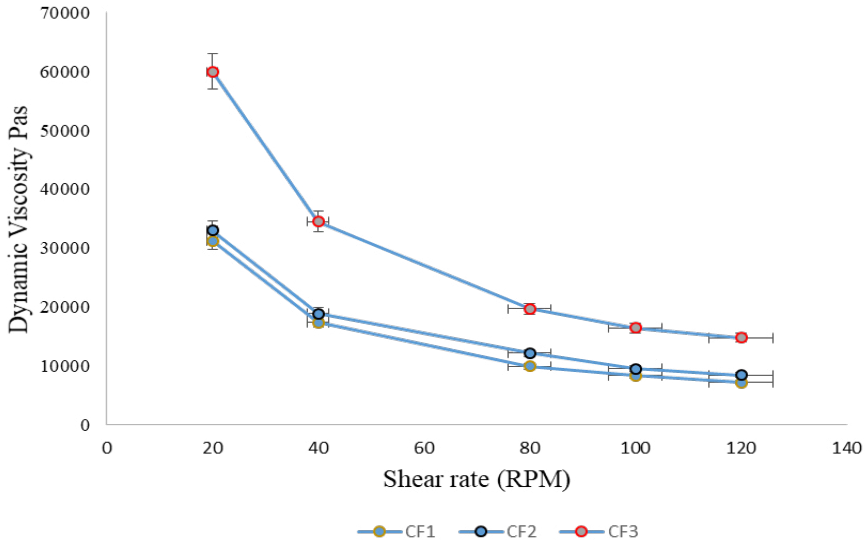

Figure 3. The rheological plot of dynamic viscosity in Pascal against shear rate in rotation per minute for formulations CF1-CF3. Data are shown as mean ± standard deviation.

In Figure 3, it can be deducted that there was a decrease in dynamic viscosity with an increase in shear rate, indicating that the flow resistance decreased on an increase in shear strain. This implies that the formulations are easily spread on topical application. All formulations showed a shear-thinning behaviour[31].

Table 2. Physicochemical properties of formulations, CF1, CF2, and CF3

Hydrogel Formulation	Dynamic Viscosity (20 rpm, PaS)	pH	Swelling Index %	Skin Irritancy
CF1	31250 ± 1.97***	5.8 ± 1.72*	85.7 ± 1.21***	Nil
CF2	32950 ± 1.01***	5.8 ± 1.11*	89.2 ± 1.95***	Nil
CF3	60000 ± 2.07***	5.8 ± 1.30*	81.4 ± 0.82***	Nil

Results are expressed as mean ± S.D (n=3). * Signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$ with regard to significant differences.

As seen in Table 2, formulation CF3 had higher viscosity due to the absence of lysine, which tends to break hydrogel polymer chains. Also, the absence curcumin loaded liposomes contributed to its high viscosity, showing that the incorporation of curcumin loaded liposomes in the polymer hydrogel affected

the texture and consistency of the overall formulation. Formulations CF1 and CF2 had a more fluid like consistency and hence lower viscosity[32].

Physicochemical properties of formulations CF1-CF3

The formulations appeared stable, with no change in texture, smell, or visual appearance, and all formulations maintained their original texture and bio adhesiveness throughout the period of this study. There was no sign of redness or swelling one hour after application in all formulations on rat skin. Formulations CF1 and CF2 were translucent and pale orange with no odour, while formulation CF3 was opaque white in colour with no odour as well. The pH test was also carried out to ascertain that the formulation was dermatologically safe. The natural pH of the skin is 5.5-5.7, and the closeness of the formulations' pH (5.8) to that of the skin also indicates its dermal safety for application. A pH below 6.0 is suitable as it creates an environment to promote angiogenesis. This is because an acidic wound bed can inhibit microbial growth and maintain a microbe free wound bed, channelling it towards its expected healing pathway[33].

The swelling index gives an indication of the level of porosity and cross-linkage that occurs on a molecular and structural level in the hydrogel. The swelling index of a hydrogel is also affected by pH and temperature. This influences liposome uptake and release from the hydrogel. Formulation CF2 had the peak swelling index, while CF3 had the least. The higher the level of cross-linkage the lower the swelling index. The relationship between drug release rate and the level of cross-linkage of hydrogel matrix is direct: the higher the level of cross-linkage, the faster the drug release rate[34].

In-vivo wound healing studies

Angiogenesis is a normal biological reaction to tissue damage. However, wound healing is not a straightforward process as it involves complicated interactions between different cell types, cytokines, mediators, and the vascular system. Bleeding at the onset of an injury is reduced by a domino effect of instant constriction of capillaries and platelet accumulation. This is followed by an infiltration of various inflammatory cells. These inflammatory cells secrete a myriad of mediators and cytokines to enhance angiogenesis, thrombosis, and re-epithelialization, leading to wound contraction. In a diabetic wound, the chronic inflammatory response at the initial stage is sub-optimal and prolonged, so it becomes excessive at the latter stages of wound healing[35]. Angiogenesis is impaired by poorly controlled diabetes, and there is a marked presence of neuropathy with a very high risk of poly-microbial infection. All these factors lead to slow wound contraction and impaired wound healing in diabetic

wounds. Although inflammation is an important element needed to confine and eliminate bacterial contamination at the earlier stages of wound healing, excessive or prolonged inflammation towards the latter stages of wound healing may result in a chronic state. Excessive inflammation was observed in DNT, as wounds presented redness and swelling, indicating excessive inflammation at latter stages at the wound site. The pictorial representation in Figure 4 shows that diabetic wounds treated with formulation CF2 had attained complete wound closure by day 7 post-incision. This proves that the treatments enhanced the facilitation and progression of wound healing. Wounds treated with formulation CF3 also showed improved wound healing progression, though not as impressive as those treated with CF2. This may be due to the absence of a strong wound healing enhancing agent, curcumin. Curcumin interferes positively with every stage of wound healing. It enhances epithelisation, collagen infiltration, formation of new tissues and capillaries, healthy remodelling, and rapid wound contraction. Curcumin acts by recruiting M2-like macrophages into white adipose tissues. This leads to the production of anti-inflammatory cytokines that are important for response to the presence of foreign bodies. It then sufficiently lessens inflammation by the stimulation of a prototypical proinflammatory signalling pathway known as the NF-KB pathway at the final stages of wound healing.

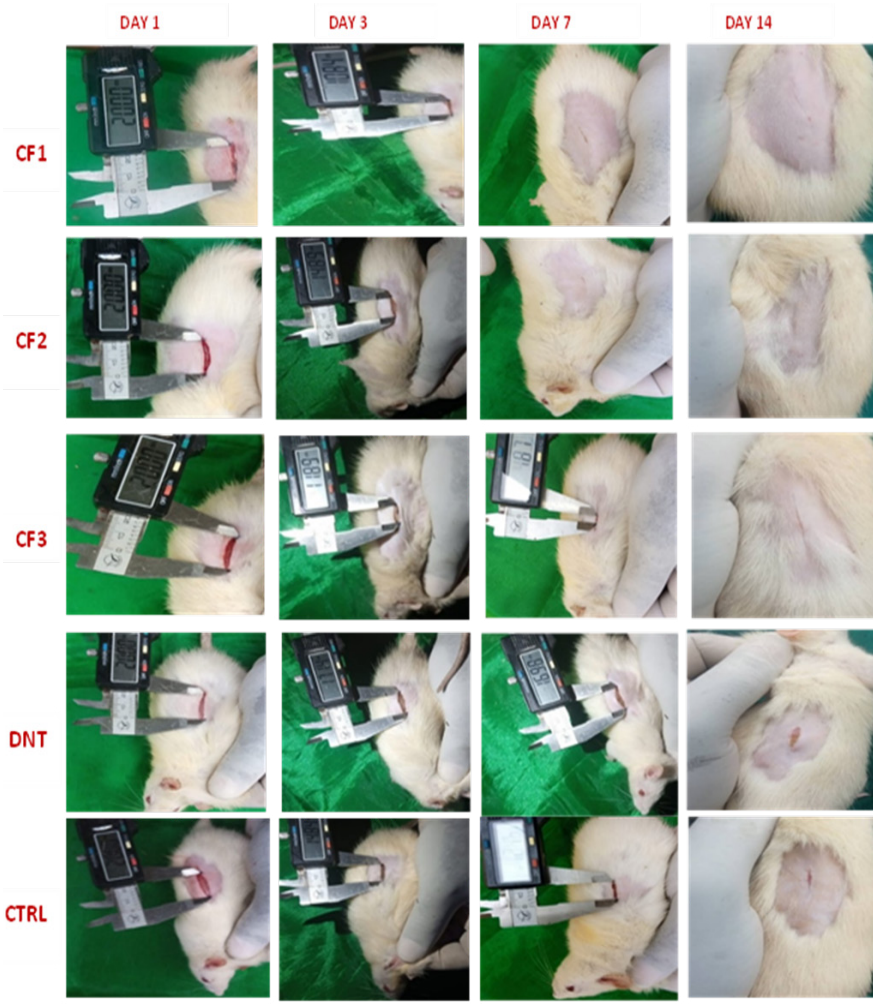

Figure 4. Pictorial representations of the contraction and re-epithelialization of wounds in rat groups treated with formulations (CF1, CF2, CF3), DNT (diabetic wound that were not treated with any formulation) and CTRL (non-diabetic wound that were not treated with any formulation).

The presence of curcumin along with other wound healing enhancing agents like lysine, collagen, and valsartan creates a synergistic effect responsible for facilitating wound healing. The absence of curcumin in formulation CF3 reflected in the slower wound healing progression in wounds of rats treated with these formulations. For all the treated wounds, complete closure was achieved by day 14 with no scarring observed. DNT healed with evidence of scars, indicating abnormal pattern of wound healing and remodelling. The control also

healed by day 14 with minimal scarification observed. Wound closure was rapid with no scars in chronic diabetic wounds treated with the developed formulations (CF1-CF3) compared to the control, which was not treated with any formulation. This indicates that tissue regeneration and re-epithelization rates were better in treated wounds. Complete re-epithelization and angiogenesis occurred within seven days for rats treated with formulation CF2, with regrowth of fur at the healed wound, as seen in Figure 4[36].

Relative wound size reduction and histological evaluation

Microscopic images of the haematoxylin and eosin staining of tissue sections from wound areas are shown in Figure 5A-E. All treated wounds showed the normal architecture of the skin, with the five layers of the epidermis intact, as well as the dermis[37]. DNT showed an abnormal tissue structure, showing clear perversion in the layers of the epidermis, the papillary dermis, and the reticular dermis (Figure 5E).

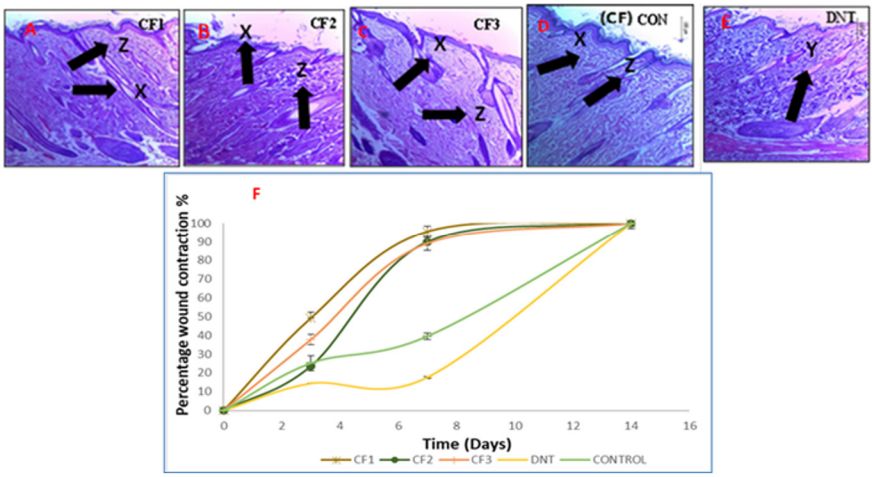

Figure 5. The microscopic images of the haematoxylin and eosin staining of tissue sections of wound areas of representative rats (A-E). The percentage relative wound size reduction from days 1 to day 14 (post-surgery) for chronic diabetic wounds (F). Data are shown as mean ± standard deviation.

The graph of percentage wound contraction against time, shown in 5F, showed that healing took place over a period of fourteen days and most of the wound contraction took place after the initial inflammation and proliferative phases[37]. All the rats used for the study survived through the post-operative process till euthanasia. Figures 5F shows wound healing curves similar to that of biological growth curves for all treated wounds. A different curve pattern was observed for DNT and control groups, proposing abnormal wound healing trajectory in these groups[38].

In Figure 5A-E, it can be seen that there is regrowth of the epidermis and presence of matured granulation tissue in diabetic wounds treated with formulation CF1, CF2, CF3, and the control. The arrow pointing to the spot marked X shows the wholly restructured epidermal and dermal layers, while spot Z shows the presence of newly formed connective tissues and capillaries, showing evidence of healthy wound healing. In Figure 5E, the spot Y shows a deformed tissue architecture for the DNT group, with abnormal layers of the epidermis and dermis, indicating poor wound healing and a lack of healthy regeneration of the dermal layers[39-41].

Histomorphometric analysis

Histomorphometry is described as the quantitative measurement of the shape or form of tissue[42]. It involves the quantitative analysis of parameters such as number of micro-vessels in the granulation tissue, percentage of collagen present in the granulation tissue, rates of re-epithelization, number of inflammatory tissues present and the thickness of the central region from the epidermis to dermis. These values are quantitative pointers to how well a wound has healed. The percentage re-epithelization rates show the level of migration of epithelial cells toward the wound bed for tissue repair, the epithelial cells achieve tissue repair through thick tissue formation[43]. The number of microvessels in granulation tissue shows the structural depth to which healing has occurred. Granulation tissue is basically composed of new connective tissue and micro blood vessels that form on the surface of a wound during the healing process. Granulation tissues progress from the base of the wound upwards to form the surface of a wound during the healing process[31]. Marked microvascular regeneration also indicates that proper neovascularization occurred in the vascular tissue at the wound bed[40]. The thickness of the central region of the epidermis and the dermis after wound healing shows how restructured the microenvironment of the wound bed is, whilst a high number of inflammatory cells in granulation tissue may mean that the wound is infected[41, 42].

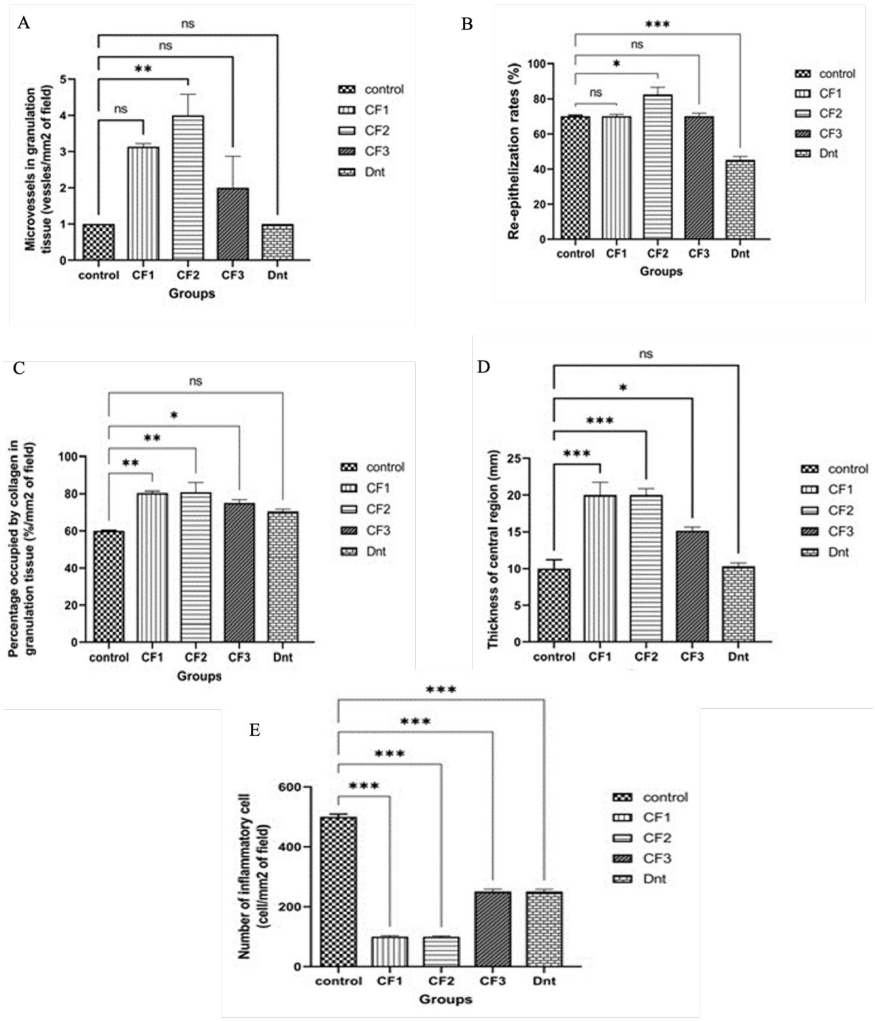

Figure 6. Histomorphometrical values for diabetic wound tissues fourteen days post wound incision (A - E). Data are shown as mean ± standard deviation. (*** means $p<0.001$, ** means p is between 0.001- 0.01, * means p is between 0.01 to 0.05, ad ns means $p \geq 0.05$).

Figures 6 (A-E) shows that the number of inflammatory cells were higher in the control group, indicating a likelihood of high microbial load at the wound site in the control group. The disparity between the control and other groups in terms of number of inflammatory cells is statistically significant. The re-epithelization rates obtained were highest for CF2 compared to the control and DNT. All wounds treated with formulations CF1, CF2, and CF3 showed re-epithelization rates above 70%, and they also contained higher collagen tissues. There was a clear and statistically significant difference between treatment group CF2 and

the control group in regard to the re-epithelization rates. The most therapeutically effective formulation, which enhanced rapid re-epithelization, contained curcumin, lysine, collagen, and valsartan (CF2). The formulations containing curcumin gave more rapid tissue regeneration[43]. This is primarily because the curcumin is favourably associated with the cellular events that occur in the inflammatory and proliferative phases[44]. Collagen is also known to contribute to the tissue tensile strength during angiogenesis at the wound bed[45]. Valsartan accelerates wound contraction, increases tensile strength of new tissue, regulates immune responses, as well as molecular and cellular processes in wound healing[13]. The number of micro vessels in the granulation tissue and the thickness of the central region of the epidermis to dermis indicate the depth of structural wound healing taking place as formation of micro vessels is necessary for vascular function at the wound site. Diabetic wounds treated with formulations CF1-CF3 showed a larger amount of micro vessels and a thicker central region of the epidermis and the dermis compared to the control and DNT (Figure 6). CF2 showed the highest level of significant difference when compared to the control in terms of number of micro vessels in tissue granulation and the thickness of the central region of the epidermis to dermis[36]. All formulations had hydrogel as base, however, curcumin-loaded liposome in lysine collagen hydrogel embedded with valsartan is preferred for management of diabetic chronic wounds due to its good swelling index, ideal viscosity, excellent in-vitro drug release, and encapsulation efficiency. Curcumin loaded liposome in lysine-collagen hydrogel embedded with valsartan demonstrated the peak wound contraction and was effective in promoting wound healing as it contained curcumin, collagen, lysine, and valsartan. The synergistic effect of these components had a clearly pronounced effect on angiogenesis at the wound site. Therefor this formulation can serve as an archetype for subsequent development as it portrays excellent therapeutic capabilities as a formulation for smart wound dressing for the management of diabetic chronic wounds.

STATEMENT OF ETHICS

Ethics approval with an ethical approval number CMUL/ACUREC/08/21/923 obtained and approved for animal studies via the Health Research Ethics Committee of College of Medicine, University of Lagos.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflicting interests.

AUTHOR CONTRIBUTIONS

Cardoso-Daodu Ibilola and Ilomuanya Margaret: Conceptualization, Methodology, Software. Cardoso-Daodu Ibilola and Azubuike Chukwuemeka: Data Curation, Writing - Original Draft Preparation. Cardoso-Daodu Ibilola: Visualization, Investigation. Azubuike Chukwuemeka and Ilomuanya Margaret: Supervision. Cardoso-Daodu Ibilola: Validation. Cardoso-Daodu Ibilola, Azubuike Chukwuemeka and Ilomuanya Margaret: Writing - Reviewing and Editing.

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Study the effect of thyroid disorder on Paraoxygenase -1, Malondialdehyde (MDA) and reduced Glutathione (GSH) in a sample of Iraqi patients

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ABSTRACT

Paraoxonase-1 (PON-1), it is an antioxidant enzyme that associate with high-density lipoproteins, plays a crucial role in mitigating lipid peroxidation. Three paraoxonase forms (PON-1, 2, and 3), with PON-1 synthesized in the liver and transported with HDLs in the plasma. This study aimed to estimate the levels of paraoxonase-1, malondialdehyde (MDA), and reduced glutathione (GSH) in the sera of patients with thyroid dysfunction that include 50 patients; 25 with hypothyroidism and 25 with hyperthyroidism which compared with 25 healthy controls in a study conducted between May and July 2023, utilized the enzyme-linked immunosorbent assay (ELISA) for PON-1, the thiobarbituric acid (TBA) method for MDA, and Ellman's method for GSH which revealed that there is a significant reduction in PON-1 activity and GHS levels in both hypothyroid and hyperthyroid groups compared to controls, with a significant increase in MDA levels in the hyperthyroid and hypothyroidism groups which indicates that thyroid dysfunction influences oxidative stress markers (MDA and GSH) and PON-1 activity, which can be used as a potential biomarker for early diagnosis or as a therapeutic target for monitoring thyroid dysfunction's clinical status.

Keywords: paraoxonase-1, malondialdehyde, lipid peroxidation, glutathione, thyroid

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INTRODUCTION

The average growth and development of bodily organs rely on the presence of thyroid hormones produced by the thyroid gland. However, when the thyroid becomes excessively active and releases excessive T3 and T4 hormones into the bloodstream, a condition called thyrotoxicosis occurs[1]. On the other hand, hypothyroidism is the opposite condition, where the thyroid fails to produce adequate amounts of hormones. Primary hypothyroidism is a thyroid dysfunction that occurs when the defect resides in the thyroid. In contrast, secondary hypothyroidism is caused by a defect outside the thyroid glands, related to an abnormality in the hypothalamic-pituitary-thyroid axis[2]. Moreover, iodine deficiency resulting from ingesting an inadequate amount of iodine in the diet can also cause hypothyroidism, which affects the production of the thyroid hormone. Hypothyroidism can be also caused by certain medications such as lithium. Globally, the deficiency of iodine can be considered the most common cause of hypothyroidism[3]. Previous studies have demonstrated significant variations in T3, T4, and TSH levels during different thyroid states with different oxidative stress markers' levels, indicating an assumed relationship between thyroxin levels and oxidative stress[4,5]. Several animal studies have shown increased lipid peroxidation and malondialdehyde (MDA) in patients with overt hypothyroidism[6,7].

Additionally, dyslipidemia was consistently linked with the peroxidation of lipids in hypothyroidism[8, 9]. Other studies reported that protein carbonylation (PCO) was identified as a measure of oxidative damage to proteins in patients with overt hypothyroidism[10,11]. lipid peroxidation considered as a catalyst for the creation of protein carbonyls which may prove the link between the oxidative stress and thyroid dysfunction[12,13]. This process is intensified by elevated levels of thyroid-stimulating hormone (TSH), which triggers oxidative stress in cases of hypothyroidism[5,14]. However, there is a lack of comprehensive research regarding oxidative stress in cases of subclinical hypothyroidism, with conflicting reports on whether or not there is a change in oxidative stress and lipid peroxidation compared to control groups[8,15]. Interestingly, there is also a shortage of information regarding the levels of protein carbonyls in the subclinical hypothyroidism group. Recent interest in studying oxidative stress in thyroid disorders has emphasized the role of thyroid hormones in regulating antioxidant activity[16].

Reduced glutathione (GSH) has a crucial function in scavenging free radicals and inhibiting lipid peroxidation, as it acts as a detoxifier for hydrogen peroxide (H_2O_2) through the enzyme glutathione peroxidase (GPx)[17]. Superoxide radicals

undergo dismutation by the superoxide dismutase enzyme that also serves as an antioxidant enzyme due to its role in scavenging the superoxide radicals[18]. In hypothyroidism, the GSH level has been inadequately reported[5,18]. A few controversial findings reported different results in that some of the studies demonstrated an elevation in the activity of SOD, CAT7, and GPx [5,19]. In contrast, other studies reported a decline in the activities of SOD, CAT, and GPx enzymes[16,20]. In contrast, no alteration was reported in the activities of SOD, CAT7, or GPx21 in overt hypothyroidism patients. To our best knowledge, GSH evaluation and antioxidant enzymes in subclinical hypothyroidism patients have hardly been reported. In most research, the obtainable literature on oxidative stress in hypothyroidism, contrasting with most other studies, focuses on overt hypothyroidism patients. Therefore, the present study aimed to evaluate the markers of lipid peroxidation and antioxidant defense code by measuring MDA, GSH, and enzymes such as PON1 in the patients' group compared to controls.

METHODOLOGY

Subjects

This study comprised Fifty patients with thyroid disorders (25 patients with hypothyroidism and 25 with hyperthyroidism) who recruited from an endocrine clinic in Al-Imamain Al-Kadhimain Medical City from May-July 2023. The patient group were compared with 25 age and sex matched healthy subjects who represent the control group.

Inclusion criteria

Patients with multinodular enlargement of the thyroid gland upon examination with a history of exaggeration of previous symptoms like weight loss, fatigue, and difficulty in swallowing.

Exclusion criteria

Patients who are currently undergoing treatment for autoimmune disorders, pregnant women, and individuals with conditions such as diabetes mellitus, rheumatoid arthritis, renal impairment, and liver diseases are not eligible for participation in the present study.

Blood sampling

Patients and control participants were subjected to blood sample collection, where 7 milliliters of blood were obtained using serum separator. The sera were isolated and promptly stored at a temperature of -20 °C until they were analyzed.

Paraoxygenase -1 activity measurement

The activity of paraoxygenase -1 was determined quantitively by using an ELISA kit supplied by AVISCERA BIOSCIENCE INC and the procedure performed according to the manufacturer instructions.

Thyroid function test

Enzyme immunoassay and colorimetry (Accu-Bind VAST KITS) were used to quantify T3, T4, and TSH levels. Each sample was tested twice, with measurements showing a difference of less than 10%. The average value was then determined and utilised for statistical analysis.

Serum MDA measurement

The levels of MDA in the serum were evaluated using the thiobarbituric acid (TBA) method developed by Buege and Aust[22]. Malondialdehyde, which is produced by the degradation of polyunsaturated fatty acids, functions as a valuable indicator of peroxidation reactions. The Buege and Aust (1978) method utilizing thiobarbituric acid to quantify MDA levels, as it undergoes a reaction with thiobarbituric acid resulting in the formation of a pink color. Measurements of absorbance were conducted at a wavelength of 532 nm.

Estimation of reduced glutathione

The concentration of reduced glutathione (GSH) was measured using Ellman's technique, as described by Ellman in 1959[23]. To do this experiment, 1.0 ml of plasma was combined with 0.5 ml of Ellman's reagent, which contains 19.8 mg of 5.5-dithiobisnitrobenzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate. Additionally, 3.0 ml of phosphate buffer with a concentration of 0.2 M and a pH of 8.0 was added. Afterwards, the intensity of light absorption was determined at a wavelength of 412 nm.

Statistical analysis

The data obtained in the current study were analyzed statistically by using the Statistical Package for the Social Sciences (SPSS) software 26. Continues variables were expressed as mean ± standard deviation (SD) and all statistical comparisons were made by student t-test and Analysis of variance (ANOVA) test with the using of post-hoc Tukey test to assess the difference between each two groups within more than two groups with $P \leq 0.05$ was considered statistically significant.

RESULTS and DISCUSSION

The results of this study showed decreases in the level of paraoxygenase -1 activity in hypothyroidism compared with hyperthyroidism and healthy controls, and statistical analysis showed a significant difference ($p<0.01$) between whole patients and the control group, as shown in Table 1 and Figure 1. Moreover, in a similar manner to that obtained with PON-1, GSH levels in patients with hypothyroidism were significantly lower than that in patients with hyperthyroidism and also than that in controls, it also demonstrated that the levels of GSH in whole patients were significantly lower than that in controls. On the other hand, the results obtained in the current work elucidate that the patients with hyperthyroidism and hypothyroidism had significantly higher levels of MDA than the healthy controls, as shown in Table 1 and Figures 1&2.

Table 1. The anthropometric and biochemical variables among the three studied groups

Parameters	Control	Hypothyroidism	Hyperthyroidism	P(ANOVA)-(T-Test)
NO.	25	25	25	.....
Age (yrs) (Mean± SD)	41.35 ± 7.08	43.89 ± 6.38	42.87 ± 7.66	NS
BMI (Kg/m²) (Mean± SD)	29.81 ± 6.55	26.86 ± 5.17	24.88 ± 4.37	Patients x Controls: P ≤ 0.01 hyper x hypo: p ≤ 0. 05
PON1 (U/ml) (Mean± SD)	378.93 ± 39.33	179.84 ± 18.53	269.38 ± 17.88	Patients x Controls: P ≤ 0.001 hyper x hypo: p ≤ 0. 05
MDA (µmol/l) (Mean± SD)	0.57 ± 0. 35	1.29 ± 0.83	1.18 ± 0.66	Patients x Controls: P ≤ 0.001 hyper x hypo: p ≤ 0. 05
GSH (mmol/l) (Mean± SD)	3.88 ± 0.17	1.37 ± 0.13	2.19 ± 0.31	Patients x Controls: P ≤ 0.001 hyper x hypo: p ≤ 0. 05
T3 (ng/ml) (Mean± SD)	1.18 ± 0.07	0.29 ± 0.03	3.58 ± 0.17	Patients x Controls: P ≤ 0.01 hyper x hypo: p ≤ 0. 05
T4(ng/ml) (Mean± SD)	8.08 ± 0.57	3.88 ± 0.11	11.99 ± 1.07	Patients x Controls: P ≤ 0.01 hyper x hypo: p ≤ 0. 05
TSH (ng/ml) (Mean± SD)	4.18 ± 0.38	15.72 ± 1.31	0.27 ± 0.09	Patients x Controls: P ≤ 0.01 hyper x hypo: p ≤ 0. 05

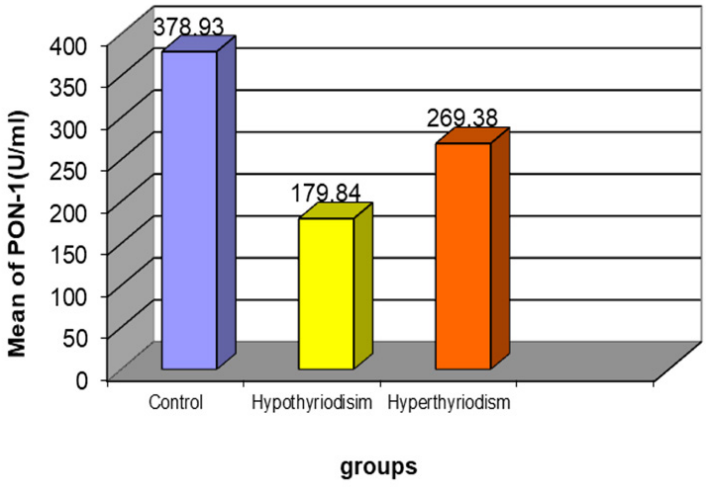

Figure 1. Levels of PON-1 in thyroid dysfunction patients in comparison with controls

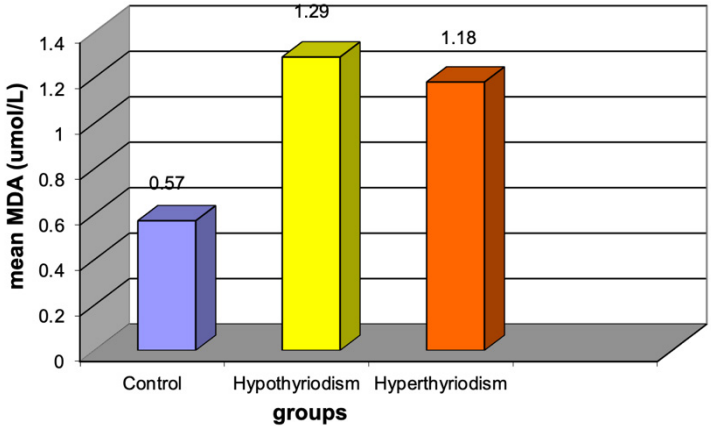

Figure 2. Levels of MDA in thyroid dysfunction patients in comparison with controls

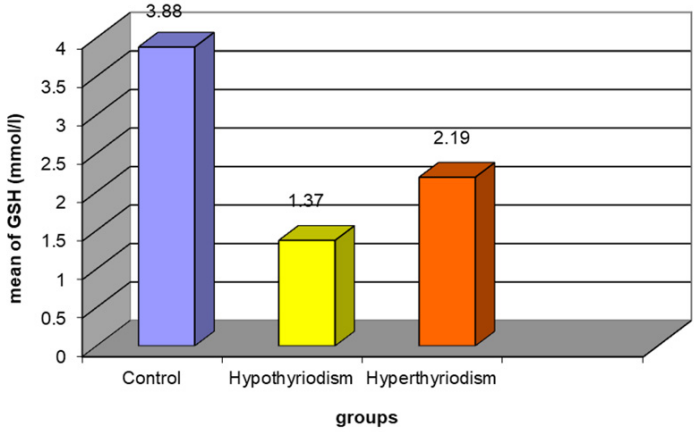

Figure 3. Levels of GSH in thyroid dysfunction patients in comparison with controls

The regulation of antioxidants by thyroid hormones is a crucial aspect, as these hormones have been found to promote the generation of free radicals within mitochondria[24]. Additionally, research indicates that thyroid hormones can exhibit a prooxidant effect on specific cells[25]. Notably, hyperthyroidism has been associated with increased oxidative stress and subsequent damage to lipids and genomic DNA in the aortic wall[26]. In the present study, patients diagnosed with hyperthyroidism or hypothyroidism showed a decrease in serum paraoxonase-1 (PON-1) activity. Existing literature on PON-1 activity in hypothyroidism has shown conflicting results[27-30]. Some studies reported decreased PON-1 activity and increased oxidative stress in patients with subclinical hypothyroidism[27,29], which aligns with our findings. However, the differences between our healthy control group and hypothyroidism patients were significantly higher than in previous studies[27,29], indicating that PON-1 activity could be a potential biomarker for assessing cardiovascular risk in subclinical hypothyroidism. It's worth noting that other studies found no difference in PON-1 activity between control groups and hypothyroidism patients[28,30], possibly due to genetic variations. Similarly, we observed a notable decrease in PON-1 activity among hyperthyroid patients compared to the control group. This finding is consistent with research by Azizi et al. who also reported a significant reduction in PON-1 activity in both hyperthyroid and hypothyroid patients[32]. Another study showed that PON-1 activity decreases in hyperthyroid patients but returns to normal levels after achieving euthyroidism[33,34]. Moreover, the decrease in PON-1 activity in hyperthyroidism may be due to the heightened generation of free oxygen radicals, potentially leading to oxidative damage[31].

The current investigation also focused on measuring serum MDA levels as an indicator of lipid peroxidation. Both hyperthyroidism and hypothyroidism patients exhibited significantly higher blood MDA levels compared to healthy individuals ($p<0.001$). However, hyperthyroidism patients had lower serum MDA levels compared to hypothyroidism patients ($p<0.05$), though still significantly higher than the control group. These findings align with other research that has demonstrated increased lipid peroxidation products in hyperthyroid tissues and increased MDA concentrations in patients with hypothyroidism, indicating oxidative damage[35-37]. Research conducted by Sewerynek et al. has demonstrated that the ratio of CD/MDA is lower in hyperthyroid patients compared to the control group[38] and another research findings suggest a significant increase in lipid peroxidation during hyperthyroidism[39].

In terms of glutathione (GSH), both hyperthyroidism and hypothyroidism patients showed a significant decrease in GSH levels compared to the control group. This decrease in GSH may be attributed to the accumulation of free radicals caused by oxidative stress associated with hyperthyroidism and the changes or modifications caused by both hyperthyroidism and hypothyroidism[40]. Previous research has shown that hyperthyroidism, with its high metabolism, leads to increased production of free radicals and oxidized fats. The differences in antioxidant levels between the study groups could also be related to body mass index, indicating lower antioxidant levels in both hyperthyroidism and hypothyroidism patients compared to the control group[41]. These results were in agreement with the results obtained in the current work. The reason is related to the hormones in the gland, oxidation of the thyroid gland's physiological state, as well as the dose and length of thyroid treatment for stress, doesn't work by speeding up the metabolism; instead, it has an effect based on how oxidation works[42]. The reason lies in the state of the gland hormone and the fact that there are a number of oxidative stress effects on the physiological state of the thyroid gland, in addition to the dose and duration of thyroid treatment for stress, not through stimulation of the metabolism process, but whose effect depends on the mechanics of oxidation. The study showed that toxic multinodular goitres with hyperthyroidism had higher levels of oxidative stress markers and increased activities of SOD and CAT, whereas their plasma GPx and GR activities were lower compared to the control group[43].

The results of the present work revealed that there was a significant elevation in the levels of MDA with a significant decrease in GSH levels and PON-1 activity in both hyperthyroidism and hypothyroidism patients compared to healthy individuals. This underscores the importance of considering effective antioxidant therapy, particularly augmenting PON-1 activity, as a potential therapeutic approach for individuals with hyperthyroidism and hypothyroidism.

STATEMENT OF ETHICS

The study received approval from the Scientific-Ethical Committee College of Science, Mustansiriyah University in November 2023, Number 7134.

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

Design – Hamidy AA, Abdul Jabbar NA, Shnawer NJ; Acquisition of data – Hamidy AA; Analysis of data – Abdul Jabbar NA, Shnawer NJ; Drafting of the manuscript – Hamidy AA, Abdul Jabbar NA, Shnawer NJ; Critical revision of the manuscript – Hamidy AA, Abdul Jabbar NA; Statistical analysis – Hamidy AA; Technical or financial support – Hamidy AA, Abdul Jabbar NA, Shnawer NJ; Supervision – Abdul Jabbar NA, Shnawer NJ.

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The use of risk score test of American Diabetes Association in the prediction of diabetes risk in Iraq

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ABSTRACT

The objective of this study was to evaluate the risk of developing diabetes mellitus (DM) in a sample of 900 non-diabetic volunteers who were randomly selected from various cities and towns in Baghdad and Babylon Governorates. These volunteers underwent a risk assessment test developed by the American Diabetic Association (ADA). The researchers completed the test forms based on the subjects' responses to assess the likelihood of developing type 2 diabetes mellitus. The findings indicated that approximately 20.56% of all participants in the study exhibited a high risk of developing DM. Additionally, the results demonstrated a strong and statistically significant inverse relationship between age and the risk of DM. The findings also indicated a higher prevalence of high-risk scores in males compared to females. However, women with a previous diagnosis of gestational diabetes have an elevated risk of developing diabetes. Additionally, the study demonstrated a significant correlation between the occurrence of hypertension and a family history of diabetes mellitus with the risk of developing diabetes. Physical activity shown a substantial correlation with the risk of DM, as seen by the significant association between weight status and DM risk. This suggests that all the aforementioned factors influence the risk of DM.

Keywords: American Diabetes Association, diabetes mellitus, risk score test

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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a diverse disease that encompasses intricate metabolic, behavioral, and genetic elements. Prospective research has enhanced the comprehension of modifiable risk factors associated with T2DM. Nevertheless, the way individuals respond to behavioral and lifestyle risk factors differs, possibly due to several factors such as variations in physiology, adherence to interventions, and the potential for intricate gene-environment interactions that are not fully comprehended[1]. The prevalence of diabetes has significantly increased in both developing and wealthy countries over the past few decades, making it a major global health concern[2]. Around 463 million persons between the ages of 20 and 79 are affected by diabetes[3]. This number is projected to increase to 700 million by the year 2045. Hence, there is an urgent requirement for an evaluation instrument that can assist healthcare practitioners in monitoring the easily identifiable risk factors for diabetes in a convenient, effective, and non-intrusive manner. This can be achieved by utilizing data gathered during routine medical visits, including details such as gender, age, body mass index (BMI), family medical history, smoking history, and the usage of steroidal and anti-hypertensive medications[4]. Utilizing a generic risk calculator for population-wide diabetes screening is preferable to employing intrusive tests such as blood glucose or HbA1c[5].

The diabetes risk test, created by the American Diabetes Association (ADA), serves as a screening tool to accurately identify persons in the community who are at a heightened risk for developing diabetes. The primary objective is to enhance consciousness of alterable risk factors and advocate for a health-conscious way of living. The ADA diabetes risk test is scored based on seven questions that cover gender, age, gestational diabetes mellitus (GDM), family history of diabetes, blood pressure, physical activity, and obesity (measured using BMI and a weight-height chart). The total score ranges from 0 to 11. Individuals who obtain scores of 5 or above are categorized as being at a heightened risk of developing diabetes. Studies have shown that the probability of acquiring diabetes increases in direct relation to the progression of age[6]. The occurrence of diabetes is more prevalent among older individuals due to the simultaneous rise in insulin resistance associated with obesity and sedentary lifestyle[7]. Prior research has also indicated that males have a greater susceptibility to developing diabetes compared to females, as evidenced by a higher incidence rate among men under the age of 55 for both heart disease and diabetes[8].

Studies have shown that women with a history of GDM are at a higher risk of developing diabetes and cardiovascular diseases in the future than those with

a normal blood sugar level during pregnancy. In fact, they have a risk of developing T2DM that is more than seven times higher. Multiple population-based studies have established a strong correlation between a family history of diabetes and the likelihood of developing diabetes. The most prominent indicators of diabetes are being overweight or obese. Research undertaken in several nations has revealed a more robust correlation between anthropometric indicators and the incidence of T2DM. Significantly, individuals with hypertension (HTN) were found to have a 50% higher likelihood of acquiring T2DM [3].

METHODOLOGY

In this screening study 900 person (584 females and 316 males) were subjected to a risk test designed by ADA as illustrated the Figure 1. The subjects recruited randomly from several cities and towns in Baghdad and Babylon Governorates and the forms were filled by the researchers according to the subjects' answers to estimate the risk of T2DM. Age of subjects ranged from 22 - 68 years old and categorized according to gender, age, family history, health status, physical activity and weight status. According to the risk test established by the ADA, the summation of ≥ 5 means that the subject is at a high risk to get T2DM.

ADA diabetes risk test

The ADA diabetes risk test (Figure 1) is a risk assessment tool that utilizes seven parameters to estimate the likelihood of developing diabetes. These criteria include gender, age, BMI, physical activity level, history of GDM, family history of diabetes, history of HTN. Participants were obligated to respond affirmatively or negatively to all inquiries, with the exception of BMI and age which are scored with range of 0 to 3. The scoring system for age is as follows: individuals aged 39 years or below are assigned a score of 0, those aged 40-49 are assigned a score of 1, those aged 50-59 are assigned a score of 2, and those aged 60 years or above are assigned a score of 3. Regarding BMI, a score of 0 was assigned to individuals with a normal or underweight status (BMI ≤ 25), a score of 1 to those who were overweight (BMI > 25), a score of 2 to individuals classified as obese class I (BMI > 30), and a score of 3 to those classified as obese class II (BMI > 35). The scoring system assigned a value of 1 to males and 0 to females for gender. A score of 1 was assigned to a negative answer, while a score of 0 was assigned to a positive response for the physical activity level. Regarding the remaining parameters, a negative reaction was assigned a score of 0, while a positive response was assigned a score of 1. The overall ADA diabetes risk test score was calculated by adding together the scores from all seven questions. Individuals who had a score of five or higher were classified as being at a high risk of getting diabetes[3].

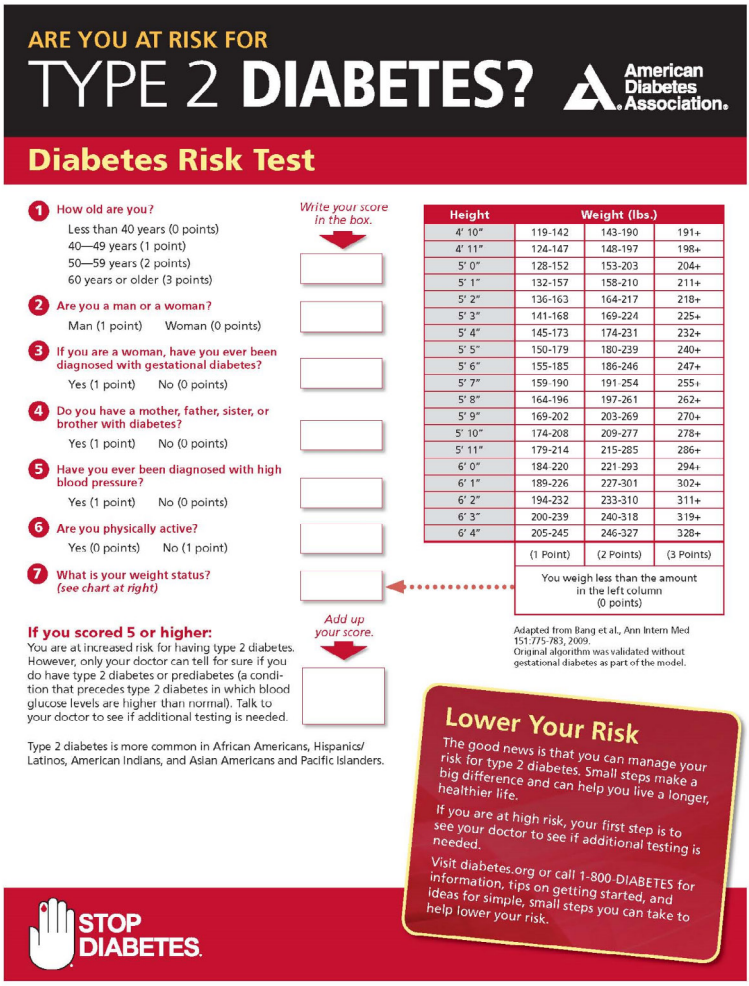

Figure 1. ADA diabetes risk test form[3]

Statistical analysis

The study's data were analyzed utilizing the SPSS program version 20. The categorical variables were quantified and examined by cross tabulation to determine the frequency and percentage of each variable within the groups being researched. Chi square (Chi^2) test was conducted to examine the correlation between all parameters. A significance level of $P \leq 0.05$ was used to determine statistical significance. The strength of the association was measured using Phi, which is a chi square-based measure. Values ranging from 0 to 0.5 were considered indicative of weak association, while values above 0.5 were considered indicative of strong association.

RESULTS and DISCUSSION

Results obtained in the current study revealed that 185 subjects out of 900 volunteers subjected to this study showed a high risk of getting DM which is representing about 20.56% of all subjects. This finding is nearly consistent with previous studies conducted in Iraq which showed that Approximately 1.4 million Iraqis are afflicted with diabetes. The reported prevalence of T2DM in Iraq varies from 8.5% (adjusted for age according to the International Diabetes Federation) and 13.9%[9]. A recent study conducted in Basrah, Southern Iraq, including around 5400 participants. The study found that the age-adjusted prevalence of diabetes in individuals aged 19 to 94 years was 19.7%[9,10].

Results illustrated in Table 1 showed that risk of DM increased with age and the results also revealed a highly significant negative correlation between the age and the risk of DM ($p<0.001$) which is in agreement with previous studies which demonstrated that the risk of diabetes increases with age[6,11]. Diabetes is more prevalent in elderly individuals due to the simultaneous increment in insulin resistance which is associated with obesity and a lifestyle which is devoid of physical activity[7].

Table 1. Cross tabulation of age versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Age (year)	Less than 40	No. of cases	55	614	669
		%	8.22%	91.78%	100.0%
	40-49	No. of cases	93	87	180
		%	51.67%	48.33%	100.0%
	50-59	No. of cases	26	14	40
		%	65%	35%	100.0%
	60 and more	No. of cases	11	0	11
		%	100.0%	0.0%	100.0%
Total		Count	185	715	900
		% Within Age	20.56%	79.44%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.554		

Results illustrated in Table 2 showed that the gender was correlated weakly (Phi = 0.096) but significantly with the risk of DM (p=0.042) in that the percentage of male subjects with high risk (27.85%) was more than the percentage of high risk female subjects (17.47%) which is compatible with the previous results which reported that Men exhibit a higher susceptibility to developing diabetes compared to women[12,13].

Table 2. Cross tabulation of gender versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Gender	Female	No. of cases	102	482	584
		%	17.47%	82.53%	100.0%
	Male	No. of cases	88	228	316
		%	27.85%	73.15%	100.0%
Total		Count	184	716	900
		% Within Gender	20.44%	79.56%	100.0%
Chi^2		p-value	0.042		
		Phi	0.096		

Table 3 showed that 54.8% of female subjects were strongly (0.627) and significantly (p<0.001) associated with previous GDM were at high risk for DM whereas only 11.6% of females who didn't experience GDM were at high risk of DM. Studies have shown that women with a history of GDM are at a higher risk of developing diabetes and cardiovascular diseases in the future than those with a normal blood sugar level during pregnancy. The risk of developing T2DM is more than seven times higher for these women[14,15].

Table 3. Cross tabulation of previous history of GDM versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Previous GDM	No	No. Of cases	94	654	748
		%	12.57%	87.43%	100.0%
	Yes	No. Of cases	85	67	152
		%	55.92%	44.08%	100.0%
Total		Count	179	721	900
		% Within Previous GDM	19.89%	80.11%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.627		

Data listed in Table 4 showed a weak (Phi = 0.177) but significant ($p<0.001$) correlation between the family history of DM and the risk of DM which is owned to that 26.5% of subjects with a family history showed a high risk of DM whereas only 11.8% of subjects without family history were at high risk of DM and this result aligns with multiple population-based studies that have indicated a strong correlation between a family history of diabetes and the onset of diabetes[16,17].

Table 4. Cross tabulation of family history of DM versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Family History	No	No. Of cases	21	157	178
		%	11.8%	88.2%	100.0%
	Yes	No. Of cases	72	200	272
		%	26.5%	73.5%	100.0%
Total		Count	183	717	900
		% Within Family History	20.7%	79.3%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.177		

Another factor that may be affect the risk of DM is the HTN and the results of the current research showed that 47.5% of subjects with HTN are at high risk of DM whereas only 12.9% of normotensive subjects were at high risk of DM which is obviated by the significant correlation between the incidence of HTN and the risk of DM (Table 5) which is compatible with the previous epidemiologic studies which demonstrated that the prevalence of HTN in individuals with diabetes is nearly twice as high as in those without diabetes that prove the strong correlation between DM an HTN[18,19,20].

Table 5. Cross tabulation of HTN incidence versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
HTN	No	No. of cases	45	304	349
		%	12.9%	87.1%	100.0%
	Yes	No. of cases	48	53	101
		%	47.5%	52.5%	100.0%
Total		Count	No. Of cases	357	450
		% Within HTN	20.7%	79.3%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.357		

Table 6 revealed that the physical activity can be considered as a protective factor against DM that reduce its incidence in a population which is clarified by the significant correlation between physical activity and the risk of DM in that only 8.5% of physically active subjects showed a high DM risk while 30.4% of physically inactive subjects faced a high risk of getting T2DM. Studies reported that the sedentary behaviors with low physical activity are a risk factor for T2DM which defined as insufficient physical activity to meet the World Health Organization 2010 recommendations[21], that reported to be is responsible for 7% of the burden of T2DM[22].

Table 6. Cross tabulation of physical activity versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Physical Activity	Yes	No. Of cases	17	183	200
		%	8.5%	91.5%	100.0%
	No	No. Of cases	76	174	250
		%	30.4%	69.6%	100.0%
Total		Count	93	357	450
		% Within Physical Activity	20.7%	79.3%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.269		

Finally, the data obtained in the present research revealed that the weight status was significantly associated with the risk of DM in that the ratio of subjects with high risk for DM increased with the increase in the weight status to reach 85.7% in subject that showed weight status of 3 as illustrated in Table 7. Many researches demonstrated that the obesity is considered as one of the major risk factors for DM. The risk of developing diabetes due to excessive body fat, as assessed by BMI or anthropometric indicators like skinfold thickness or waist circumference, increases in a continual manner. The clinical risk categories for BMI, which include normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obesity (≥ 30 kg/m^2), are linked to a gradual rise in the chance of developing diabetes[23]. Individuals who are overweight or obese have a higher susceptibility to developing T2DM, particularly if they carry excess weight in their abdominal region. Abdominal obesity triggers the release of 'proinflammatory' substances by fat cells, which can reduce the body's sensitivity to insulin. This interference affects the function of insulin-responsive cells and their capacity to properly respond to insulin. This condition is known as insulin resistance[24].

Table 7. Cross tabulation of weight status versus the risk of DM

			Risk of DM		Total
			At High Risk	At low risk	
Weight status	0.00	No. Of cases	1	98	99
		%	1.0%	99.0%	100.0%
	1.00	No. Of cases	26	212	238
		%	10.9%	89.1%	100.0%
	2.00	No. Of cases	60	46	106
		%	56.6%	43.4%	100.0%
	3.00	No. Of cases	6	1	7
		%	85.7%	14.3%	100.0%
Total		Count	93	357	450
		% Within Weight status	20.7%	79.3%	100.0%
Chi^2		p-value	<0.001		
		Phi	0.555		

According to risk test designed by American Diabetic Association (ADA), there is significant correlations between the age, gender, family history, GDM, HTN, physical activity and weight status with the risk of DM.

STATEMENT OF ETHICS

The study was approved by the "Scientific Committee at the Baghdad College of Medical Sciences" (Approval number: 8S, date: 15th November 2023).

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Design – Alrubaye YSJ, Albadri HMB, Yousif OA; Acquisition of data – Alrubaye YSJ, Albadri HMB; Analysis of data – Albadri HMB, Yousif OA; Drafting of the manuscript – Alrubaye YSJ; Critical revision of the manuscript – Albadri HMB, Yousif OA; Statistical analysis – Albadri HMB; Technical or financial support – Yousif OA; Supervision – Alrubaye YSJ, Albadri HMB, Yousif OA.

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In vivo antidiarrheal and *in vitro* antimicrobial activities of the aerial part extracts of *Waronia saharae* Benthem ex Benth. & Coss.

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ABSTRACT

A North African endemic plant known as *Warionia saharae* Benth. & Coss is frequently used in Morocco for treating digestive problems. In this work, the possible *in vivo* antidiarrheal effect of the aqueous extract of aerial part of this plant was evaluated to confirm its traditional use, also, the organic fractions were tested *in vitro* on antimicrobial activity. The results obtained from the *in vivo* study of the antidiarrheal activity of the aqueous extract showed significant inhibition against the diarrheal effect induced by castor oil with 75.07% at a 400 mg/kg dose. Additionally, this extract significantly inhibited the amount of fluid that accumulated in the intestinal lumen with 38.45% at 400 mg/kg, as well as the intestinal transit of activated charcoal. The antibacterial activities were tested for the aqueous extract and four organic fractions against five

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strains for the Gram-negative and the Gram-positive bacteria. Also, these extracts were tested for antifungal activity. The results of the study reveal that the aqueous extract and the dichloromethane fraction demonstrated the greatest levels of activity against the tested bacteria. This work provides a possible strategy for treating bacterial infections, which can be used to take advantage of this antimicrobial activities promising in the treatment of diarrhea caused primarily by these bacteria.

Keywords: *Warionia saharae,* antidiarrheal effect, antibacterial effect, antifungal effect

INTRODUCTION

Diarrheal diseases are the fourth leading cause of death among children under five, accounting for more than one million deaths per year globally[1].

Pharmacologic agents used to treat diarrhea includes several categories. Anti-inflammatory agents, such as glucocorticoids, salicylates, and indomethacin, aim to reduce inflammation. Organic anions, including gallic acid (tannin), d-galacturonic acid (pectin), and nicotinic acid. Neuroactive drugs, such as catecholamines, somatostatin, propranolol, phenothiazines, local anesthetics, and opiates, including derivatives like synthetic opiates (e.g., diphenoxylate and loperamide) and enkephalins, also contribute to managing diarrhea[2], in addition, diarrhea is a very common symptom of gastrointestinal infections, which can be caused by a huge variety of pathogens, with microorganisms accounting for the majority of the causes. Humans with fungus infections have also been known to develop diarrhea. However, many antibiotics are developed to treat diarrhea due to microbial infections.

Several drugs on the above list have posed toxicity risks and undesirable effects, and the misuse of antibiotics is also the root cause of the emergence of multidrug-resistant bacteria or superbugs. To stop this process of developing toxicity or resistance, it is essential to explore natural alternative approaches to reduce or eliminate it without relying on synthetic products.

Medicine plants are an alternative in primary care systems, and as a result, a promising pathway for drug development has historically improved. *Cotula cinerea* Del.[3], *Artemisia campestris* L. subsp. *glutinosa*[4], *Dissotis thollonii* Cogn.[5], *Zygophyllum gaetulum* Emberger[6], and *Calpurnia aurea*[7] are examples of plants herbs used in tradionnal medicine for treating diarrhea. In this study, we came to choose *Warionia saharae* Benthem ex Benth. & Coss., a

plant species mentioned in the world of traditional pharmacopeia as a treatment for digestive problems[8].

W. saharae is a North African endemic shrub. This plant is native to Morocco and Algeria, and it is the only species in the genus *Warionia* (Asteraceae)[9]. The leaves infusion of *W. saharae* are used in Moroccan pharmacopeia for their gastrointestinal properties, inflammatory diseases, and epilepsy problems[8]. Previous studies on specific parts of this plant have shown their immense strength as an antioxidant[10], anti-inflammatory, and cytotoxic properties against the cancer cell line "KB cells"[11,12].

According to the literature, these biological effects are mainly due to the presence in the *W. saharae* plant of several varieties of substances called secondary metabolites belonging to several classes such as phenolic compounds (flavonoids, chlorogenic acids), terpenes, and alkaloids, depending on the extract used; the hydrometanolic extract contains hydroxycinnamic acid derivatives and flavonoids[13], The chloroform extract contains the β-sitosterol as a major component, The ethyl acetate extract contains also the esculetin and cirsimaritin[14], Hilmi in 2002 reported the first-time isolation of 12 sesquiterpene lactones (SLs) of the guaianolide type from this plant. These SLs include dehydroleucodin, reynosin, 1,2-didehydro-3-oxo-costic acid, along with the flavonoid hispidulin[15], the essential oil contains in increasing order, nerolidyl acetate (21.44%), β-eudesmol (19.47%), and linalool (16.48%), which account for 57.39% of the total composition[16]. The primary goal of this study was to learn for the first time about the antidiarrheal and antimicrobial properties of *W. saharae* from southern Morocco.

METHODOLOGY

Plant material

W. saharae aerial parts were collected from the southeastern region of Morocco (Errachidia: 31°55′53 N′′, 4°25′35′′ W) on November 2020, identified by Professor Mostafa Elachouri and a specimen was registered in the herbarium of the Faculty of Sciences, Mohammed First University Oujda, Morocco, with the number UHPOM 450. Before the extraction process, the dried plant material is stored in a laboratory at 25° C in a dry and dark environment.

Preparation of the aqueous extract and the organic fractions

A quantity of 28 g of *W. saharae* aerial parts was infused in boiled distilled water (100 mL) for 1 hour, the aqueous extract (AEWS) was filtered, dried using a rotary evaporator (Buchi B-480, Switzerland), and then kept in a freezer until needed (-20°C). The various fractions of *W. saharae* were obtained following filho method[17] using a Soxhlet extractor, which dissolves the chemical compounds contained in a solid powder (100 mg) into a liquid or organic solvent (500 mL); the extraction takes place over a series of 6 h of cycles. The different solvents used have an increasing order of polarity, starting with hexane, followed by dichloromethane, ethyl acetate, and ending with methanol. The following formula employed to determine the extraction yield is:

$$\textbf{Extraction Yield}\ (\%) = \left(\frac{\text{Weight of extracted compounds}}{\text{Weight of dry plant material}}\right) \text{x } 100$$

Antidiarrheal effect of the aqueous extract

Animals

Albino mice weighing 25 to 30 g of both sexes were used in these experiments of antidiarrheal effect. They were placed under standard conditions in the animal house of the Faculty of Sciences, Oujda, Morocco, with free access to drinking water ad libitum. Maintained at controlled lighting (12h - 12h light-darkness cycle), humidity, and temperature. All the animals were fasted 18 hours before the day of the experiment with free access to the water. All animals were treated following the US National Institutes of Health's Guide for the Care and Use of Experimental Animals[18].

Castor oil-induced diarrhea

The protocol of Degu et al.,[19] was applied. Five groups of six mice each were treated according to the following repartition: Negative control: a group that received distilled water orally (1 mL/100g of body weight). The Positive control group received the loperamide hydrochloride (10 mg/kg) and the other groups received 50, 200, and 400 mg/kg of AEWS. For all groups, castor oil (0.5 mL) was administered 1 h after the first treatment, and finally, each animal was individually placed in cages with the ground coated with transparent paper and changed every 30 min with 4 h of observation. The parameters studied were the time of onset of first diarrhea, the total number of wet feces for 4 h, the total number of solid feces for 4 h, the total number of defecations for 4 h, and the number of mice with wet feces.

The percentage (%) of diarrhea inhibition is calculated as follows:

$$\% \text{ of diarrhea inhibition} = \left[\frac{(\text{WFC} - \text{WFT})}{\text{WFC}}\right] \times 100$$

WFC: average of wet feces in the negative control group.

WFT: average of wet feces in the treated group.

Small intestinal transit study

The protocol of Karim et al.[20] was applied. The following four groups of six mice each were randomly to perform the intestinal transit, treatment is carried out orally by gavage for all groups. A group that was given distilled water (1 mL/100g, body weight) served as the negative control. A group that received Loperamide hydrochloride (10 mg/kg) served as the positive control. The AEWS doses for the other groups were 200 and 400 mg/kg.

Fifteen min after this treatment, the mice in each group received 0.2 mL of an activated charcoal solution (3%) suspended in 0.5% methyl cellulose. After 30 min, all the mice were sacrificed by cervical dislocation. The abdominal cavity was opened, and the entire intestine was rapidly and carefully removed from the beginning from the duodenum to the end of the ileum. The results were expressed as a percentage of the distance traveled by the activated charcoal over the total length of the intestine:

$$\% \text{ of IP} = \frac{\text{Distance of intestine traveled by the activated charcoal (cm)}}{\text{The whole length of the intestine(cm)}} \times 100$$

With IP: Percentage of the intestinal propulsion.

From this formula, we will calculate the percentage of inhibition of intestinal transit (IT):

$$\% \text{ of IT} = \frac{\% \text{ of IP (test)} - \% \text{ of IP (negative control)}}{\% \text{ of IP (negative control)}} \times 100$$

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This study was conducted on the same groups described previously; treatment is carried out orally by gavage for all groups. A group that was given distilled water (1 mL/100g, body weight) served as the negative control. A group that received Loperamide hydrochloride (10 mg/kg) served as the positive control. The AEWS doses for the other groups were 200 and 400 mg/kg. One hour after the treatment, all these animals received orally 0.2 mL of castor oil. After

30 min, the abdominal cavity was opened and two ligatures were performed, one at the level of the pylorus and the other at the level of the cecum. We took the entire intestine from these two nodes and weighed respectively with (W1) and without (W2) its intestinal liquid. We measured also the total length of intestine (L). The desired parameter in this study is the ability of the extract to inhibit intestinal secretions caused by the castor oil (Enteropooling)[21]. This parameter can be calculated from the following formula:

$$\text{Enteropooling} = \frac{\text{W1} - \text{W2}}{\text{L}} \times 100$$

Antimicrobial assay of the aqueous extract and organics fractions

Inoculums standardization

The antibacterial activities of the AEWS, hexane fraction (HFWS), dichloromethane fraction (DFWS), ethyl acetate fraction (EaFWS), and methanol fraction (MFWS) of *W. saharae* were examined against five strains: *Escherichia coli* (ATB:57) B6N; *Escherichia coli* (ATB:97) BGM; *Pseudomonas aeruginosa; Klebsiella pneumonia* for the Gram-negative bacteria and the strains: *Staphylococcus aureus* for the Gram-positive bacteria. These extracts were tested for their antifungal activity against *Candida albicans* ATCC10231 and *Saccharomyces cerevisiae* ATCC9763. These different strains were obtained from the Microbiology Laboratory, Faculty of Medicine and Pharmacy Fez, and Hassan II Hospital Fez, and were preserved in Muller-Hinton agar under refrigeration (4°C). The antibiotic Streptomycin and the antifungal: Fluconazole have been used as positive controls.

Preparation of the microbial suspension

The microbial inoculum was prepared by the direct suspension method from 2 to 3 colonies of a fresh culture aged 24 hours which were collected aseptically and suspended in 0.9% sterile physiological saline solution (NaCl), turbidity was adjusted to 0.5 McFarland[22t]. Bacterial suspensions contain approximately 1-2 x 10^8 CFU/mL, while the yeast suspension contains approximately 1-5 x 10^6 CFU/mL. The McFarland standard was prepared with a mixture of 99.5 mL of a sulfuric acid solution (H_2SO_4, 0.36 N) with 0.5 mL of a dehydrated barium chloride solution (0.048 M).

Disc diffusion method

The sensitivity was tested by disc diffusion method in accordance to the standard method by Bauer et al.[23]. Mueller Hinton agar plates were inoculated by the standardized suspensions. Whatman paper discs (6 mm) were placed on

the surface of preinoculated agar, which had been impregnated with 10 µL of the test compound extracts (1 mg/disc). All plates were incubated at 37° C for the bacteria and 30°C for the yeasts for 24 hours. After incubation, the growth inhibition zones were measured in mm. The test was repeated three times to ensure reliability[24].

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC was determined by the broth microdilution method following the guideline of the National Committee for Clinical and Laboratory Standards Institute[25]. Successive dilutions (dilution of factor ½ in each well) of the test compound (1 mg/mL) were prepared directly in a 96-well microplate containing a Muller Hinton (MH) used for bacteria or Yeast Peptone Glucose (YPG) used for yeasts to obtain various concentrations. The different fractions of *W. saharae* were diluted in 10% dimethyl sulfoxide (DMSO) in such a way, these later did not exceed 1% in the wells. On the other hand, the control positives and microbial suspensions were diluted in a culture medium. The microplate was incubated under agitation for 24 hours at 37°C for the bacteria and 30°C for the yeasts. To read the results, 20 µL of 2,3,5-triphenyltetrazolium chloride (TTC) (1%) purchased from the company BIOKAR, was added to all the wells, the wells containing bacterial growth became pink due to the activity of the dehydrogenases, while the well without bacterial growth remained color less after 2 h incubation[26].

Statistical analysis

The results are presented on the average plus or minus the standard error expressed SEM, the different tests were carried out using the GraphPad Prism software (5 Software, San Diego, CA, USA). To compare the different means with each other and with the control, Tukey's post-test was used. A difference is considered significant if the probability P is less than 5% with * $p \leq 0.05$; ** $p \leq 0.01$, ***: $p<0.001$.

RESULTS and DISCUSSION

Yield of extraction

For the aqueous extract, a yield of 8.10% was obtained from the extraction of the aerial parts of *W. saharae*. The extraction processes made by Soxhlet apparatus starting with hexane and finishing with methanol, made it possible to reap four organic fractions of *W. saharae*. A fraction received by way of hexane whose yield is 3.41%, a fragment acquired by means of exhaustion with dichloromethane whose yield is 5.12%, a fragment of ethyl acetate received with 2.38% of yield and in the end a methanolic fraction of 8.8%.

Antidiarrheal effect of the aqueous extract

Castor oil-induced diarrhea

The effect of the AEWS (50, 200, 400 mg/kg) on castor oil-induced diarrhea in mice caused an extension of latency time, reduced defecation frequency with 21.76%, 40.52% and 49.9% respectively, as well as the number of wet feces in comparison with the untreated group (distilled water) with 34.83%, 69.96% and 75.07% respectively. The AEWS therefore exerted significant antidiarrheal activity in a dose-dependent manner with a maximum effect at 400 mg/kg (Table 1).

Table 1. Effect of the aqueous extract of *Warionia saharae* and loperamide (positive control) on castor oil-induced diarrhea in mice

Treatment	Onset of diarrhea (min)	Total number of defecations	Inhibition (%)	Total of wet feces (g)	Inhibition (%)
Distilled water	33.81 ± 3.81	5.33 ± 0.27	-	3.33 ± 0.18	-
Loperamide (10 mg/kg)	148 ± 5.01	0.83 ± 0.23***	84.42	0.33 ± 0.18**	90.09
AEWS (50 mg/ kg)	52.83 ± 5.53	4.17 ± 0.61NS	21.76	2.17 ± 0.61NS	34.83
AEWS (200 mg/kg)	85.50 ± 10.41	3.17 ± 0.64*	40.52	1 ± 0.27*	69.96
AEWS (400 mg/kg)	126.50 ± 2.76	2.67 ± 0.41**	49.9	0.83 ± 0.34**	75.07

AEWS: Aqueous extract of *Warionia saharae*. NS not significant; * p≤ 0.05; ** p≤ 0.01; *** p≤ 0.001. The difference is statistically significant at the control. Results are presented as mean ± SEM with n=6.

The castor oil used in the present study is a trigger for diarrhea thanks to its inflammatory action on the intestinal mucosa, release of prostaglandins, nitric oxide, cyclic adenosine monophosphate, and platelet activating factors[27]. The prostaglandin synthesis inhibitors are considered antidiarrheal agent[7]. Pretreatment of mice with the AEWS elicited a significantly prolonged latency time, reduced defecation frequency, and decreased number of wet feces in comparison with the untreated group. However, the AEWS (200 mg/kg) reduced the number of fecal episodes by 40.52%, while the 400 mg/kg dose reduced the number of animals suffering from diarrhea by reducing the defecation frequency by 49.9%. Loperamide (10 mg/kg) profoundly reduced the

occurrence of castor oil-induced diarrhea and the number of diarrheal episodes by 84.42%. This latter was employed as a positive control, known for its antidiarrheal properties[28], and inhibitors of muscarinic and calcium receptors[29]. In terms of protection against diarrhea induced by castor oil, the AEWS (400 mg/kg) is capable of causing about half the protection induced by Loperamide. This could be due to the raw nature of the *W. saharae* extract as opposed to the pure form of Loperamide.

Small intestinal transit study

According to the results of this study (Table 2), we note that the AEWS has significantly reduced the distance traveled by activated charcoal by comparison to control and therefore bowel propulsion with 47.93 ± 1.98% at 400 mg/Kg. In the presence of Loperamide, the latter was reduced twice compared to the control.

Table 2. Effect of the aqueous extract of *Warionia saharae* (AEWS) and loperamide (positive control) on intestinal transit induced by castor oil in mice

Treatment	Length of the intestine (cm)	Distance covered by charcoal meal (cm)	IP (%)	(IT) (%)
Distilled water (Control)	32.58 ± 1.19	25.38 ± 0.92	77.9 ± 4.85	-
Loperamide (10 mg/ kg)	35.37 ± 1.71	10.50 ± 1.29***	29.97 ± 2.81***	61.53
AEWS (200 mg/kg)	31 ± 0.41	17.33 ± 0.52***	55.85 ± 1.20*	28.3
AEWS (400 mg/kg)	33.25 ± 1.46	15.94 ± 0.43***	47.93 ± 1.98**	38.45

AEWS: Aqueous extract of *Warionia saharae*. **IP:** intestinal propulsion, **IT**: inhibition of intestinal transit. *p≤ 0.05; ** p≤ 0.01; *** p≤ 0.001. The difference is statistically significant at the control. Results are presented as mean ± SEM with n=6.

In this intestinal motility test, we wanted to know the mechanism of action of the AEWS on castor oil-induced diarrhea. We notice that the AEWS (200 and 400 mg/kg) considerably delays the intestinal transit of activated charcoal in mice compared to the positive control. For mice that received these doses of the extract, intestinal propulsion was 55.85% and 47.93%, respectively, while in mice given just water, it was 77.9%. In the case of mice treated with Loperamide (10 mg/kg), only 29.97% of the entire intestine has been passed through by activated charcoal. It was also observed that the antidiarrheal effect of the extract was increased with increasing doses. These results allow us to suggest

that the AEWS might have antimotility properties, so we categorize it as an antidiarrheal agent. These results are in agreement with a previous work showing the antispasmodic activity of the aqueous extract of this same plant on rat and rabbit jejunums[30].

Enteropooling essay

Treatment of mice with castor oil resulted in an increase in the volume accumulation of fluids in the intestinal lumen (Figure 1); on the other hand, treatment with loperamide (10 mg/kg) and AEWS (200 - 400 mg/kg) significantly reduced this last with 13.17% and 7.47% respectively.

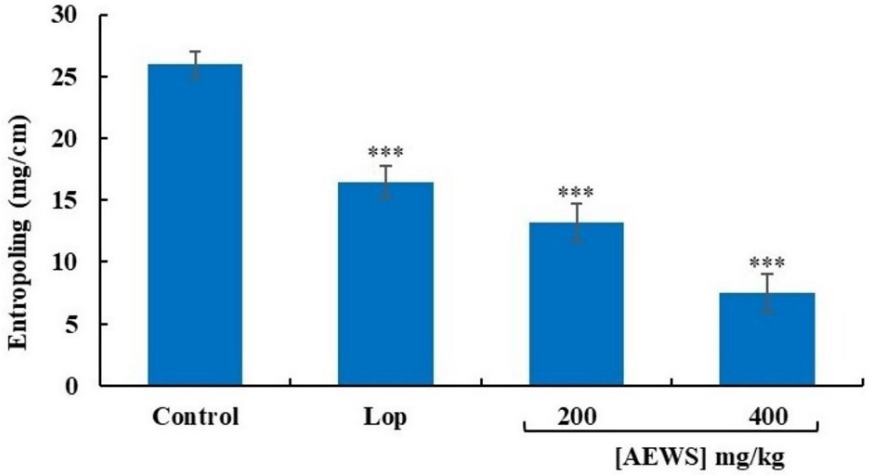

Figure 1. Effect of the AEWS on the accumulation of fluids in the intestinal lumen on castor oil-induced gut motility in mice

***: p≤0.001. The difference is statistically significant at the control. The results are presented in mean ± SEM with n=6. Lop: Loperamide.

Still within the framework of the same objective of clarifying the mechanism of action of the AEWS on castor oil-induced diarrhea, we tried to see the effect of this extract on the accumulation of intestinal fluids. The AEWS (200 and 400 mg/kg) and the antidiarrheal agent Loperamide both reduced the volume of fluids accumulation in the intestinal lumen. It seems that the AEWS has antisecretory and antimotility activities, which are due to the presence of one or more relaxant components in this extract. Antimuscarinic drugs and calcium channel blockers may be antispasmodics, antimotilities, and antidiarrheals[29,31]. In addition, in our previous studies, we have discovered that the aqueous extract and essential oil of this plant possessed an antispasmodic activities[16,30], which contributes to its effectiveness in treating diarrhea and abdominal

spasms. Similar results with other extract plants have been found in aqueous and methanolic leaves extracts of *Dissotis thollonii* Cogn[5], *Rubia tinctorum* L.[20], and *Streblus asper*[32].

Antimicrobial assay of the aqueous extract and organics fractions

Antibacterial activity

The antibacterial activity tested in the present study has been assessed quantitatively and qualitatively based on the presence or absence of zones of inhibition compared to the streptomycin used as a reference antibiotic. These later values ranged from 9 ± 0.2 to 20 ± 0.03 mm for Gram-positive bacteria and from 7 ± 0.30 to 14 ± 0.60 mm for Gram-negative bacteria (Table 3).

Table 3. Diameters of the zones of inhibition (mm) of the aqueous and organic extracts of *Warionia saharae* and streptomycin (positive control) against Gram-positive and Gram-negative bacteria

	Gram (-) bacteria				Gram (+) bacteria
	Escherichia coli 57	*Escherichia coli 97*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
	Diameter (mm)				
AEWS (1 mg/disc)	10 ± 0.40	9 ± 0.11	11 ± 1.04	10 ± 0.60	16 ± 1.10
MFWS (1 mg/disc)	7 ± 0.80	11 ± 1.05	8 ± 1.60	7 ± 0.30	14 ± 1.02
DFWS (1 mg/disc)	14 ± 0.60	-	10 ± 0.24	11 ± 0.07	14 ± 2.40
EaFWS (1 mg/disc)	10 ± 0.60	13 ± 1.03	-	12 ± 0.50	20 ± 0.03
HFWS (1 mg/disc)	11 ± 0.60	13 ± 1.40	-	-	9 ± 0.2
Streptomycin (0,02 mg/disc)	-	-	-	-	9 ± 1.00

AEWS: aqueous extract, **MFWS**: Methanolic fraction, **DFWS**: Dichloromethanic fraction, **EaFWS**: Ethyl acetate fraction, **HFWS**: Hexanic fraction, of *Warionia saharae*. Data are expressed as mean ± SEM (n=3 trials for each sample). **-:** no activity.

The AEWS showed a satisfactory result in inhibiting all the microorganisms test (*Escherichia coli* 57, *Escherichia coli* 97, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) with an inhibition diameter around of 9 ± 0.11 to 16 ± 1.10 mm. The MFWS, showed the inhibition of these five bacteria but by an inhibition diameter lower than that due to the AEWS (7 ± 0.80 à 14 ± 1.02 mm). While the EaFWS and DFWS were only active against

four organisms test (*Escherichia coli* 57, *Escherichia coli* 97, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*) and (*Escherichia coli* 57, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*) respectively. The hexane extract was only active against three strains: *Escherichia coli* 57, *Escherichia coli* 97, and *Staphylococcus aureus*. Whereas, Streptomycin showed no activity for these microorganisms. The greatest and minimum inhibitory diameters of *W. saharae* extracts were from, 20 ± 0.03 mm with EaFWS against *Staphylococcus aureus* and 7 ± 0.30 mm with MFWS against *Pseudomonas aeruginosa* (Table 3).

The antibacterial activity of *W. saharae* was also assessed by the MIC assay. In this study, all the extracts displayed a broad spectrum of antimicrobial activity, with MIC values ranging from 0.009 to 0.625 mg/mL for Gram-positive bacteria and from 0.009 to 25 mg/mL for Gram-negative bacteria (Table 4). For gram (-) bacteria, the best activities were expressed by the DFWS (0.009 to 0.312 mg/mL) and AEWS (0.02 to 0.40 mg/mL) against all microorganisms tested. MFWS had only been active against *Pseudomonas aeruginosa* (0.09 mg/mL), while EaFWS and HFWS were weakly active (5 to 10 mg/mL). Streptomycin was active against all of these microorganisms except *Pseudomonas aeruginosa* (Table 4).

Table 4. MICs values of the aqueous and organic extracts of *Warionia saharae* and streptomycin (positive control) against Gram-positive and Gram-negative bacteria

	Gram (-) bacteria				Gram (+) bacteria
	Escherichia coli 57	Escherichia coli 97	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staphylococcus aureus
	MIC (mg/mL)				
AEWS	0.20	0.02	0.20	0.40	0.009
MFWS	8	5	25	0.09	0.009
DFWS	0.156	0.039	0.009	0.312	0.009
EaFWS	5	5	2.5	10	0.078
HFWS	10	10	10	10	0.625
Streptomycin	0.25	0.5	0.003	-	0.062

AEWS: aqueous extract, **MFWS**: Methanolic fraction, **DFWS**: Dichloromethanic fraction, **EaFWS**: Ethyl acetate fraction, **HFWS**: Hexanic fraction, of *Warionia saharae*. Data are expressed as mean ± SEM (n=3 trials for each sample). **-:** no activity.

For the first time, Rechek et al.[13] allowed the identification of 24 compounds from the hydromethanolic extract of *W. saharae* including derivatives of hydroxycinnamic acid and flavonoids. However, only ten compounds were isolated from the chloroform and ethyl acetate extracts from the aerial sections of the Algerian plant *W. saharae* using UV, IR, and NMR (1 H and 13 C) spectroscopies. These isolated compounds are β-sitosterol, stigmasterol, scopoletin, esculetin, hispidulin, cirsimaritin, chryseriol, luteolin, taxifolin, and quercetin[14]. Flavonoids are recognized for their antidiarrheal activity, which can be attributed to various mechanisms including antimicrobial action, inhibition of intestinal motility, and an antisecretory effect[7]. For instance, quercetin has demonstrated a significant decline in the Na^+/K^+–ATPase activity of enteropathogenic *Escherichia coli*-induced infectious diarrhoea, potentially leading to a notable reduction in the reabsorption of ions and water[33]. Additionally, this antidiarrheal effect may be associated with the presence of caffeic acid[34] and thymol[35]therefore, used for standardization in the German pharmacopoeia (0.03% phenols calculated as thymol in their composition. This activity can also be linked to the chemical components found in the essential oil, which possess spasmolytic properties. Examples includes cineol, 1-terpinen-4-ol[36], terpineol acetate[37] and linalool[38].

The different fractions of *W. saharae* and the aqueous extract demonstrated a broad spectrum of antimicrobial activity against the bacteria tested; Gram-positive and Gram-negative, with microbial growth inhibition ranging from 7 ± 0.30 to 20 ± 0.03 mm. Those extracts have also displayed a large spectrum of antimicrobial activity, with MIC values starting from 0.009 to 0.625 mg/mL for Gram-positive bacteria and from 0.009 to 25 mg/mL for Gram-negative bacteria. In the literature, numerous studies have been conducted to classify the distinct plant extracts consistent with their MIC value[39,40]. Kuete[41] classified crude extract activity as significant if the MIC is less than 100 µg/mL, moderate if the MIC is between 100 and 625 µg/mL, and low if the MIC is greater than 625 µg/mL. In this study, extracts with MIC values ranging from 0.009-0.4 mg/mL were considered to have good activity against the bacterial strains tested.

The best inhibitory activities were exerted by the DFWS and the AEWS, while the MFWS, EaFWS, and HFWS showed weak activity when considered with them. The DFWS showed a high level of antibacterial activity. It has been reported that the phenolic diterpenoids, which can be the primary compounds of the apolar fraction of plant extracts, are responsible for the antibacterial action[42]. These compounds are particularly lipophilic in nature and are extracted with low-polarity solvents such as hexane. However, the hexane fraction of this study showed antibacterial activity that was relatively low compared to other

extracts. Therefore, the size and load of the particles present in such an extract influence this activity. When compared to the fractions (MFWS, EaFWS, and HFWS) and streptomycin, the AEWS was also active against the five bacterial strains tested. It could be due to secondary metabolites (polyphenols and flavonoids) found in many crude extracts, which have a variety of pharmacological activities, including antibacterial activity[43]. It has also been demonstrated that these secondary metabolites have a large site capable of causing the rupture of the lipopolysaccharide layer from the plasma membrane of Gram-negative bacteria such as *Escherichia coli*, allowing for the alteration of vital intracellular enzyme systems in bacteria[44].

Our results are in agreement with those already mentioned. Gilbert et al.[5] for example, confirmed that the aqueous extract of *Dissotis thollonii* Cogn. exhibited a stronger antibacterial impact than the ones of hexane, methanol, and ethyl acetate from this plant. At the same time, Guadie et al.[45] proved the opposite case.

The EaWS recorded the highest levels of antibacterial activity for *Staphylococcus aureus*. This last one is a pathogenic Gram-positive bacterium that is found primarily in the intestinal lumen and can cause diarrhea[39]. Likewise, *Escherichia coli* and *Pseudomonas aeruginosa* are among the Gram-negative bacteria inhabiting the human gastrointestinal tract[46], and have presented a good range of sensitivity, specifically through the FDWS (MIC = 0.156-0.312 mg/ml) and the AEWS (MIC = 0.20-0.40 mg/ml) (Table 4). However, *Pseudomonas aeruginosa* is renowned for having an excessive level of intrinsic resistance, which was acquired against the majority of antibiotics[47].

Antifungal activity

Regarding the antifungal activity, the aqueous extract and the organic fractions of *W. saharae* all exhibited inhibitory activities towards *Candida albicans* and *Saccharomyces Cerevisiae* with inhibitory diameters ranging from 10 to 15 mm (Table 5), and MIC values ranging from 0.009 to 0.625 mg/mL (Table 6). In fact, the best antifungal activities detected as decreasing have been those of the DFWS, the MFWS, the AEWS, and subsequently the EaFWS, whose activities are greater than those of the HFWS and fluconazole.

Table 5. Diameters of the zones of inhibition (mm) of the aqueous and organic extracts of *Warionia saharae* and Fluconazole (positive control) against Gram-positive and Gram-negative bacteria

		Candida albicans	*Saccharomyces Cerevisiae*
	Dose	**Diameter (mm)**	
AEWS	1 mg/disc	10	11
MFWS	1 mg/disc	14	13
DFWS	1 mg/disc	15	12
EaFWS	1 mg/disc	12	11
HFWS	1 mg/disc	11	12
Fluconazole	5 mg/disc	21	27

AEWS: aqueous extract, **MFWS**: Methanolic fraction, **DFWS**: Dichloromethanic fraction, **EaFWS**: Ethyl acetate fraction, **HFWS**: Hexanic fraction, of *Warionia saharae*. Data are expressed as mean ± SEM (n=3 trials for each sample). -: no activity.

Table 6. MICs values of the aqueous and organic extracts of *Warionia saharae* and Fluconazole (positive control) against Gram-positive and Gram-negative bacteria

	Candida albicans	*Saccharomyces Cerevisiae*
	MIC (mg/mL)	
AEWS	0.3	0.1
MFWS	0.1	0.1
DFWS	0.018	0.009
EaFWS	0.312	0.312
HFWS	0.625	0.625
Fluconazole	0.4	0.2

AEWS: aqueous extract, **MFWS**: Methanolic fraction, **DFWS**: Dichloromethanic fraction, **EaFWS**: Ethyl acetate fraction, **HFWS**: Hexanic fraction, of *Warionia saharae*. Data are expressed as mean ± SEM (n=3 trials for each sample)

Each of these extracts demonstrated antifungal activity, as shown by the results of inhibition experiments using *W. saharae* aqueous extract and organic fractions on *Candida albicans* and *Saccharomyces cerevisiae*; no signs of resistance were found. Regarding the aqueous extract, we observe that it has very

strong antifungal activity. We list the dichloromethane fraction, the methanolic fraction, the ethyl acetate fraction, and lastly the hexanic fraction for the organic fractions in decreasing order of antifungal activity. This effectiveness may be connected to the extract's chemical composition[48], the solvent's ability to solubilize a number of substances found in the crushed plant, or the study itself[49]. Similar studies in the literature have proven that organic fractions and aqueous extracts of the plants showed good antifungal activity[50].

STATEMENT OF ETHICS

This study was approved by The Faculty of Sciences, Mohammed First University, Oujda (Morocco) under Trial Registration Number: 08/24-LBBEH-08 and 01/08/2024.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contribute the work equally throughout.

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In vitro investigation of the toxicological mechanisms of gemcitabine in colorectal cancer cells

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ABSTRACT

Colon cancer is the third most common cancer type in the world. Gemcitabine (2'deoxy-2'2'-difluorocytidine monohydrochloride) was found to be very effective against small cell lung cancer, pancreatic cancer, bladder cancer and breast cancer and was approved for the treatment for these cancers. Although it is similar to cytosine arabinoside (Ara-C) in terms of structure, metabolism and mechanism of action, the spectrum of antitumor activity of gemcitabine is much wider. Autophagy is a general term that refers to the degradation of cytoplasmic components within lysosomes. Autophagy plays a critical role in many disorders such as neurodegenerative diseases, cancer, infection, cardiovascular, metabolic, pulmonary diseases and aging. In this study, we evaluate the gemcitabine alone and its' combinations with autophagy inhibitor (chloroquine) and activator (rapamycin) effect on cell cycle, apoptosis and autophagy on human colorectal cancer cell line (HCT-116). We exposed the cells to gemcitabine (0,625 mM, 12,5 mM, 2,5 mM, 5 mM), rapamycin (0,5 μM) and chloroquine (20 μM) for 24 hours. Gemcitabine, alone or in combination with chloroquine caused cell cycle arrest at G1 and G2. However, the combination with rapamycin doesn't cause any significant change in the cell cycle of the exposed

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cells. Gemcitabine-chloroquine group also significantly increased the apoptosis. Consequently, combining gemcitabine with chloroquine increased the cell death when comparing gemcitabine alone. Therefore, gemcitabine and chloroquine combination could increase the efficacy of chemotherapeutic treatment of colorectal cancer.

Keywords: colorectal, cancer, gemcitabine, autophagy, cytotoxicity

INTRODUCTION

Colon cancer is the third most common cancer type in the world[1,2]. In Europe, 250,000 new cases of colon cancer are diagnosed each year, accounting for 9% of all diseases. The incidence of colon cancer is increasing with industrialization and urbanization. The incidence of colon cancer in individuals under the age of 45 is very rare (2 per 100,000 people per year). While it is 20 per 100,000 people between the ages of 45-54, this rate increases much more as the age increases to be 55 per 100,000 for the 55-64 age group, 150 per 100,000 for the 65-74 age group, and 250 per 100,000 after the age of 75[3].

Surgical intervention is considered the first in patients with colorectal cancer who have a chance of recovery. Adjunctive therapy is a systemic therapy used to reduce the risk of cancer recurrence and death. The risk of recurrence can be estimated by pathological staging[4]. While adjuvant chemotherapy has become the standard for third-stage patients, it does not play much of a role in the second stage. In the case of metastatic cancer, the most important goal of chemotherapy is to prolong and improve life expectancy[4].

Chemotherapeutic treatment of colorectal cancer contains several drugs like fluoropyrimidine, irinotecan, oxaliplatin, bevacizumab, cetuximab, panitumumab, capecitabine[5]. Generally, first-line chemotherapy includes fluorouracil (5-FU) or capecitabine or combining them with leucovorin (LV) or oxaliplatin for alleviating the symptoms and increasing the quality of life. In second-line chemotherapy, patients will be selected based on resistance of chemotherapeutic drugs[6]. Gemcitabine (2' deoxy-2'2'-difluorocytidine monohydrochloride) was approved for the treatment of small cell lung cancer[7], pancreatic cancer[8], and breast cancer[9].

Gemcitabine inhibits DNA synthesis at G1/S cell cycle and represses cell proliferation. Gemcitabine is not approved for the colorectal treatment, however, some studies reported that some chemotherapeutics like capecitabine, oxaliplatin in combination with gemcitabine can be a therapeutic option for refractory

advanced or progressive colorectal cancer[10,11]. In addition, gemcitabine has been found to be effective in oxaliplatin-resistant colorectal cells an *in vitro* study[12].

Gemcitabine (Gem) is a potent and specific analogue of deoxycytidine. After being taken up by malignant cells, gemcitabine is phosphorylated by deoxycytidine kinase to form gemcitabine monophosphate. This monophosphate form is then converted to gemcitabine diphosphate and gemcitabine triphosphate, which are the active metabolites of gemcitabine. Consequently, these active metabolites are responsible for the antitumor activity of gemcitabine[13].

Autophagy is a general term that refers to the degradation of cytoplasmic components within lysosomes. Autophagy plays a critical role in many disorders such as neurodegenerative diseases including cancer, infection, cardiovascular, metabolic, pulmonary diseases and aging[14]. Autophagy has effects on carcinogenesis that can go both ways. While autophagy helps to prevent the transformation into malignancy by removing damaged organelles, accumulated proteins on normal cells, reducing DNA damage, reactive oxygen derivatives (ROS) and mitochondrial abnormalities; it also contributes to tumor formation by enabling the tumor cell to reach nutrients, prevent cellular death and increase drug resistance[15,16]. The response of cells to autophagy during cancer metastasis is phase-of-cancer dependent. In the early stages, autophagy inhibits tumor cell metastasis by producing inflammatory responses against tumors. In addition, autophagy limits tumor necrosis and transformation of dormant cancer cells into micro-metastases[17]. In advanced stages, autophagy increases the survival of metastatic cells in the extracellular matrix and promotes the spread of cancer cells to distant organ sites[17,18]. Some studies showed that both activation and inhibition of autophagy with the specific chemicals increased anticancer activity of the chemotherapeutics[19-24].

In this study, it is aimed to determine the effects of gemcitabine and its' combinations (autophagy inhibitor, chloroquine and autophagy activator, rapamycin) on HCT-116 human colorectal cancer cell line and to investigate the role of autophagy in the anticancer activity of gemcitabine in colorectal cancer cells.

METHODOLOGY

Cell culture

HCT-116 (Human colorectal carcinoma-CCL-247) cell line was purchased from ATCC, USA. Cells were cultured in RPMI-1640 medium contained 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic and 1% non-essential amino acid. Cells were subcultured when they reach 60-70% confluence.

Drug treatment

Cells were exposed to gemcitabine for 24 hours. 1.25 mM gemcitabine was used for the combination studies. In the study, rapamycin (RAPA) was used as an autophagy activator, and chloroquine (CQ) was chosen as an autophagy inhibitor. The concentration of the drugs was determined as 0.5 μM for rapamycin[25,26,27] and 20 μM for chloroquine[28,29,30] according to the literature. To validate autophagy activation/inhibition in our conditions LC3B II/I protein expression level was investigated with western blot after 0.5 μM rapamycin and 20 μM chloroquine exposure for 24 hours. After 0.5 μM rapamycin and 20 μM chloroquine exposure LC3B II/I protein expression enhanced significantly, and the increase was found significantly higher after chloroquine exposure than rapamycin (Figure 1). Chloroquine impairs autophagosome degradation by affecting autophagosome-lysosome fusion, so LC3B II accumulates in the cell[31], which is supported by other studies[28,32,33]. However, the cell viability was not affected significantly (data not shown) at these concentrations. Inhibitor of autophagy, CQ induced the formation of the autophagosome, but inhibited the degradation of autophagosome in the last stage of autophagy[34].

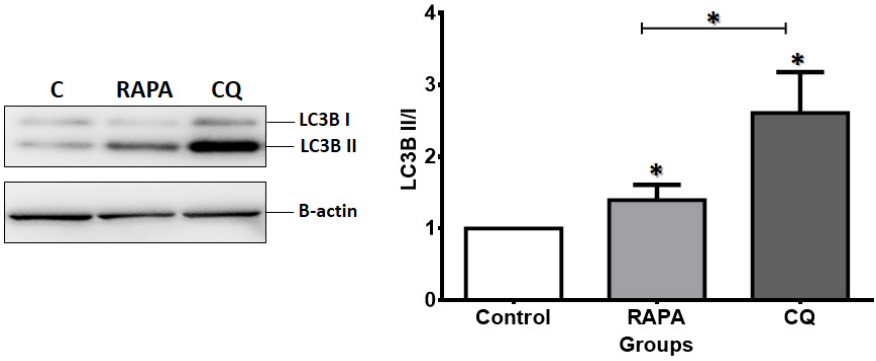

Figure 1. Changes in LC3B II/I expression after 0.5 nM RAPA (rapamycin) and 20 μM chloroquine (CQ) exposure for 24 h
*p<0.05

Cell viability assay

Cell viability was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. HCT-116 cells were treated with gemcitabine only (1.562-50 mM), combinations of gemcitabine 1.25 mM) with chloroquine (20 μM) and rapamycin (0.5 μM), for 24 hours. At the end of exposure time, MTT (5 mg/mL) solution was added to each well and incubated at 37°C

for 3 hours. Then, the medium and MTT dye in the wells were removed and formazan was dissolved with DMSO in, Optical density (OD) was measured at 590 nm using a multiwell plate reader (Biotek, Bad Friedrichshall, Germany).

Detection of apoptosis

Apoptosis was determined with a commercial kit (Biolegend, California, USA) following the manufacturer's rules. After 24 hours exposure, the cells were collected with trypsin and resuspended in Annexin V binding buffer (100 µL). Then Annexin V (5 µL) and PI (10 µL) dye solutions were added to the cell suspension and incubated at room temperature. After 15 min incubation, 400 µl of Annexin V Binding Buffer was added and fluorescence signals were determined in the FITC channel (FL-1) and PE channel (FL-2) by ACEA flow cytometry (Agilent, California, USA). The results were analyzed with Novoexpress software (Agilent, California, USA).

Cell cycle analysis

Cell cycle analysis was performed with a commercial kit (Elabscience Biotechnology, Houston, USA) following the manufacturer's instructions. After drug treatments, cells were collected by trypsinization and washed with PBS. Then, cells were transferred to tubes containing 1.2 mL absolute ethanol and incubated at -20 °C for 1 hour. After centrifugation and washing steps, 100 µL of RNase A reagent was added to each tube and incubated in a water bath at 37 °C for 30 minutes. At the end of the incubation time, 400 µL propidium iodide (PI) staining solution was added to each tube and incubated at 2-8°C for 30 minutes. Fluorescence intensity was determined by a flow cytometry in FL-2-A channel (Agilent, California, USA) and results were calculated using Novoexpress software (Agilent, California, USA).

Statistical analysis

Data were analyzed using GraphPad prism software (version 6) with one-way ANOVA followed by Tukey test. $p<0.05$ values were considered as statistically significant. All data were represented as mean ± standard deviation (SD).

RESULTS and DISCUSSION

Cell viability

According to cell viability assay, gemcitabine decreased cell viability starting from 2.5 mM dose (cell viability 71.16% ± 1.86) and IC_{50} value of gemcitabine was calculated to be 5.50 mM ± 0,2. Cell viability decreased in gemcitabine (1.25 mM)-rapamycin (0.5 µM) combination at the same doses comparing to the control group, however it was not found to be statistically significant differ-

ence when comparing with gemcitabine group. Gemcitabine (1.25 mM)-chloroquine (20 μM) combination inhibited cell viability more than gemcitabine group at 1.25 mM, 2.5 mM and 5 mM concentrations (Figure 2).

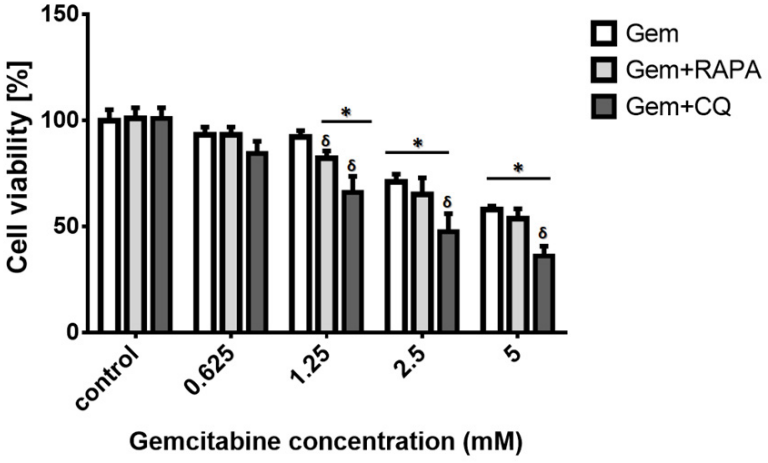

Figure 2. Changes in the cell viability following 24h Gem, Gem+RAPA and Gem+CQ exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine

Apoptotic and necrotic cell death

Gemcitabine (1.25 mM) induced 1,5-fold more apoptotic cell death (p<0.05) but not necrotic cell death. Apoptotic cell death was 1.36-fold less in gemcitabine-rapamycin (0.5 μM) group comparing to gemcitabine group. Apoptosis significantly increased (1,5-fold) in gemcitabine-chloroquine (20 μM) group in comparison with the control group but there was no significant difference with the gemcitabine-alone group (Figure 3).

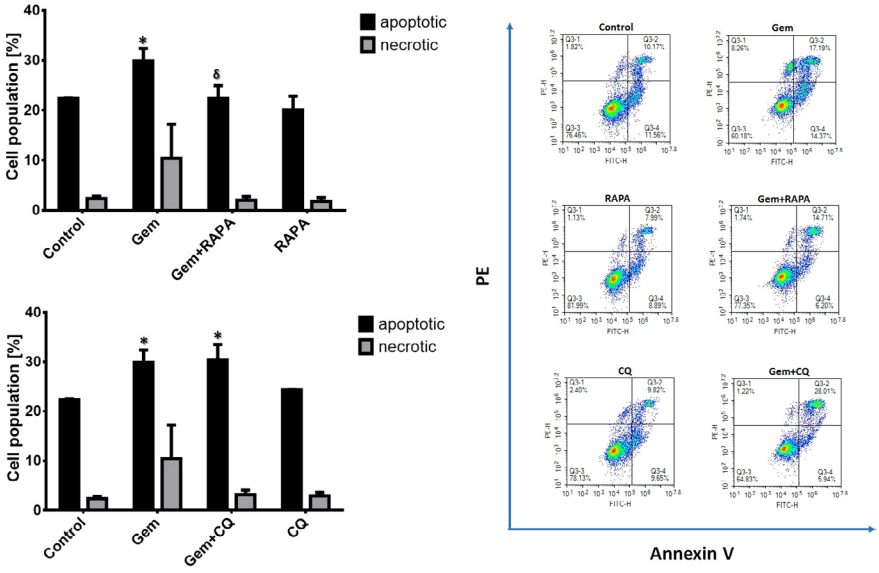

Figure 3. Changes in apoptotic and necrotic cell population following 24 hours Gem (1.25 mM), Gem+RAPA (0.5 μM) and Gem+CQ (20 μM) exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine.

Cell cycle analysis

It was observed that gemcitabine led to G1 and G2 arrest in HCT-116 cell line. Although rapamycin (0.5 μM) also induced G2 arrest compared to the control group (1.25-fold), there were no significant changes in gemcitabine-rapamycin combination group in comparison with gemcitabine group. However, G1 and G2 arrest was exacerbated (1.5-fold) in chloroquine (20 μM)-gemcitabine (1.25 mM) combination compared to the control group and gemcitabine-alone (1.25 mM) group also cause to the cell cycle arrest, but arrest of gemcitabine-chloroquine combination group was higher than chloroquine-alone group probably due to the synergistic effect (Figure 4).

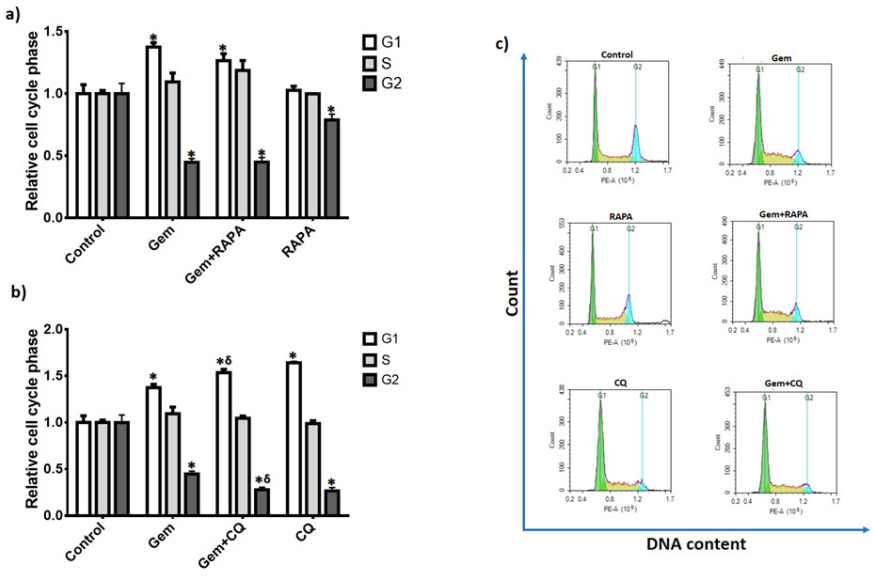

Figure 4. Changes in G1, S and G2 cell cycle phases following 24 hours Gem (1.25 mM), Gem+RAPA (0.5 μM) and Gem+CQ (20 μM) exposures *p<0.05 versus control group, $^{\delta}$p<0.05 versus Gem group. Gem: Gemcitabine, RAPA: Rapamycin, CQ: Chloroquine.

Colorectal cancer is the third most common and fourth deadliest cancer type in the world[1,2,35]. In 1996, Food and Drug Administration (FDA) approved gemcitabine for first line treatment of advanced and metastatic pancreas[36]. Although gemcitabine has no FDA approval in the treatment of colorectal cancer, some studies suggest that adding gemcitabine into the traditional chemotherapy improves the anticancer effect of treatments[8,9]. Autophagy activators/inhibitors that added to conventional chemotherapy has been shown to increase anticancer effect of the chemotherapy[37,38]. Besides, the combination therapies can be used to overcome chemotherapy resistance[39]. In the study, anticancer activity of gemcitabine was investigated in the presence of rapamycin (autophagy activator) and chloroquine (autophagy inhibitor) using HCT-116 cell line.

According to the findings, both rapamycin and chloroquine combinations decreased cell viability compared with gemcitabine group and the decrease was found statistically significant for chloroquine combination but not for rapamycin.

Apoptosis is a programmed cell death that protects the entire organism against more serious damages, such as cancers. In normal cell, when there is a damage on DNA and it can't be repaired, apoptosis is triggered, and the abnormal cell dies as programmed. When there is a problem in apoptosis induction, the

abnormal cell continues to proliferation and finally, cancer cells will occur. In cancer, there is an imbalance between proliferation and programmed cell death. Most treatments like chemotherapy, radiation, hormonal treatments generally aim to create an irreparable cellular damage and trigger their apoptosis[40]. Gemcitabine has been shown to induce apoptosis on many cancer cell lines like, pancreatic, breast, and human osteosarcoma cells[41,42,43]. Similarly, it was also found that gemcitabine increased the apoptotic cell death after 24 h exposure in the study. It has been noted that gemcitabine-induced autophagy has been shown to prevent apoptosis in lung cancer cells. Thus, adding of autophagy inhibitors to gemcitabine treatment increased apoptosis in lung cancer cells[38]. Another study also showed that autophagy inhibition increased hypoxia-induced apoptosis in HCT-116 cells[44]. Similarly, gemcitabine and chloroquine combination induce apoptotic cell death in HCT-116 cell comparing to gemcitabine group in the present study.

Activating the autophagy with rapamycin (50 nM, for 24 hours exposure) has been reported to induce the apoptosis in human osteosarcoma cells[19]. Furthermore, anticancer effect was increased the anticancer drug efficiency when rapamycin (10.3 nM, for 48 hours exposure) was added to the regimen through stimulating autophagy, apoptosis and cell cycle arrest in breast cancer cells[21]. In our study, rapamycin did not increase apoptotic cell death in HCT-116 cells and autophagy activation by rapamycin treatment alleviated the gemcitabine-induced apoptotic cell death in HCT-116 cells.

Eukaryotic cell division is regulated by different mechanisms to prevent uncontrolled cell proliferation under physiological conditions. interphase and M phase are major components of the mitotic cell division. After all, separation of cellular content duplication during interphase occurs and two genetically identical daughter cells are formed. DNA replication is performed in S phase. The phase that separates end of mitosis from S phase is G1 and separates S phase from M phase is G2, which are also called gap phases since they have been considered as gaps between DNA duplication and DNA segregation. Additionally, these phases play crucial role for the regulation of cell cycle[45,46]. It is known that the regulation of the cell cycle plays a crucial role in influencing the proliferation, metastasis, and recurrence of tumor cells. In cancer treatment, many chemotherapeutic drugs show anticancer effect via inducing cell cycle inhibition[47]. It has been reported that gemcitabine (30 nM for 24-48 hours exposure) caused to cell cycle arrest at G1, S and G2 phases in some cancerous cell lines[48,49]. In the present study, gemcitabine (1.25 mM) also induces cell cycle disruption at G1 and G2 phases in HCT-116 cells.

The antimalarial drug chloroquine has demonstrated anticancer effects on some cancer cells[50,51]. In the present study, chloroquine caused to disruption of G1 and G2 phases on colorectal cancer cells. Furthermore, when used in combination with conventional chemotherapies, Chloroquine has been found to enhance the anticancer effect of the treatment and sensitized the tumor cells to chemotherapeutic agent or radiotherapy[24,52,53]. Similar to these studies, chloroquine combination potentiates gemcitabine-induced G1 and G2 arrest when compared with gemcitabine group.

Our findings indicate that combining gemcitabine with chloroquine results in a higher rate of HCT-116 cell death compared to using gemcitabine alone, likely due to disturbance in the cell cycle, but there is no significant change in cell death for gemcitabine-rapamycin group. Therefore, gemcitabine and chloroquine combination could be a therapeutic option in the treatment of colorectal cancer.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Design: G.Ö., T.B., E.T., Ö.S.Z. Acquisition of data: T.B., E. T., Ö.S.Z. Analysis of data: T.B., E. T., Ö.S.Z. Drafting of the manuscript: E.T. Supervision: G.Ö. Statistical analysis: T.B.

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Topical mupirocin-steroid for wound care in an era of rising antibiotic resistance

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ABSTRACT

Wound infections are characterized by antibiotic-resistant bacteria, a growing issue. When these microbes infect a wound, it's treated aggressively. A few therapeutic antibiotics make this therapy problematic. Mupirocin and other newly discovered methods show promise as bacterial wound-killing agents. This study includes three topical mupirocin-steroid therapies that were tested for their ability to treat antibiotic-resistant clinical isolates. Wounds may be treated using mupirocin-based liquid, cream, and dressing coating. The reduction in viable bacterial population after mupirocin exposure was used to compare the bactericidal efficiency of different mupirocin treatments. Results showed that each ingredient had the potential to reduce germ reproduction. The mupirocin-coated bandage was the most efficient strategy to kill antibiotic-resistant bacteria, whereas the liquid mupirocin was the least efficient. The mupirocin-coated steroid bandage swiftly killed the tested germs and showed promise against other bacterial strains. Mupirocin might be a therapeutic and prophylactic medication for wound colonization by organisms that hinder healing.

Keywords: mupirocin, wound care, antibiotic resistance

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INTRODUCTION

Despite being home to fungus and bacteria, the human skin functions as a barrier against germ penetration and infection of underlying tissues. Human skin is thicker and more impermeable than other surfaces[1]. Once a wound breaks this barrier, the likelihood of bacterial penetration of an intact tissue rises. In traumatic, thermal, or chronic wounds, the colonization and infection potential rise due to the compromised barrier and the development of avascular eschar, which allows unrestricted microbe growth[2] which leads to uncontrolled microorganism growth that permits germs to proliferate unrestrictedly. Infection is usually related to the kind of wound, wound management, and host factors[3] including the patient's age, diet, immune system health, and underlying sickness. If proper treatment techniques are followed, wound infection rates may be kept low[4].

Antibiotic prophylaxis is necessary to reduce the wound's microbial load and hasten healing. A slow-healing wound increases the patient's medical expenditures and worsens his or her health, leading to recurrent hospitalizations[5]. Antibiotic resistance has increased due to the widespread use of antibiotics, which has led to a rise in the isolation of antibiotic-resistant organisms from wounds. Implementing strategies to minimize patient-to-patient transmission and manage nosocomial outbreaks may result in an antibiotic-resistant wound infection[6]. When treating an antibiotic-resistant illness, it is normal practice to minimize patient contact to prevent the infection from spreading. Infections are often treated with medicines that are effective against the causing organism. Medical treatments are followed. This makes managing resistant organisms with antibiotics problematic. Antibiotics should be used with caution. Hospitals and communities have taken stringent efforts to prevent the spread of antibiotic-resistant microorganisms. To counteract wound colonization or infection by antibiotic-resistant bacteria, a means of prophylaxis that minimizes the risk of resistant organisms must be found. The chosen technique must be effective against many species and destroy intruders quickly[7]. This article discusses the effectiveness of topical antibiotic-resistant bacterial treatments with mupirocin and steroids. As potential topical mupirocin-steroid treatments, a solution, cream, and dressing were studied.

Mupirocin, also known as pseudomonic acid A, is a compound that is synthesized by the soil bacterium *Pseudomonas fluorescens*, which belongs to the Gram-negative group of bacteria. As an antibiotic for the skin, it works by attaching to bacterial isoleucyl-tRNA synthetase (IleRS) and stopping the production of proteins. This medication is used to treat infections caused by microorganisms such as *Streptococci* and *Staphylococci* strains, including those that are resistant to methicillin. It is commonly employed to treat methicillin-

resistant *S. aureus* (MRSA), a bacterium that mostly causes bloodstream infections acquired in hospitals and is a significant contributor to wound infections. Prior studies have indicated that mupirocin has wound healing properties, mostly attributed to its antibacterial action against bacteria commonly seen in wounds. The process of wound healing is multifaceted and involves various cellular activities and molecular interactions, with growth factors playing a significant role. While most wound healing studies have primarily examined the antibacterial properties of mupirocin, a limited number of studies have investigated the impact of mupirocin on inflammation and cell migration. These investigations have revealed that mupirocin promotes the production of tumour necrosis factor (TNF)-α in RAW 264.7 cells. TNF-α has a crucial role as a cytokine in the inflammatory phase of wound healing. In addition, the administration of anti-TNF-α monoclonal antibodies in mice caused a delay in the wound healing process. This delay was accompanied by a reduction in the number of inflammatory cells and fibroblasts in the wound area. In contrast, the administration of TNF-α greatly improved the healing of the wound. This indicates that mupirocin possesses wound healing capabilities that are not only attributed to its antibacterial properties[8].

METHODOLOGY

Microorganisms

Clinical isolates were obtained from Baghdad University and Basra University and Table 1 lists resistance microbes and medications as provided from the source. The strains' stock cultures were preserved at 0°C throughout the operation. The frozen stock was thawed, and the bacterial strains were grown overnight in tryptic soy broth. This made the strains usable. They might then be used. After a day of growth in tryptic soy broth, Gram-negative bacteria were isolated by centrifuging at 13000 rpm for five minutes. After developing in broth, the bacteria were isolated. The bacteria were then washed with physiologic saline and resuspended until their optical density at 750 nm was 0.25 to 0.40. In one test, the bacteria were resuspended in 60% calf serum diluted with physiologic saline. Calf serum is used because it contains low levels of antibodies and other growth-inhibiting components and it can also protect cells from harmful disruptions, including large pH shifts. So, the bacteria's optical density will be compared with calf serum that considered as a control. Gram-positive organisms were washed and resuspended in sterile water to ease recovery. The next paragraphs describe this technique and the recovery of Gram-positive organisms from test materials. After inoculating the organism in Mueller-Hinton Agar plates and examining bacterial growth, high-quality cultures were obtained.

Table 1. Clinical isolates, isolation sites, and resistant antibiotics

Organism	Strain	Sample type	Antibiotic resistance
Burkholderia cepacia	UT363	Wound sample	1,2 3,4,5,7,10,20,22
P. aeruginosa	137366	Traumatic Wound sample	1,2,3,4,5,6,7, 11,13
Pseudomonas spp (P cepacia, P. stutzeri, P. maltophilia and P. putrefaciens)	150938-1	Fluid of body (saliva, blood, interstitial fluids)	5,6,7,8,9,22,23,24
E. faecium	118271	Urine sample	3,4,5,6,716,17,18,22,23
S. aureus	141960	Fluid of body (saliva, blood, interstitial fluids)	1,2 3,4,5,6,7,8,9,10
S. aureus	140277	Urinary catheter	17,18,19,20,21,22,23,24
Acinetobacter	150938-2	Fluid of body (saliva, blood, interstitial fluids)	16,17,18,19,20,21,22,23
Enterococcus faecalis	118271	Feces sample	5,6,7,8,9, 16,17,18,22,23
Citrobacter koseri	150938-3	Fluid of body (saliva, blood, interstitial fluids)	11,12, 13,14,15,16,17,18
Klebsiella pneumoniae	147225	Wound sample	14,15,16,17,18,22,23,24
Alcaligenes	144218	Fluid of body (saliva, blood, interstitial fluids)	1,2 3,4,5,6,7,8,9,10

1- Ampicillin, 2- Ciprofloxacin, 3- Erythromycin 4- Teicoplanin, 5- Tetracycline, 6- Vancomycin, 7- Teicoplanin, 8- Cephazolin, 9- Cloxacillin, 10- Penicillin, 11- Cefazolin, 12- Cefotaxime, 13- Ceftriaxone, 14- Cefuroxime, 15- Gentamicin, 16- Tobramycin, 17- Ceftazidime, Piperacillin, 18- Cephalothin, 19- Clavulan, 20- Clindamycin, 21- Cloxacillin, 22- Ofloxacin, 23- Imipenem, 24- Amikacin

Coating

At each and every step of the product development process, including the conceptualization of the dressings and the determination of the ideal number of antibacterial agents to include, the most current clinical best practices were taken into careful account. The rayon and polyester absorbent core of the dressing that was made for the building of the mupirocin dressing was placed between two sheets of high-density polyethylene mesh. This was done in order to complete the construction of the mupirocin dressing. After that, the dressing was divided into squares with dimensions of about three centimeters each.

The concentration of silver sulfadiazine ultimately reached a value of 0.6 percent after the inclusion of the bacterial inoculum, and it has remained at that value ever since. In order to produce the mupirocin dressing, a dressing was

used that had the very same components and in the exact same proportions as the mupirocin dressing itself. As a coating for the dressing, a very thin layer of mupirocin cream with a concentration of two percent was applied. It was administered as a cream. The layer's overall weight was somewhere around 0.61 grams. The high-density polyethyleneused in the mupirocin-coated dressing was coated with nanocrystalline mupirocin (Neopharma Pharmaceuticals, Abu Dhabi, UAE), but the high- density polyethylene used in the mupirocin nitrate dressing was not. This was the sole difference between the two dressings. The fabrication of both kinds of dressings used the same kinds of components for the rest of the dressing. Both the control dressing and the mupirocin dressing werepreciselythe same size (three centimeters by three centimeters), and they were both constructed using the exact same components.

Design of study

Both the control and test dressings were made in triplicate and placed singly on separate pieces of plastic sheeting that were somewhat larger than the test item. The control dressing served as the standard for comparison. The dressings were inoculated using an aliquot of a bacterial solution of a concentration of 6,200 CFU/ml. Each component of the dressing, with the exception of the mupirocin which did not absorb the inoculum, was covered with an additional piece of plastic sheet and pressed down to ensure that the inoculum came into contact with the active components of the dressing. The inoculate and the dressings were both left to incubate for twenty minutes at a temperature of 37°C. Following the completion of the incubation procedure, the dressings were removed from the incubator and the dressings were then immersed carefully in a bacterial recovery solution that included salt, polysorbate, and sodium thioglycolate [9].

The percentage of sodium chloride in the sodium thioglycolate solution was increased to 10 percent so that the staphylococci and enterococci could be recovered more easily from the dressings. This was done in order to simplify the process of regaining control of the bacteria. In order to gradually dilute the dressings as well as the sodium thioglycolate, vigorous vortexing and the use of phosphate buffered saline were both used. The method that was used to determine the number of viable bacteria that were still present after being exposed to the various dressings was to place the serial dilutions of the bacteria on Mueller-Hinton Agar and count the number of colony-forming units after 24 to 72 hours of incubation at 37°C.

The recovery was verified by simultaneously pouring the same amount of bacterial suspension into a sealed test tube and incubating the culture for a length of time that was comparable to the incubation duration for the inoculum that was applied to the test articles for 24hrs. There was not a discernible difference in the results of the experiment regardless of whether the germs were taken from the test tubes or the control dressing pieces. This would imply that the kind of bacteria that was present at the time may have a significant impact on the degree of healing that a person experienced.

The approach thatwas described above was modified in order to create a time course for the death of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. The dressings were createdin the exact same way as they had been in the past, with the one key difference being that an enough quantity of each form of dressing was manufactured. In order to make it possible to analyze duplicate samples at intervals that had been randomly determined, this step was taken (1, 3, and 4 hours). In addition, the bacteria were first cultivated in the manner that was described earlier, and then they were resuspended to the desired optical density in new tryptic soy broth. This was done so that the tests could be carried out. After everything was accomplished, the inoculum was given to the rats to ensure accurate results.

RESULTS and DISCUSSION

Activity of mupirocin-steroid assay

The bactericidal test that was performed in order to establish the efficacy of topical silver treatments made it possible for an accurate estimate of the impact that the combination of mupirocin and steroid had on the infection. The process also makes it possible to assess the rate of bacterial mortality.

Activity of mupirocin-steroid against bacteria

We investigated whether or not applying silver to wounds using one of three novel approaches was effective against clinical isolates of bacteria that are resistant to antibiotics (Table 2). The mupirocin-steroid coated and mupirocin-steroid cream formulations were able to demonstrate antimicrobial efficacy against a subset of the isolates. However, clinical isolates occurred in each of these varied kinds of mupirocin-steroid treatments, and these products were essentially ineffective against these clinical isolates over the whole-time range of the investigation. The findings also suggest that, with the exception of *Staphylococcus aureus*, mupirocin-steroid sulfadiazine appears to produce a more significant reduction in the number of recoverable live cells than does mupirocin-steroid cream. This is in contrast to the situation with mupirocin-

steroid cream, which produces only a moderate reduction in the number of recoverable live cells as illustrated in the Table 2 which showed that the bacterial number reduced non-significantly after 20 and 30 minutes. When the results of the two therapies were compared, it was clear that this was the case. The nanocrystalline that was included within the mupirocin-steroid-coated dressing was able to exert a significant level of control ($p<0.05$) over the organisms in each of these situations. It is essential to take note of the fact that the method that was employed for the enumeration of live cells did not permitthe detection of less than 200 viable organisms in the dressing material. This is an important pointto take into consideration. Because of this, providing an exact number indicating the degree to which the number of organisms in a particular test was decreased was not possible because it wasnot practical to do so.

In addition, a test was carried out in order to ascertain the impact that serum proteins have on the efficacy of the six distinct formulations of mupirocin and steroid that were used in the study. When the bacteria were suspended in either 60% serum in saline or in 100% normal saline, the results showed that the silver-coated dressing was able to achieve a significant ($p<0.05$) reduction in the number of recoverable organisms that was greater than 8 log10 in magnitude. This was the case despite the fact that a variety of potential solutions were explored (*Pseudomonas aeruginosa 150938-1*). Since this result consistently transpired, the presence or absence of serum in the saline solution was irrelevant to the investigation. When the two different formulations were tested against bacteria that were suspended in serum that was 60% concentration, there was non-significant ($p>0.05$) difference between them.There was no noticeable difference between the two formulations when they were evaluated against each other, and neither mupirocin-steroid cream nor mupirocin-steroid liquid had a significant influence when tested in saline (Table 2).

Table 2. The number of bacteria (CFU) after treatment with antibiotic

Bacteria	Strain	Control	20 min	30 min	p-value
Mupirocin-steroid- coated					
Burkholderia cepacia	UT363	8.1 ± 0.03	<4.0	<3.0	<0.05
P. aeruginosa	137366	8.3 ± 0.02	<4.0	<3.0	<0.05
Pseudomonas spp. (P. cepacia, P. stutzeri, P. maltophilia, and P. putrefaciens).	150938-1	8.2 ± 0.01	<4.0	<3.0	<0.05
E. faecium	118271	8.5 ± 0.04	<4.0	<3.0	<0.05
S. aureus	141960	8.7 ± 0.07	<4.0	<3.0	<0.05
S. aureus	140277	8.3 ± 0.06	<4.0	<3.0	<0.05
Acinetobacter	150938-2	8.9 ± 0.04	<4.0	<3.0	<0.05
Enterococcus faecalis	118271	8.2 ± 0.04	<4.0	<3.0	<0.05
Citrobacter koseri	150938-3	8.8 ± 0.03	<4.0	<3.0	<0.05
Klebsiella pneumoniae	147225	8.6 ± 0.02	<4.0	<3.0	<0.05
Alcaligenes	144218	8.3 ± 0.01	<4.0	<3.0	<0.05
Mupirocin-steroid- cream					
Burkholderia cepacia	UT363	8.1 ± 0.03	<8.0	<6.0	p > 0.05
P. aeruginosa	137366	8.3 ± 0.02	<8.0	<6.0	p > 0.05
Pseudomonas spp. (P. cepacia, P. stutzeri, P. maltophilia, and P. putrefaciens).	150938-1	8.2 ± 0.01	<8.0	<6.0	p > 0.05
E. faecium	118271	8.5 ± 0.04	<8.0	<6.0	p > 0.05
S. aureus	141960	8.7 ± 0.07	<8.0	<6.0	p > 0.05
S. aureus	140277	8.3 ± 0.06	<8.0	<6.0	p > 0.05
Acinetobacter	150938-2	8.9 ± 0.04	<8.0	<6.0	p > 0.05
Enterococcus faecalis	118271	8.2 ± 0.04	<8.0	<6.0	p > 0.05
Citrobacter koseri	150938-3	8.8 ± 0.03	<8.0	<6.0	p > 0.05
Klebsiella pneumoniae	147225	8.6 ± 0.02	<8.0	<6.0	p > 0.05
Alcaligenes	144218	8.3 ± 0.01	<8.0	<6.0	p > 0.05
Mupirocin-steroid- liquid					
Burkholderia cepacia	UT363	8.1 ± 0.03	<15	<10.0	p > 0.05
P. aeruginosa	137366	8.3 ± 0.02	<15	<10.0	p > 0.05
Pseudomonas spp. (P. cepacia, P. stutzeri, P. maltophilia, and P. putrefaciens).	150938-1	8.2 ± 0.01	<15	<10.0	p > 0.05
E. faecium	118271	8.5 ± 0.04	<15	<10.0	p > 0.05
S. aureus	141960	8.7 ± 0.07	<15	<10.0	p > 0.05
S. aureus	140277	8.3 ± 0.06	<15	<10.0	p > 0.05
Acinetobacter	150938-2	8.9 ± 0.04	<15	<10.0	p > 0.05
Enterococcus faecalis	118271	8.2 ± 0.04	<15	<10.0	p > 0.05
Citrobacter koseri	150938-3	8.8 ± 0.03	<15	<10.0	p > 0.05
Klebsiella pneumoniae	147225	8.6 ± 0.02	<15	<10.0	p > 0.05
Alcaligenes	144218	8.3 ± 0.01	<15	<10.0	p > 0.05

Eradication graph

An isolate of methicillin-resistant *S. aureus* and an isolate of vancomycin-resistant *E. coli*, both of which were resistant to vancomycin, were evaluated in order to discover which of the three potential combinations of mupirocin and steroids was the most successful. Both of these bacterialstrains exhibited resistance to the antibiotic vancomycin. The data, which are shown in (Figure 1 and Figure 2), demonstrate the rapidity with which these serious infections were cleared up by using any one of the several mupirocin-steroid therapy protocols studied. In every instance, the use ofmupirocin-steroid cream as well as mupirocin-steroid coated led to a significant decrease ($p<0.05$) in the number of organisms that could be collected and were still alive. This was the case whether or not the organisms had been exposed to the cream or the coated medication. This was the case irrespective of the kind of mupirocin-steroid preparation that was carried out in the experiment. The outcomes were the same regardless of the kind of mupirocin-steroid combination that was used in the experiment.

There was no difference in the result regardless of whether or not the organisms had been given the treatment since this was always the case. There was no difference in the outcome. On the other hand, in none of these situations did the number of organisms reduced to the point where they could no longer be located. Instead, the situation turned out to be the exact reverse of what was expected to occur in accordance with the predictions that were made. As a direct consequence of the mupirocin and the steroid-coated dressing, the number of live organismshad been decreased to an undetectable level in less than twenty minutes. According to these figures, it would appear that using silver in this method makes it easier to quickly eradicate live organisms, which would be consistent with the hypothesis that using mupirocin-steroidin in this manner makes it simpler to quickly eradicate living organisms.

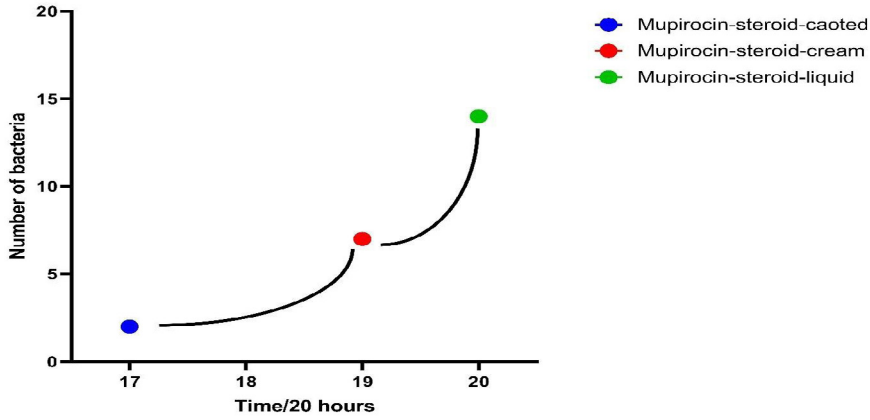

Figure 1. Number of bacteria (CFU) according to the time/ 20 hours

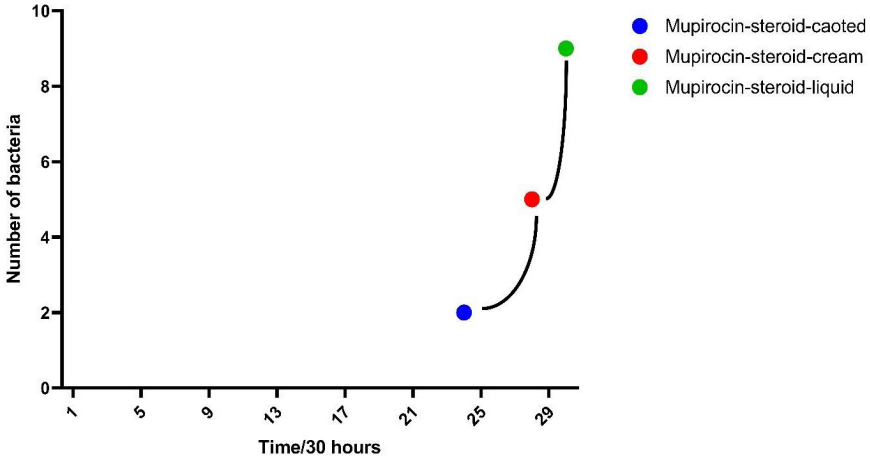

Figure 2. Number of bacteria (CFU) according to the time/ 30 hours

Antibiotic resistance is a worldwide issue that is having the most significant impact on medicine.The identification of organisms that have limited or limited susceptibility to antibiotics is currentlybeing place[1,2]. The issue of antibiotic-resistant microbes colonizing wounds is a concern for the medical profession, particularly in immunocompromised patients. Infections that are resistant to antibiotic treatment are potentially another use for topical mupirocin-steroid[2,3]. This is feasible despite the fact that it is rare for clinical isolates to simultaneously exhibit antibiotic and noble metal resistance[3,4]. The clinical using of isolates for mupirocin-steroid and antibiotic-resistant bacterial strains is not shown in any of the published literature[10]. Since the 19th century, therapeutic

applications of mupirocin-steroid have been carried out. Over the course of a century of clinical use, the safety of mupirocin-steroid combination therapy has been established[11]. Both the mupirocin-steroid cream dressing and the mupirocin- steroid liquid produced no adverse effects when subjected to cutaneous sensitization and irritation tests. Experiments conducted *in vitro* revealed that mupirocin-steroid-coated dressing was muchless hazardous to cells than mupirocin-steroid alone[12]. In a manner similar to that of other heavy metals, mupirocin-steroid poisons respiratory enzymes as well as components of microbialelectron transport systems and interferes with DNA activity[13]. To kill bacteria, mupirocin-steroid must be in the form of a solution, and the efficacy of the solution is directly proportional to the amount of mupirocin-steroid present in it[14]. As a result of their high reactivity, many of the ions found in body fluids, particularly chloride, consume silver ions[15]. In order to treat this condition, topical mupirocin-steroid coated solutions are often used more than one time per day[16]. It's possible that the solution may irritate and tighten the tissues[17]. When using silver nitrate, Klein et al. found that there was limited eschar penetration and browning of the tissue[18]. It was decided to include silver sulfadiazine in order to prevent specific issues that might arise when working with solutions containing silver nitrate[19]. To reduce the number of treatments required to maintain an effective concentration close to a wound, mupirocin-steroid combination medication that isincorporated in a cream base is used[20]. When applied to wounds, mupirocin-steroid coated sulfadiazine causes the tissue to form Pseudo-Eschar and become dry[21]. The removal of cream might be unpleasant for some individuals[22]. Wetting of wounds is encouraged by mupirocin-steroid sulfadiazine, whereas epithelialization is slowed down[23]. Mupirocin-steroid-coated dressings provide a silver concentration that is both effective and long-lasting in the vicinity of a wound[24].

Westaim Biomedical has created a method to sputter silver ions onto a variety of surfaces using their own proprietary technology. It has been shown that an active species of silver included inside the mupirocin-steroid-coated dressing is capable of eliminating a broad variety of germs[25]. According to the data shown in Table 2, physiological chloride concentrations did not have an effect on the efficacy of the mupirocin-steroid-coated dressing. Antimicrobials based on mupirocin and steroids have a reaction with plasma proteins, which disrupts their ability to kill bacteria[26]. In order to determine whether or not serum proteins altered the efficacy of mupirocin-steroid containing wound care products, bacteria suspended in 60% calf serum were studied[27]. As has been stated before, nanocrystalline mupirocin-steroid was shown effective in combating bacterial suspension. According to these findings, serum proteins

may not be able to deactivate nanocrystalline mupirocin-steroid, at least not throughout the time period that was examined[28]. The effect of serum proteins on the other two topicals that were evaluated did not provide any conclusive results since those topicals did not exhibit a significant amount of antibacterial activity after coming into contact with the bacteria under investigation for 20 and 30 minutes[29].

As was hypothesized, Table 2 and Figure 1 and Figure 2 demonstrate that the combination of mupirocin and steroid is effective against a broad variety of antibiotic-resistant bacteria. Because the plasmid for heavy metal resistance, particularly mercury resistance, is associated to antibiotic resistance, other heavy metal antimicrobials are ineffective against antibiotic-resistant bacteria[30]. This is especially true of mercury resistance. In spite of the fact that certain instances of acquired microbiological resistance to silver do occur, particularly with antibiotic, the likelihood of these events happening with mupirocin-steroid coated dressings is much lower[31]. The speed, breadth, and thoroughness with which nanocrystalline mupirocin- steroid kills bacteria should eliminate the possibility of bacterial resistance[5-8]. In the fight to manage antibiotic-resistant infections in wound care, modern mupirocin-steroid treatments may show to be effective tools, particularly those that provide rapid killing of a broad range of bacterialspecies[1-4].

STATEMENT OF ETHICS

The study received approval from the "Scientific Committee in the Department of Pharmacy, Al-Kut University College, Wasit, Iraq" on May 6, 2023 (51/2023).

CONFLICT OF INTEREST STATEMENT

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

Design – Joudah MS, Abbood AZA; Acquisition of data – Ajeel ZH, Hadi HS; Analysis of data – Joudah MS, Abbood AZA; Drafting of the manuscript – Abbood AZA, Ajeel ZH; Critical revision of the manuscript – Ajeel ZH, Hadi HS; Statistical analysis – Joudah MS, Abbood AZA; Technical or financial support – Ajeel ZH, Hadi HS; Supervision – Joudah MS, Abbood AZA, Ajeel ZH, Hadi HS.

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Rasagiline mesylate mucoadhesive buccal microsphere-loaded gel formulations: A new candidate for non-oral Parkinson's treatment

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ABSTRACT

Parkinson's disease (PD) is one of the most common neurological diseases worldwide and affects over 10 million people around the world. Mucosal administration as an alternative to oral administration is very important for the patient's compliance with the treatment. The purpose of this study Rasagiline mesylate (RM) microspheres (MS) and RM MS-loaded gel formulations were developed and evaluated. Particle size, encapsulation efficiency, and loading capacity of MS were evaluated. For buccal administration, mucoadhesive RM MS was dispersed in chitosan (Chi) gels and characterized from the point of viscosity, mechanical, mucoadhesive, rheological, and release properties. The *in-vitro* cytotoxic effects of RM microspheres and RM MS-loaded gel formulation were tested against human embryonic kidney epithelial cells (HEK-293T) and mouse embryonic fibroblast cells (NIH/3T3) lines. The tested formulations did not have toxic effects on cells after 12 hours. RM MS-loaded gel formulation was successfully prepared and evaluated.

Keywords: Parkinson's disease, Rasagiline mesylate, mucoadhesive, buccal delivery, microspheres

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INTRODUCTION

Parkinson's disease (PD), also known as Paralysis Agitans, is characterized as an idiopathic neurodegenerative disorder affecting the central nervous system. It arises from the demise of dopamine-containing cells in the substantia nigra, a central region of the brain. Furthermore, the monoamine oxidase (MAO) enzyme diminishes dopamine levels by catalyzing its breakdown. MAO inhibitors are commonly employed to impede dopamine degradation. In the early stages of Parkinson's disease, monoamine oxidase type B (MAO-B) inhibitors may assist in maintaining synaptic dopamine levels, potentially delaying the initiation of levodopa intake by patients. Particularly in advanced PD cases with levodopa-induced response fluctuations, MAO-B inhibitor drugs could enable the use of lower levodopa doses. Rasagiline mesylate (RM) is a potent, selective, irreversible MAO-B inhibitor devoid of tyramine-enhancing effects and exhibiting neuroprotective activity[1]. It is utilized either as monotherapy in early PD without dose titration or as an adjunct to levodopa in later stages, administered at a daily dose of 1 mg. Literature suggests that rasagiline has an oral bioavailability of 35%, with a relatively short half-life ranging from 1.5 to 3.5 hours. Additionally, RM undergoes extensive liver metabolism mediated by cytochrome P450 type 1A2 (CYP1A2)[2]. Many researchers and clinicians acknowledge that oral therapies for symptom control in Parkinson's disease may become less effective as the condition progresses. Dysphagia is often implicated, but the impact of gastrointestinal issues is increasingly emphasized. Gastrointestinal dysfunctions, including delayed gastric emptying and reduced absorption of oral medications, can contribute to motor and non-motor fluctuations in patients. Given these challenges, physicians must explore alternative treatment approaches for PD patients[3,4]. To address this need, numerous studies have been conducted to develop non-oral drug delivery systems. Non-oral drug delivery through mucosal linings of the nasal, rectal, vaginal, ocular, and oral cavities provides distinct advantages over traditional oral administration for achieving systemic drug delivery. Key benefits include the potential avoidance of presystemic elimination within the gastrointestinal tract and the circumvention of the first-pass hepatic effect[5,6]. The buccal area is a highly acceptable route for patients with excellent accessibility in comparison with other mucous membranes. Buccal mucosa shows good permeability as it contains quite a lot of blood vessels. In addition, it is known to have a robust and rapid healing feature[7]. Therefore, it is an attractive region for drug administration[8]. Because the buccal mucosa has large smooth muscle and relatively immobile mucosa, it is a suitable site for the administration of controlled-release dosage forms. Disadvantages of drug delivery by this route are the low perme-

ability and a smaller surface area. Due to saliva secretion, the persistency of the drug in the region is shortened, and swallowing the saliva prevents the loss of the active substance and its absorption from the targeted area. In addition, involuntary ingestion of the dosage form may lead to suffocation. It is also possible that when using the dosage form, the patient may feel uncomfortable while eating[9]. The use of mucoadhesive drug delivery systems can prevent these problems, and efficiency can be increased by preparing vesicular or particulate systems such as liposomes, nanoparticles, or microspheres (MS) with mucoadhesive properties[10]. Among these systems, MS are larger in size and can provide higher loading capacity. In addition, MS prepared with mucoadhesive polymers can adhere strongly to the mucosa and remain in that area for a long time. However, mucoadhesive gels are seen as a suitable carrier for MS in order to prevent separation from the mucosa by salivary secretion and to provide long-term drug release when applied to the buccal mucous. The main aim of this study was to develop bioadhesive RM MS prepared with Carbopol 2020 NF and Eudragit E100 (EE100) for PD non-oral treatment. Afterward, MS were dispersed in chitosan (Chi) gel to prolong buccal residence time, provide sustained release, and enhance efficiency and bioavailability. Finally, the biocompatibility of novel formulations was tested against mouse embryonic fibroblast (NIH/3T3) and human embryonic epithelial (HEK-293T) cell lines by *in-vitro* cell culture studies.

METHODOLOGY

RM was gifted from Ali Raif Pharmaceutical Industry, Turkey. Chi (viscosity 0,2-0,8 Pa.s) was purchased from Sigma-Aldrich (St Louis, MO, USA). Eudragit E100 and Carbopol 2020 NF were gifted from Evonik Industries (Essen, Germany) and Lubrizol (Wickliffe, OH, USA), respectively. Magnesium stearate was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade. NIH/3T3 and HEK-293T cell lines were purchased by American Type Culture Collection (ATCC), USA. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA were purchased by Biowest, France. Trypan Blue solution was purchased by Biological Industries (Israel), Sterile cell culture plastics and ThinCert inserts were purchased by Greiner Bio-One, Germany. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) cell proliferation reagent was purchased by Roche Diagnostics, Germany.

Fourier transform infrared spectrum analysis

RM, Eudragit E100, Carbopol 2020 NF, Chi and their mixtures (1:1) were mixed homogeneously with potassium bromide and compressed under pressure. The compressed powder mixture was scanned using IR spectrometry at a wavelength of 400-4000 cm^{-1} (Spectrum 100; PerkinElmer, Waltham, MA, USA).

Preparation of rasagiline mesylate mucoadhesive MS

MS were prepared with the solvent-evaporation method[1,2]. After dissolving 3 g of EE100 in 16 mL of acetone, 2 g of Carbopol 2020 NF was then added to this solution as a mucoadhesive polymer. Magnesium stearate (0.6 g) and RM (1 mg of RM in 2 g of gel) were suspended in 8 mL of acetone and added to the polymer dispersion. The resulting dispersion was cooled to 5°C and added slowly to 80 mL of liquid paraffin at the same temperature with stirring at 750 rpm. It was then mixed at 40°C for 50 minutes at 750 rpm. Then it was cooled to room temperature and mixed at 750 rpm and 30 mL of *n*-hexane was added. MSs formed in liquid paraffin were filtered and washed five times with 50 mL of *n*-hexane and dried at room temperature overnight. Obtained MS were sieved with 75, 125 and 250 μm sieves, and MS at 125 μm were used for further testing due to high production efficiency.

Characterization of microspheres

Particle size distribution

Mean diameters of RM MS were concluded with a Malvern Mastersizer 2000 (Malvern Instruments, Malvern, UK). The measurement was made while the MS suspended in *n*-hexane were mixed at 2000 rpm. All trials were run in triplicate.

Scanning electron microscopy

First, MSs were coated with gold palladium (Au/Pd) using a vacuum evaporator, and the surface morphology was examined by scanning electron microscopy at 5 kV pressure. (Thermo Scientific Apreo S, Germany).

Encapsulation efficiency and drug loading of microspheres

Drug-loaded MSs (0.5 g) were dissolved in 40 mL ammonium acetate buffer solution (pH 5.8) and mixed at 200 rpm for 24 hours at ambient temperature with horizontal shaker. The solution was filtered through a 0.45 μm syringe filter and the filtrate analyzed by high performance liquid chromatography (HPLC) (Thermo Scientific Accela, USA) to determine the amount of RM loaded in the MS. Encapsulation efficiency (%) and drug loading (%) were calculated according to the following equations.

Encapsulation efficiency (%)[1]: (Total Free RM-Total RM/ Total Free RM) 100

Drug loading (%)[2]: (Total RM-Free RM/Total amount of formulation components) 100

A fully validated HPLC method was used for RM assay using a Kromasil C18 (2504.6 mm, 5 µm) column at 25°C. Ammonium acetate buffer: Acetonitrile mixture (40:60) was used as mobile phase from HPLC studies. 50 µL of sample was injected into the system at a flow rate of 1 mL/min. The wavelength was 265 nm[3].

Preparation of Chi gels

To prepare mucoadhesive Chi gel, 3% (w/w) Chi was dissolved in 1% acetic acid solution. In order to obtain a clear gel, it was kept at ambient temperature for 24 hours and then, the calculated amount of MSs according to the encapsulation efficiency results were mixed and dispersed in the gel formulation until it became homogeneous (0.045 g MS/2 g gel)[2].

Characterization of MS loaded gel formulation

Mechanical and mucoadhesive properties

Mechanic tests and bioadhesion studies have been done with software-controlled penetrometer (TA-XT Plus texture analyzer; Stable Micro Systems, Godalming, UK). Test conditions are stated Table 1[2].

Table 1. Test conditions

Conditions of mechanic test	Conditions of mucoadhesion study
Prob: Perspex (10 mm diameter)	Prob: Perspex (10 mm diameter)
Cell load: 0.5 kg	Cell load: 0.5 kg
Speed of probe before test: 2 mm/s	Speed of probe before test: 1 mm/s
Speed of probe during test: 2 mm/s	Speed of probe during test: 1 mm/s
Speed of probe after test: 2.5 mm/s	Speed of probe after test: 2.5 mm/s
Target mode: Distance	Return distance of the probe: 20.000 mm
Penetration distance of the probe: 15.0 mm	Contact time between gel and buccal mucosa: 60 s
Force that the probe should feel to start the test: 0.05 N	Force applied during the contact of the gel with the buccal mucosa: 6000 mN

*Dx (10) refers the particle diameter of 10% of the sample volume, Dx (50) refers the particle diameter of 50% of the sample volume Dx (90) refers the particle diameter of 90% of the sample volume.

Rheological properties

The rheological properties of the formulations were characterized as described in the literature using a TA TX Discovery HR1 rheometer (TA, USA) at 25 and 37 ± 0.1°C[4].

To determine the storage modulus (G') and loss modulus (G"), oscillatory analysis of the formulations in the linear viscoelastic region was performed as indicated in the literature. Studies were carried out at 25 and 37 ± 0.1°C using a 40 mm diameter steel probe, 0.3 mm gap[5].

In-vitro release studies

Chi gels (2g) containing 1.56 mg of RM MS were placed into dialysis membranes (MW 12,000–14,000 Da). These membranes were then immersed in 35 mL of artificial saliva fluid (composed of disodiumhydrogenphosphate dihydrate 2.38 g, Potassium dihydrogenphosphate 0.19 g, sodium chloride 8 g, with distilled water q.s. to 1000 mL) to simulate the conditions of the buccal area and stirred at 300 rpm. The amount of artificial saliva fluid and the formulation introduced into the dialysis membranes was determined based on sink condition calculations.

At predetermined time intervals, samples were withdrawn. To maintain a constant volume of the medium, an equal volume of ambient liquid was added to the medium, thereby ensuring the maintenance of sink conditions[11]. Drug content was analyzed using the developed HPLC method (n=6).

Ex-vivo permeation studies

Permeability studies were performed using vertical jacketed Franz-type diffusion cells (Logan, Germany) with a diffusion area of 0.384 cm^2. As a result of the literature review, it is seen that the bovine cheek can be used to mimic the human buccal area in buccal permeation studies[6,7]. As the membrane model, mucosa samples taken from the inner cheeks (buccal area) of freshly slaughtered bovine for human consumption obtained from the local slaughterhouse were used. After collection, samples were immediately placed in PBS (pH 7.4), transferred to the laboratory in a refrigerated transport box within 1 hour, surgically processed to remove excess fat and connective tissue within 2 hours of animal sacrifice, and then stored at -20°C. Before starting the run, samples were equilibrated at room temperature and held for approximately 1 minute in pre-warmed 60°C PBS (pH 7.4). The connective tissue was then carefully peeled from the mucosa to obtain heat-separated epithelium along with the intact basal lamina. A digital micrometer was used to see if the mucosal thicknesses were homogeneous (250 μm). The samples were then incubated in PBS for approximately 3 hours at room temperature and the medium was refreshed

with fresh PBS every 15 minutes. Appropriately sized tissue sections were placed in vertical Franz-type diffusion cells, artificial saliva fluid was added to the recipient chambers, and allowed to equilibrate for 1 hour at 37 ± 0.1°C. Formulations weighing 0.3 g were placed on the mucous membranes and the study began. At certain time intervals, samples (1 mL) were taken from the receiving chamber and added to the medium containing 1 mL of artificial saliva fluid at 37 ± 0.1°C to maintain the sink conditions. The study was carried out at 37 ± 0.1°C for 24 hours. The amount of RM passing through the bovine buccal mucosa was analyzed by validated HPLC method. And membrane integrity was evaluated. A graph was drawn between the % cumulative amount of rasagiline mesylate passing through the buccal mucosa and the time (h). The steady state flux, Jss (μg/cm^2.h), was determined from the slope of straight line of the plot. All data were presented as mean ± standard deviation (n=6)[8].

In-vitro cytotoxic assay

Novel MS and MS-loaded Chi gel formulations' effects on cell viability against HEK-293T and NIH/3T3 cells by WST-1 reagent[9]. To cell culture treatments, formulations were extracted in DMEM. For this purpose, 0.025 g MSs and 5 mL DMEM were added into a sterile tube. In another tube, 2 g MSs-loaded Chi gel was added into a 4 mL DMEM medium. Tubes were incubated in an ultrasonic water bath for 8 hours at 37°C. Then, the tube contains filtered through a 0.22 μm sterile syringe filter.

Cells were grown in 2 mM L-glutamine and 10% FBS supplemented DMEM in conventional cell culture conditions. The day before the assay, cells were counted and seeded into the 12-well ThinCert™ plates at a density of 5x10^5 cells. Plates were incubated in a conventional CO_2 incubator overnight[10]. After the medium was replaced with 1 mL of fresh DMEM, ThinCert™ inserts (0.4 μm pore size) were placed into each well. Then 0.5 mL of filtered formulation extracts were added into inserts. Cells were treated with formulations for 12, 24 and 48 hours. Then inserts were discarded. The cell medium was replaced with a 0.5 mL WST-1 reagent prepared in fresh DMEM (10%) and incubated for four hours. The intensity of the red color formed in the wells, correlated with the number of viable cells, was measured in the spectrophotometer (CLARIOstar Plus, BMG LabTech) at 450 nm. Cell viability was expressed as the percentage of formazan absorbance. Results were given as mean of three different experiments plus minus standard deviation (Mean ± SD, n=3). Statistical analysis of the results was carried out by one-way ANOVA analysis in GraphPad Prism 5.0 statistical package program. The level of significance was accepted as $p<0.05$.

Stability studies

The stability of formulation was investigated at two temperatures and relative humidity (25°C ± 2°C/60% RH, 40°C ± 2°C/75% RH). Formulations were placed in the stability cabinet (Nüve TK 252, Türkiye) in a coated aluminum tube. Organoleptic properties, RM amount, flow properties and viscosity of the optimum formulation were evaluated at 0, 30 and 90 days.

RESULTS and DISCUSSION

It is widely recognized by researchers and healthcare professionals that with the progression of PD, conventional treatments for symptom control may become less effective. There may be different reasons for this result, but the importance of gastrointestinal system-related problems such as delayed gastric emptying and decreased absorption of oral treatments is better understood over time. If alternative treatment modalities for PD by-pass the gastrointestinal tract as with non-oral treatments, it may be valuable in patients who develop motor complications such as severe motor and/or non-motor fluctuations despite optimized oral therapy, particularly in patients with gastrointestinal absorption problems including gastroparesis. The buccal route can directly deliver a drug into the systemic circulation, avoiding gastrointestinal degradation and bypassing first hepatic metabolism. In addition, the buccal area can reach the area of 50 cm^2 for drug permeation. Nevertheless, efficient drug delivery through buccal mucosa has several disadvantages such as a low drug permeability and a low drug residence time. To overcome these limitations, the use of bioadhesive MS can be a useful approach to increase drug permeation and expand residence time[12].

FTIR analysis

According to the literature, the results obtained from the FTIR analysis were found to be appropriate and it was observed that there was no interaction between RM and the polymers (data not shown).

Preparation and characterization of RM MS

RM MS were efficiently prepared by solvent evaporation method[2]. At beginning, several bioadhesive polymers used for preparation of MS. The shapes of the MS were non-uniform and asymmetrical when prepared with Eudragit RS 100 and different type of HPMC (data not shown). When MS was prepared with Eudragit E100 and Carbopol 2020NF polymers, uniform and spherical particles with narrow size distribution and good surface properties were obtained. This is our final formulation was determined for future studies. The surface morphology of MS was visualized by scanning electron microscopy and

most MS were spherical shape with uneven surface morphology and the surface was free of drug crystals (Figure 1).

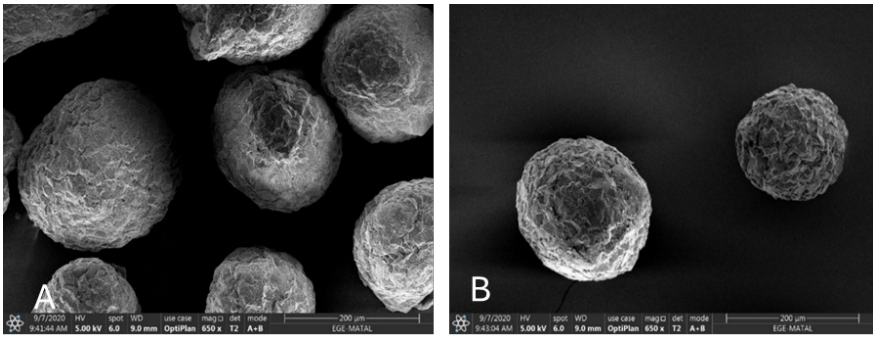

Figure 1. (A) Scanning electron microscopy of blank MS, (B) Scanning electron microscopy of RM loaded MS.

The particle size and distribution of MS were examined by laser-light scattering (Table 2).

Table 2. Particle-size distribution of MS

Formulation Code	Dx (10) µm	Dx (50) µm	Dx (90) µm	Span
Blank MS	150 µm ± 4.6	284 µm ± 5.2	503 µm ± 5.5	1.486
RM loaded MS	134 µm ± 3.2	196 µm ± 4.9	279 µm ± 5.0	4.839

When the results were examined, it was seen that 50% of the RM-loaded MSs had particle sizes of 196 µm, and 50% of the empty MSs were 284 µm in size. Span value was determined as 1,486 for blank MS and 4,839 for RM loaded MS. A span value close to 0 means that the particles are more uniform, and the dimensional consistency is better. However, the span value depends on the characteristics of the sample and there is no definite principle regarding this value. It is seen in the literature that some examples have significantly large span values[13].

MSs with RM-loaded high drug encapsulation efficiency and loading capacity could be produced. The encapsulation efficiency and loading capacity were found to be 85.20 ± 1.1% and 3.45 ± 0.04%, respectively. However, MSs were produced with high production efficiency (88.13% for total produced MSs and 59.75% for 125 µm size particles). In a study by Toksoy et al., RM solid lipid nanoparticles were prepared for nasal application. The encapsulation efficiency for RM solid lipid nanoparticles was found to be 37.8 ± 0.596%. It was deter-

mined that the remaining RM was in the aqueous phase of the formulation[14]. Upon examining the previous literature, it can be concluded that, based on the obtained values for encapsulation efficiency, loading capacity, and production efficiency, solvent evaporation is a simple and suitable technique for producing RM-loaded microspheres.

Preparation and characterization of MS loaded gel

It is requested that the applied gel adheres to the mucosa and remains there. Designing buccal formulations using bioadhesive biomaterials that strongly adhere to the buccal mucosa prevents washing of the carrier during eating and with tongue movements and increases the persistency of the drug in the target area. Therefore, MS were suspended in bioadhesive chitosan gel to prolong residence in the buccal mucosa. For this purpose, gel formulations of chitosan at different ratios and molecular weights (low, medium and high) were prepared. For this purpose, medium molecular-weight chitosan at 2% concentration was chosen as the gel base because of its strong mucoadhesiveness, proper mechanical and rheological characteristics (data not shown).

Flow properties, viscosity and oscillation properties of the formulation are important in terms of applicability. When it comes into contact with the buccal mucosa, it is desired that the formulation has a strong gel structure and high viscosity value. Thus, the formulation is desired to remain in the buccal mucosa for a long time and help the diffusion of the active substance.

Pseudoplastic flow is observed in systems whose viscosity decreases as shear rate increases. In this type of flow, a constant viscosity cannot be mentioned and generally observed in gel and emulsion systems. The selected formulation showed pseudoplastic flow (Figure 2), indicating that the viscosity of the gel formulations decreased as they were mixed, with shear thinning behavior demonstrated at both temperatures (Figure 3). When previous literatures examined, we observed that the viscosity of our MSs loaded gel formulation were suitable for buccal application[15].

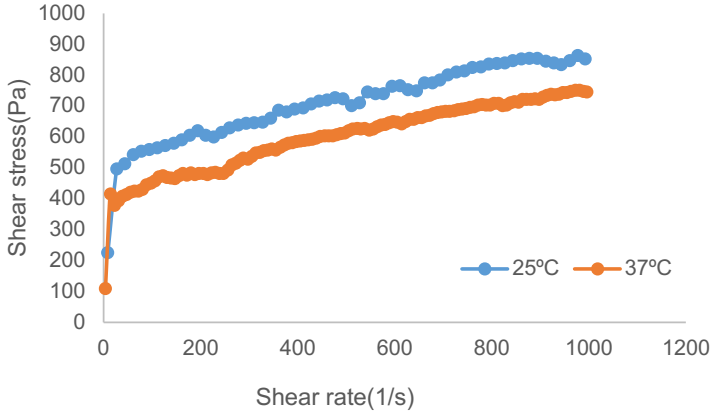

Figure 2. Shear rate–shear stress curve for the blank gel formulation at 25°C and 37°C

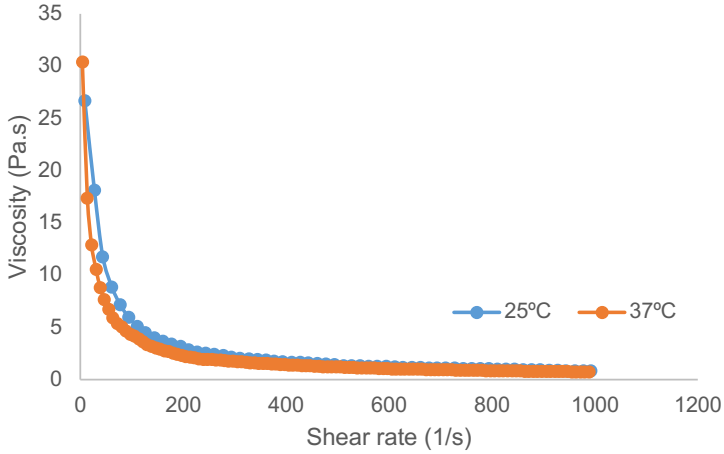

Figure 3. Shear rate–shear stress curve for the RM MSs loaded gel formulation at 25°C and 37°C

Additionally, within the scope of the rheological studies, oscillation tests that are a dynamic method were performed to determine about viscous and elastic properties of the formulations. During these measurements storage modulus G′ (elastic response) and the loss modulus G″ (viscous response) curves were obtained at two different temperatures. In Figure 4 and Figure 5 the plots of G′ and G″ were shown as a function of frequency at two different temperature values. G′ and G″ moduli of MSs loaded gel formulations were high at room temperature and decrease significantly at body temperature. At both temperature values, G′ dominated G″. It has been observed that the gap between the

two modules widens with increasing frequency. When these results were evaluated, it was seen that the prepared MS loaded gel formulation had a strong gel structure[2].

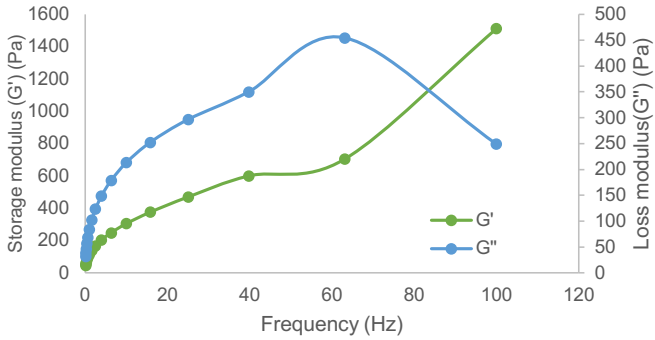

Figure 4. Frequency-dependent changes of the viscoelastic properties of RM MSs loaded gel formulation at 25°C

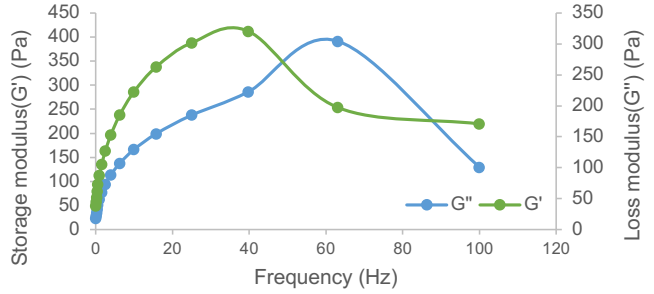

Figure 5. Frequency-dependent changes of the viscoelastic properties of RM MSs loaded gel formulation at 37°C

Gel formulations must have some suitable mechanical properties such as high adhesiveness, ease of application to the surface, low hardness and good adhesion in the application area to provide an effective treatment. TPA is a very useful device for evaluating these mechanical properties of gel formulations. Therefore, the mechanical properties of the blank and MS-loaded gel formulations were characterized in terms of hardness, compressibility, adhesiveness, elasticity and cohesiveness using TPA. The mechanical properties of the formulations are presented in Table 3.

Table 3. Mechanical properties of blank and RM MSs loaded gel formulations

Code	Hardness (g) ± SD	Compressibility (g.s) ± SD	Adhesiveness (g.s) ± SD	Cohesiveness ± SD	Elasticity ± SD
Blank gel	0.71 ± 0.01	0.99 ± 0.017	1.40 ± 0.07	0.84 ± 0.004	0.52 ± 0.059
MSs loaded gel	2.04 ± 0.01	4.31 ± 0.210	18.02 ± 1.520	0.97 ± 0.008	1.04 ± 0.06

It is important for the hardness value to be low, enabling easy application of the formulation and ensuring good spreadability. The compressibility value should be low so that the patient can easily take the formulation from the container during use. Higher adhesiveness behavior is significant to provide great adhesion and high drug retention in the buccal mucosa. Elasticity represents the return rate of the deformed sample to its beginning condition. Also, cohesiveness shows the effect of repeated shearing stresses on the formulations. As shown in Table 3, depending on adding MSs, hardness, compressibility and adhesiveness values of formulation increased significantly, and this was thought to be associated with the motion of the MSs. These results were in accordance with rheological evaluations. Based on the results, it appeared that the addition of MSs strongly improve the mechanical properties of gels. When the results of the mechanical properties were examined and their compatibility with the literature was evaluated, it was seen that prepared formulation had acceptable mechanical properties for mucosal application[16].

When the results of mucoadhesion studies presented in Table 4 were examined, adding the MSs in gel, caused to increase in the mucoadhesion. This result showed that the developed formulation can provide drug release at the buccal mucosa having the appropriate mucoadhesive properties.

Table 4. Mucoadhesive properties of blank and RM MSs loaded gel formulations

Code	Force (mN) ± SS	Mucoadhesion (mN.mm) ± SS
Blank gel	22.03 ± 2.01	12.45 ± 3.25
MSs loaded gel	39.57 ± 2.33	19.02 ± 1.21

The *in-vitro* release profile of RM MS-loaded gel formulations were examined in artificial saliva at 37 ± 0.1°C with dialysis bags, and results are displayed in Figure 6.

MS has the potential to be used for targeted and controlled release drug delivery in general; however, the addition of bioadhesive properties to MS also has significant additional advantages such as closer contact with the mucus layer,

efficient absorption of drugs and also improved bioavailability due to targeting of drugs to the absorption site[11]. Prolonged release of drugs and a reduction in frequency of drug administration can highly improve the Parkinson's patient compliance. Gel formulations are systems that reduce the release rate of the active substance compared to colloidal systems. For RM MS-loaded gel formulation, the gel system must first be eroded and then the active substance must be released from the MS. In one study, RM transfersome loaded in situ gel formulations were developed for nasal application. When RM *in-vitro* release was examined, RM release was found to be between 64.42-100.25% at the end of the 8th hour. In the same study, it was determined that the drug was completely released from the RM dispersion prepared after 30 minutes[17]. The formulation developed in our study were evaluated for their release rate. As seen in Figure 6, the formulation released ~80% after 24 hours and initial burst release was not apparent, which was related to MS structure. The slow release of the RM from the formulation suggests homogeneous entrapment of the drug throughout the systems and could have the potential to contribute to a lower dosing frequency.

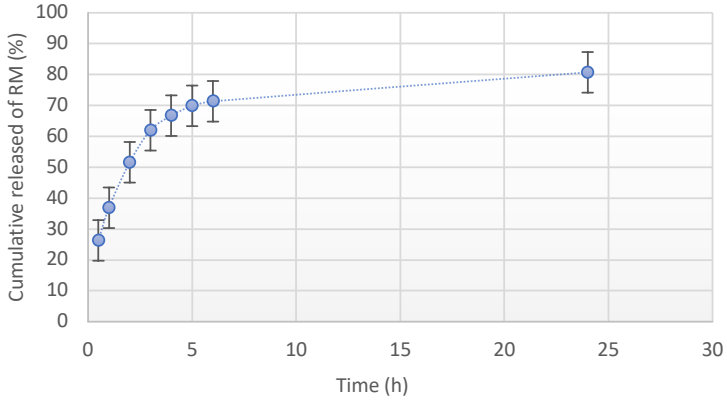

Figure 6. *In-vitro* release profile of RM MS-loaded gel formulation

Ex-vivo permeation study

Drug molecules diffuse through the buccal mucosa in two different ways: paracellular and transcellular. Paracellular transport takes place in the intercellular space between buccal epithelial cells. On the other hand, transcellular transport occurs by the transport of drug molecules from different cell layers[8]. It is known that RM is an active substance belonging to BCS class III. This shows that RM has high solubility and low permeability. In the results of permeability studies in the literature, it is seen that the pure RM solution has low flux val-

ues[18]. Within the scope of our study, the flux was found to be 86.44 μg/cm²/h ± 4.34 for the RM MS-loaded gel formulation. At the end of the 24th hour, the % cumulative permeated RM was determined as 20% ± 1.54. The permeation graph is shown in Figure 7.

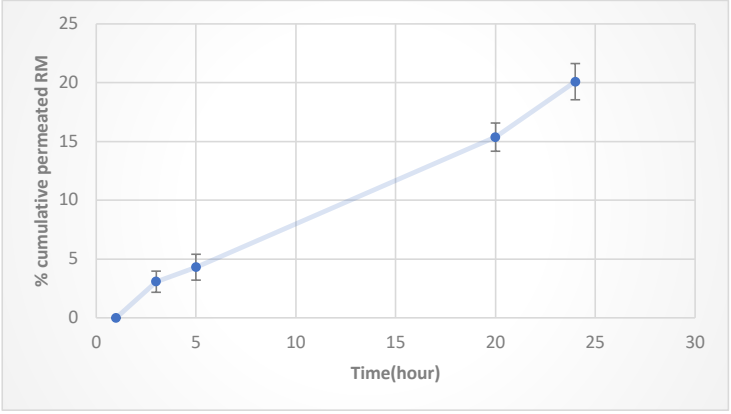

Figure 7. RM permeation profile through the buccal mucosa

During *ex-vivo* studies, it is thought that the small volume of dissolution environment in the donor chamber and the fact that the upper part of the mucosa is not moistened by saliva reduces swelling of the formulation, resulting in slower drug release in contrast to dissolution studies. Similar results have been obtained from different studies[19].

Cell culture and *in-vitro* cytotoxicity studies

To determine the biocompatibility of novel pharmaceutical carrier formulations, *in-vitro* cell culture cytotoxicity experiments provide valuable preliminary data[20]. Therefore, in this study, we investigated the cytotoxic potential of the novel MSs, RM loaded MSs, blank chitosan gel (Blank gel), blank MSs added chitosan gel (MSs-loaded gel), and RM MSs loaded gel formulations against HEK-293T cell and NIH/3T3 cells by WST-1 method. Obtained data are represented in Figure 8 and Figure 9. In experiments performed on HEK-293T cells, it was determined that the tested formulations did not show any cytotoxic effect on these cells at 12- and 24-hour incubation times ($p>0.05$). Similarly, at the end of the 48-hour treatment, no evidence of cytotoxicity was found in the blank gel and MSs-loaded gel formulations treated groups (97.68 ± 2.49% and 93.52 ± 3.19%, respectively) ($p>0.05$). In contrast, blank MSs, RM/MSs and RM/MSs-loaded gel treatment caused a significant decrease in HEK-293T cell viability compared to the control cells (93.47 ± 1.98%, 92.84

± 1.91%, and 93.52 ± 1.71%, respectively) ($p<0.05$). On the other hand, it was also determined that the cell viability in all tested groups was 90% and above.

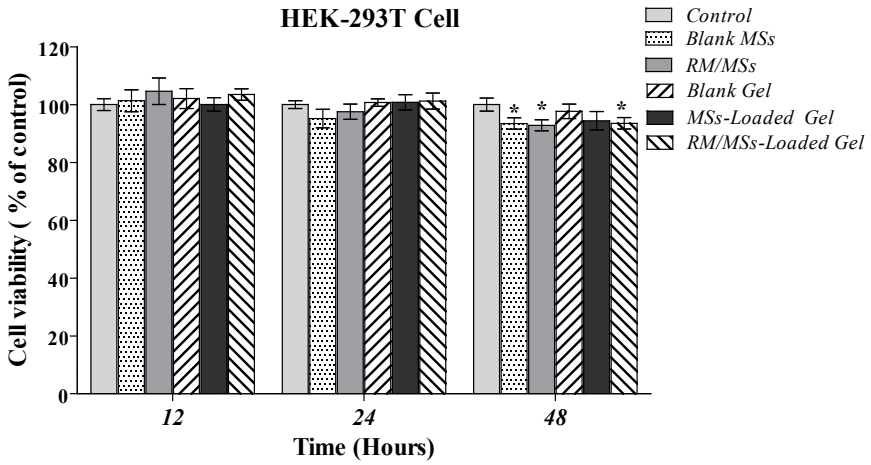

Figure 8. Cell viability evaluation following WST-1 assay on HEK-293T cells treated with tested formulations*

*The bars show the % cell viability compared to the control. Data expressed as mean ± S.D. (n=3). Cell viability significantly decreased compared to control cells, $p<0.05$.

As shown in Figure 9, in experiments performed on NIH/3T3 cells, similar to HEK-293T cell, after 12-treatment with formulations, % cell viability values were observed similar to control in all formulations tested ($p>0.05$). Among the cells treated for 24 hours, cell viability was determined to slightly decreased in the only blank gel treated group compared to the control (82.54 ± 2.58%, $p<0.05$). Controversially, it was observed that all the tested formulations were caused a significant decrease in NIH/3T3 cells at the 48 hours ($p<0.05$).

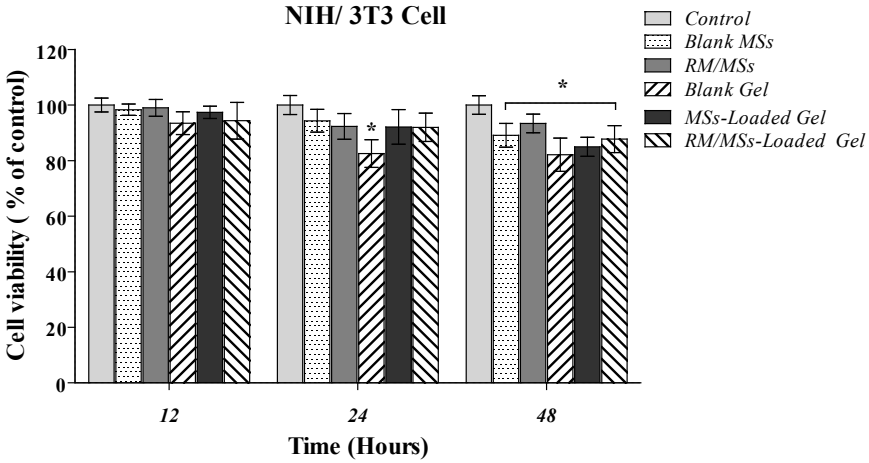

Figure 9. Cell viability evaluation following WST-1 assay on NIH/3T3 cells treated with tested formulations*

*The bars show the % cell viability compared to the control. Data expressed as mean ± S.D. (n=3). Cell viability significantly decreased compared to control cells, $p<0.05$.

Buccal formulations are drug delivery systems that stand out with their very short application times and rapid release of the active ingredient. The fact that no toxic effect of any formulation tested in the cytotoxicity studies we conducted was detected within 12 hours of the treatments reveals that these novel developed formulations are biocompatible for drug carrier systems.

Stability studies

Drugs must be effective and safe throughout their shelf life and until they reach patient use. Stability is one of the most important quality indicators. When the stability of a drug is mentioned, many factors are evaluated together. For example, the chemical properties of the active substance forming a dosage form, the interaction of the excipients in the formula with the drug molecule, the possibility of the product to encounter external environmental conditions such as high temperature, light and humidity determines the stability of the pharmaceutical product. Stability is a prerequisite for the pharmacological effect expected from an active substance[21]. In the light of the stability data obtained, it was observed that there was no significant change in the amount of active substance of the RM MS-loaded gel formulation. In addition, in the rheological examination, there was no significant difference in flow properties and viscosity (data not shown).

RM MS-loaded gel formulation could be a good alternative to conventional therapy of RM to overcome the limitations of the oral application and also reduce the dose-dependent side effects. A spherical and uniform RM MS with an

average diameter of 196 μm, a drug loading of 3.45 ± 0.04% and an encapsulation efficiency of 85.20 ± 1.1% could be successfully prepared by solvent evaporation. It was also possible to produce MS with 88.13% efficiency with this method. *In-vitro* dissolution experiments revealed that the dissolution of RM was slowly released from MS loaded gel formulation over 24 h duration. The results obtained revealed that MS-loaded gel formulations exhibited suitable properties for buccal administration of RM, with their strong gel structure, desirable mechanical, bioadhesive, and *in-vitro* properties. According to the results of *ex-vivo* permeation study, the flux was found to be 86.44 μg/cm^2/h ± 4.34. Additionally, the % cumulative permeated RM was determined as 20% ± 1.54 for the RM MS-loaded gel formulation. Permeation enhancers like Isopropyl myristate, Hyaluronidase, Ethanol, Oleic acid, Polyethylene glycol 400 and propylene glycol can be tried to increase the cumulative permeation of RM from the buccal mucosa, or it can be suggested to use a hydroalcoholic gel formulation as a gel formulation[21-22].

Cumulatively, the results suggested that mucoadhesive RM MS-loaded gel formulation could be used as a possible alternative to conventional treatment of PD disease. In addition to the results of *in-vitro* characterization studies and cell culture studies conducted in the current study, it is planned to demonstrate the effectiveness by performing *in-vivo* animal experiments in our future studies.

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 no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

MG, FAK, SYK designed the study. MG, GT, FAK worked on literature search. MG, GT, FAK, SYK conducted the experimental work and collected the data. MG, FAK, SYK analyzed and interpreted the data. MG, GT, FAK, SYK wrote the draft of manuscript. ÖÖ supervised the study. All authors involved in revising the final manuscript.

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Isolation and quantification of anthocyanins from red cabbage (*Brassica oleracea* L.) and its potential uses as antioxidant in natural food

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ABSTRACT

Red cabbage (*Brassica oleracea* L. *var. capitata*) is a widely cultivated vegetable known for its diverse varieties and health benefits. This article focuses on the extraction and analysis of anthocyanins, phenolic compounds, and antioxidant activities from red cabbage powder and paste. The physicochemical properties of red cabbage, including its nutritional composition, were evaluated. The anthocyanin content was found to be highest in red cabbage paste in water, followed by red cabbage powder in water, while the lowest level was observed in red cabbage powder in methanol. The total anthocyanin content in red cabbage was determined to be 78.47 mg/100g. The total phenolic content was highest in red cabbage paste in methanol, followed by red cabbage paste in water. The antioxidant activity was assessed using the DPPH assay, showing promising results for red cabbage extracts. The findings demonstrate the potential of red cabbage as a natural source of anthocyanins and phenolic compounds with antioxidant properties, which can be utilized in various applications, including the food industry.

Keywords: *Brassica oleracea* L., DPPH, antioxidants, anthocyanin

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INTRODUCTION

One of the most significant vegetables farmed worldwide is cabbage (*Brassica oleracea* L. *var. capitata*). It is a member of the Cruciferae family, along with Brussels sprouts, broccoli, cauliflower, and kale[1]. In terms of the size, shape, color, and texture of the leaves as well as the size, shape, color, and texture of the head, the different cultivated varieties of cabbage exhibit enormous variability. The various cultivars of cabbage are divided into three categories: white, red, and savoy cabbage[2]. People across the world use brassica vegetables in the human diet[3]. Numerous epidemiological and clinical researches have shown that cabbage is good for your health[4,5]. Red cabbage is a blooming plant that is herbaceous, biennial, and dicotyledonous. Its leaves are typically served as coleslaw, salad, and beverage[6]. Red cabbage stands out for its flavour, colour, and texture. It was first grown and harvested in Europe, but it is now grown and harvested all over the world. This vegetable contains a lot of micronutrients and phytochemicals, like glucosinolates, vitamin C and K, beta-carotene, minerals, fibre, and total polyphenols[7]. Red cabbage is becoming increasingly popular across the world, and it is consumed raw as well as after technical and home treatment[8].

The most important pigments found in vascular plants are anthocyanins (anthos, which means flower and kianos, which means blue). Because they are non-toxic and simple to absorb into aqueous mediums, anthocyanins are attractive as natural water-soluble colourants[9]. Aglycone, acylated and glycosidic anthocyanins are all absorbed by people. Anthocyanins are ingested by humans at levels that may be physiologically relevant as components of plant food items. A rise in interest in these polyphenols was caused by this discovery as well as the pro-health qualities of anthocyanins (anticancer, cardioprotective, eyesight-improving, antidiabetic)[10]. Anthocyanins have been shown to have several potential health benefits, including a reduced risk of chronic illnesses like cancer and coronary heart disease[11], obesity and type 2 diabetes prevention[13], vision and acuteness enhancement[12], and anti-allergic and antibacterial activities[14]. Red cabbage has a distinct anthocyanin pattern. It includes a substantial number of anthocyanins, the primary structure of which is cyanidin glycosides usually. However, most anthocyanin compounds in red cabbage are acylated[15]. When compared to anthocyanins from other natural sources, red cabbage anthocyanins represent the colour throughout a wide range of pH values, which is extremely appealing to the food industry as natural food colourants. It is critical to pick a suitable approach with high anthocyanin recovery from red cabbage, with organic solvent extraction being the most often used extraction method[16]. Red cabbage's anthocyanins can be used in neutral foods as well as acidic ones because they range in colour from red at low pH to blue and green at high pH. They therefore have the poten-

tial to offer a natural substitute for artificial colourants[17].

Red cabbage contains more than 150 flavonoids, among which anthocyanins are abundant. Among the Brassica species, red cabbage is the vegetable with the best antiradical defence system. Food oxidation can be inhibited or controlled by antioxidant substances. The main antioxidants in red cabbage are phenolic compounds. Their chemical activity is proportional to the quantity of hydroxyl groups they contain. They are natural antioxidants with numerous roles, including free radical scavengers and potential pro-oxidant producers[18]. During a reaction, the activation energy of the radial molecule is very less it is composed of a chain reaction of kinetic property. The free radicals are prevented from causing any harm with the help of antioxidants. Since the antioxidants are composed of phenolic hydroxyl groups, these groups react with the present free-radical molecule, thus producing a stable product i.e., imiquimod radical. Another way to stop the free radical from creating harm is to reduce itself and donate an electron to the radical[19,20].

The purpose of the current study is to isolate and quantify the anthocyanins from red cabbage along with their antioxidant activity.

METHODOLOGY

Plant material

Three fresh red cabbages were acquired at a nearby grocery, cash and carry Punjab society in Lahore, Pakistan. The red cabbages were washed and cleaned under running water to eliminate any signs of dust and contaminants[21]. Following that, raw red cabbage (1770g) was weighed and sliced into small pieces. The chopped cabbage was then placed in a polythene bag and refrigerated for later use.

Chemical and reagents

All additional chemicals used in the current experiment were analytical graded, except for the following: sodium carbonate, the Folin-Ciocalteu reagent, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), ethanol, and methanol, which were all acquired from sigma Chemical Co.

Extraction procedure

Two procedures were used to extract the red cabbage powder:

In the first method, fresh red cabbage was sliced and weighed before being placed in a tray and dried in a dry oven at 50 degrees Celsius for 56 hours. After drying, the dried cabbage was converted into a fine powder.

The second method used to create red cabbage powder was solvent extraction.

In this procedure, 200g of chopped cabbage was placed in a flask along with a 1:1 combination of 250 mL ethanol and 250 mL water (v/v) and covered with foil and plastic wrap for 1 week. After a week, the red cabbage colour was released into the solution, which was then dried and pulverized in a dry oven. A red cabbage paste was obtained at the end[22].

Physiochemical analysis

AOAC techniques were used to measure physicochemical properties such as moisture, ash, fat fibre, and protein content of fresh red cabbage and red cabbage powder, and then the measured parameters were compared.

Isolation and quantification of anthocyanins

The colours (purple, blue, and red) in many plants are caused by anthocyanins, which are water-soluble flavonoid pigments that come in a spectrum of hues ranging from red to blue depending on the pH of the vacuole water. Anthocyanins are becoming more and more important as antioxidants in addition to serving as food colouring. Several therapeutic benefits, including as Vaso protective and anti-inflammatory properties, have been associated with anthocyanins[23].

The total monomeric anthocyanin was calculated using the pH differential method. Using pH 1 (0.025 M potassium chloride) and pH 4.5 (0.4 M sodium acetate) buffers, the extract was diluted 100 times. 15 minutes were given for the solutions to equilibrate in the dark. On cuvettes with a 1 cm path length, absorbance was measured at 530 and 700 nm using a Shimadzu UV/visible spectrophotometer[24].

The following formula is used to calculate the anthocyanin content:

$$Abs = (A_{530} - A_{700}) - (B_{530} - B_{700})$$

$$\frac{Abs \times Molar\ Facror \times 1000 \times (dilution\ factor)}{29600 \times 1}$$

Determination of total polyphenols content

A modified Folin-Ciocalteu technique was used to measure the number of total polyphenols in the sample[25]. 250 L of Folin-Ciocalteu reagent and approximately 50 L of each sample's diluted extract were blended with minor adjustments. 1.5 mL of sodium carbonate solution (7%) was added after 5 minutes, and the mixture was shaken occasionally for the next 30 minutes. The absorbance of the resulting solution was measured at 760 nm. Milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW) were used to assess the amount of phenol in the sample[26].

DPPH assay

The hue of the organic nitrogen radical, DPPH free radical, is dark purple. When DPPH reagent is introduced in a solution that includes an antioxidant, this then changes from purple hue to yellow. The DPPH test is also regarded as the simplest colorimetric technique for such uses. It is a stable radical in solution, and when present in methanol, it looks purple in colour. The DPPH test works on the idea that when the DPPH ion absorbs a hydrogen atom from the antioxidant, the absorbance decreases and the colour changes from purple to yellow[27].

The approach recommended by Brand-Williams was modified to determine the overall free radical-scavenging activity of the samples under analysis. In this experiment, 25–100 L of each red cabbage extract was mixed with 2.95 mL of a methanolic solution containing 0.4 mmol/L of DPPH. The absorbance at 517 nm was measured after standing at room temperature in the dark for 30 minutes[28,29].

Using the equation, the percentage of DPPH scavenging (RSA%) was calculated:

$$\% \text{ Scavenging of DPPH} = [(A0 - A1)/A0] \times 100$$

Where A0 = absorbance of the control and A1 = absorbance of the test extracts[30].

Statistical analysis

The average and standard deviation (SD) of the three replicates were used to express the experimental results. Using the SAS v. 9.1.3 programme, the results of each analysis were compared using an ANOVA and Duncan's multiple range test.

RESULTS and DISCUSSION

Colour is a sensory property of food that makes it more appealing, enticing, and tasty to customers. Colour has a strong impact on flavour perception and is frequently recognized before fragrance[31]. In addition to the food industry, the textile, and cosmetics sectors both heavily rely on colour. These sectors typically employ artificial colours, which represent a health risk, and as the globe changes, everyone is gravitating toward natural and organic goods. Keeping that in mind, the present research involves the extraction of natural colour from red cabbage. In this study, red cabbage powder was extracted (Figure 1) and tested for anthocyanin, phenolic, and antioxidant activities.

Figure 1. Red cabbage flakes, extract and powder

Physiochemical analysis

Red cabbage powder was assessed for its physiochemical properties that include its nutritional value shown in Table 1. The nutritional composition of red cabbage which includes moisture, ash, carbs, protein, fats, and fibre was assessed. The findings revealed that 100 g of edible red cabbage contains the following nutritional value: calories (31 kcal), water (90 g), protein (2.0 g), and fat (0.2 g)[32]. The nutritional value of red cabbage powder was also assessed, and it was then compared to the nutritional value of fresh cabbage. red cabbage powder has the following nutritional value: red cabbage powder contains 8.3% moisture, and this value was like Drozdowska's study who also reported 8.33% of moisture in dehydrated red cabbage powder[33].

Table 1. Nutritional composition of red cabbage

Parameters	Value (Fresh red cabbage)	Value (Red cabbage powder)
Moisture (%)	89.70	8.30
Ash (%)	0.90	6.37
Fats (%)	0.12	3.21
Fiber (%)	2.20	6.50
Protein (%)	1.52	9.45
Carbohydrates (%)	5.56	66.17

Anthocyanin content in red cabbage powder and paste

Red cabbage paste and powder were tested for total anthocyanin content using a UV spectrophotometer, and the results are shown in Table 2. The result of anthocyanin content in red cabbage shows that the highest anthocyanin level was detected in red cabbage paste in water (27.55mg/100g) and then in red cabbage powder water (26.14mg/100g) and (14.93mg/100g) anthocyanin was present in red cabbage paste in methanol. The minimum level of anthocyanin was seen in red cabbage powder in water (9.85mg/100g). Total anthocyanin determined in red cabbage powder and paste was 78.47mg/100g. The total anthocyanins contents decreased in the following order: Red cabbage paste (Water) > Red cabbage powder (Water) > Red cabbage paste (Methanol)>Red cabbage powder (Methanol)[34].

Table 2. Total anthocyanin content in red cabbage

Samples	Total anthocyanins content
Red cabbage paste (Water)	27.55 mg/100g
Red cabbage paste (Methanol)	14.93 mg/100g
Red cabbage powder (Water)	26.14 mg/100g
Red cabbage powder (Methanol)	9.85 mg/100g

Red cabbage anthocyanin-rich extract improved cell viability and apoptosis by reducing H_2O_2-induced intracellular oxidative stress in human hepatocellular carcinoma (HepG2) cells[35]. Anthocyanins are water-soluble pigments that could be used to colour a variety of food goods[36]. Currently, these pigments are used to create food colorings that are produced from horticulture crops and

processing wastes[37]. Additionally, anthocyanins have been found in both in vitro and in vivo studies to have a variety of potential health advantages and to be excellent free radical scavengers, making them good antioxidant chemicals[38,39].

Total phenolic content

The total phenolic content of red cabbage pastes and powder is shown in Table 3. The TPC was determined by Folin–Ciocalteu reagent and the result obtained shows the highest phenolic content was present in red cabbage paste (methanol) at 140µg/GAE. Then the second highest was seen in red cabbage paste (Water) at 112µg/GAE. Red cabbage powder (methanol) had 107µg/GAE of phenolic content. The lowest level of phenolic content was seen in red cabbage powder (water) at 24µg/GAE. Our results align with the study reported by Izzo L. et al. 2020[40].

Table 3. Total phenolic content of red cabbage

Samples	Total phenolic content
Red cabbage paste (Methanol)	140 µg/g GAE
Red cabbage paste (Water)	112 µg/g GAE
Red cabbage powder (Methanol)	107 µg/g GAE
Red cabbage powder (Water)	24 µg/g GAE

Red cabbage extract contains 21 hydroxycinnamic acid derivatives (HCAs), the majority of which are leftovers of the organic acids p-coumaric, ferulic, and synaptic or their hydrated forms. As it is well known, different herbs and regions of the world have variable amounts of TPC in plant extracts. Numerous factors, including genetics, the impact of the environment's climate and the type of solvent employed during the extraction technique, can be utilized to explain this variation[41].

Antioxidant activity using DPPH Assay

By applying the previously reported DPPH technique in a free radical scavenging experiment, the antioxidant activity of Aloe vera extract was determined. The technique is based on a reduction in DPPH in the presence of antioxidants and a progressive change in the colour of DPPH from purple to yellow depending on the concentration of antioxidants, which is shown by a reduction in absorbance[42]. According to the results, the highest antioxidant activity is present in red cabbage paste (Methanol) which lies in the range of 57.71-69.21 in the 25-100µl sample which are in line with the study conducted by Ricci[43,44]. Table 4 shows the antioxidant activity of red cabbage.

Table 4. Antioxidant activity of red cabbage

Antioxidant activity (DPPH)	Sample (25µl)	Sample (50µl)
Red cabbage paste (Methanol)	57.71	69.21
Red cabbage paste (Water)	50.74	60.90
Red cabbage powder (Methanol)	29.47	55.44
Red cabbage powder (water)	9.10	36.35

It was found that red cabbage in paste form showed maximum antioxidant activity in methanol than in water. Similarly, red cabbage in powder form showed more antioxidant activity in methanol than in water.

The above study showed that red cabbage is the best source of antioxidants and can be used as a potential antioxidant source in food and cosmetics. The extracted anthocyanins can be used as natural colorants in different food items.

STATEMENT OF ETHICS

Ethical approval of this study was obtained from the Food and Biotechnology Research Centre, PCSIR Laboratories Complex Lahore.

CONFLICT OF INTEREST STATEMENT

There were no known conflicts of interest.

AUTHOR CONTRIBUTIONS

The authors contributed equally.

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Determination of essential oil compositions as well as phenolic and flavonoid contents of *Inula viscosa* L. and *Inula graveolens* L. from the coastal region of Latakia - Syria

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ABSTRACT

The essential oils (EOs) of dry leaves of *Inula viscosa* L. and *Inula graveolens* L. obtained by hydro-distillation were investigated using GC-MS within two different harvest seasons, namely summer and autumn (August and October, respectively). The results showed that the essential oils were mainly composed of monoterpenes and sesquiterpenes. Twenty-four and fifty active components, representing 63.22% and 85.41% of the essential oils, were identified in *I. viscosa* L., whereas, fifty-two and thirty-five active components, representing 96.46% and 58.26% of the essential oils, were identified in *I. graveolens* L throughout the two harvest seasons respectively. The quantitative determination of methanolic extracts of *I. viscosa* L. and *I. graveolens* L. leaves was performed, where the total phenolic contents (TPC) and total flavonoid contents (TFC) were measured. Methanolic extracts of *I. viscosa* L. were found to be richer in phenolic and flavonoid contents than those of *I. graveolens* L. in the two considered seasons.

Keywords: medical plants, essential oil, phenolic content, flavonoid content, harvest season

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INTRODUCTION

In the persistent attempts to improve the efficacy of drug discovery and medical practices, healthy organic foods, plants and dietary supplements enriched with medicinal ingredients have attracted attention all over the world, especially plants that are used in traditional medicine to prevent and treat many human diseases[1,2]. Medicinal plant are natural sources that are rich of active phytochemicals (secondary metabolites) that are known to have important medical properties[3].

The Asteraceae (Compositae) family is one of the largest flowering plant families, with over 1600 genera and 2500 species worldwide. Members of the Asteraceae family have been used in diets and medicine for centuries. Most Asteraceae family members demonstrate strong antioxidant, anti-inflammatory, antimicrobial and wound healing properties. Their pharmacological effects can be attributed to their range of phytochemical compounds, including polyphenols, phenolic acids, flavonoids, triterpenes and sesquiterpene lactones[4].

Inula L. is a large genus from Asteraceae family with more than one hundred species[5]. *Inula* L. species grow in Africa, Asia, Europe and the Mediterranean region. They were first used in Roman, Greek and Chinese traditional medicine to treat various diseases[6]. Extracts of the species are particularly rich in terpenoids, in particular eudesmane acids, ilicic acid and α-costic acid[7], Several *Inula* L. species are known for their therapeutic properties, for example *Inula viscosa* L. and *Inula graveolens* L.

Inula viscosa L. Aiton (Asteraceae) (Synonym: *Dittrichia viscosa* L. Greuter), is a strong-smelling perennial plant. Its leaves are sticky, oval and pointed. At the top of the stem there are numerous yellow flowered heads[8]. By contrast, *Inula graveolens* L. Desf. (Synonym: *Dittrichia graveolens* L. Greuter), is an annual aromatic plant with a foul camphor odor. Its leaves are sticky, oval and pointed, while its flowers appear in September / October and have yellow petals. Its yellow flowers do not produce seeds until September, while other plants from this genus complete their life cycles by the end of September[9,10]. Both plant species grow on cultivated land, abandoned roadsides and rural areas[11].

In traditional medicine, *I. viscosa* L. has many uses. These include antispasmodic, sedative, antiseptic, treating bronchial disorders, expectorant, antipyretic, antispasmodic, antidiabetic, diuretic, anthelmintic and anti-aging. The fresh leaves are used for wound healing by being applied to the wound area[12,13]. Meanwhile, *I. graveolens* L. is widely used for its anti-inflammatory effects and in aromatherapy for the treatment of asthma. It is also used as Broncho

spasmolytic, mucolytic, rheumatic fever, reducing blood sugar, dissolving internal blood clots, treating of urinary tract infections and wound infections[14].

Essential oil, aqueous and organic extracts of *I. viscosa* L. different parts showed antifungal and antibacterial activities in vitro[12,15,16]. In addition, antioxidant and anticancer activities were observed in ethanolic and methanolic extracts in relation to the presence of flavonoids and Sesquiterpenoids[6]. The ethanolic extract induces programmed cell death in kinetoplastids[17], and causes strong anticancer effects on Burkitt lymphoma cell line through inhibition of cell proliferation and induction of cell apoptosis[18]. Methanolic extract of the plant's leaves has an antihypertensive effect[19], essential oil and hexane extract from leaves have insecticidal properties, and play an important role in control of the bacteria that cause olive knot disease[20,21].

Essential oil, methanolic and ethanolic extracts of *I. graveolens* L. have antioxidant properties[22]. Methanolic, ethanolic and acetone extracts show antifungal properties against *Fusarium poae* that causes Fusarium head blight (FHB) disease, which is an important and insidious disease affecting mainly wheat and other cereals worldwide. Essential oils have anti candida, anti-bacterial, anti-cancer effects as well as acetylcholinesterase and tyrosinase inhibitor effects[23-26].

Both species are found in several areas in Syria and contain some pharmacologically active compounds, including phenols, flavonoids, terpenoids and tannins[11]. The present study is undertaken to report the essential oil compositions, phenolic and flavonoid contents of methanolic extracts from the leaves of the Syrian species *I. viscosa* L. and *I. graveolens* L. growing in Wadi Al-Janayin (AL-Qardaha region, Latakia - Syria). This study has not been reported previously.

METHODOLOGY

Plant material

The leaves of both plant species were collected in August (summer, before flowering) and October (Autumn, during flowering) of 2021 from populations growing wild in Wadi Al-Janayin (AL-Qardaha region, Latakia - Syria). Both plant species were identified by Prof. Aziza Ibrahim Youssef, a member of the academic staff at the Faculty of Pharmacy, Tishreen University (Syria). The leaves were dried in ambient air in the pharmacognosy laboratory and sheltered from light and moisture.

Chemicals

All of the chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA), and the solvents were from E. Merck (Darmstadt, Germany). All of the reagents were prepared in deionized distilled water to eliminate the contamination of metal ions.

Extraction of essential oils

According to the British Pharmacopoeia, essential oils were extracted from 100 g of dry leaves of each plant species (separately) by hydro-distillation in Clevenger-type apparatus for a period of 4 hours with 1 L of distilled water .The essential oils were dried over anhydrous sodium sulfate, filtered and stored at 4 °C until the time of analysis by gas chromatography-mass spectrometry (GC/MS).

GC/MS analysis

The GC/MS analysis of the oil samples were carried out at the Institute of Marine Research, Faculty of Agriculture, Tishreen University, Latakia – Syria. From Agilent technologies, A Hewlett-Packard 6890N gas chromatograph coupled to a 5975-mass spectrometry detector, and a capillary HP-5 column (5%-phenyl)-methylpolysiloxane) (30m×250μm×0.25μm) were used. The carrier gas was helium, with a flow velocity of 1.2 mL/min. The temperature of the injector and the detector was set to 250°C and 280°C respectively. The heating program started at 70°C for 2 min, then the temperature was increased to 280°C at a rate of 4°C/min. The chemical components were, then, identified based on a comparison of their retention indices relative to (C6–C24) n-alkanes with those in literatures, and by matching their mass spectra with those recorded in the mass spectral libraries.

Extraction of phenolic and flavonoid compounds

The method proposed by Oniszczuk and Podgórski was adopted[27]. A 1 g of powdered leaves of each plant species was subject to extraction using 20 mL of 80% methanol following the reflux extraction method at 70°C for 20 min. The methanolic extracts were filtered and evaporated to dryness under reduced pressure in a rotary evaporator at 40°C. The extracts obtained by evaporation have a gelatinous liquid appearance of dark brown color.

Determination of total phenolic contents

The total phenolic contents were determined using the Folin-Ciocalteu (F-C) method, which was previously described by Singleton et al.[28] with slight modi-

fications. The calibration curve was plotted using gallic acid standard (50-700 µg/mL). An amount of 0.1 mL of the plant dry extracts that are dissolved in methanol 80% was mixed with 2 mL of 2% sodium carbonate solution. 2 mL of 10% F-C reagent was added to the mixture 10 mins later and shaken well. After 15 minutes of incubation in a dark place at room temperature, the absorbance of the blue mixtures was read against the blank at 750 nm using UV/visible spectrophotometer. The total phenolic concentrations were calculated from the calibration curve (Abs = 0.0007x + 0.0365, R^2 = 0.998). The concentrations were expressed as milligram gallic acid equivalent per gram dry extract (mg GAE. g DE^{-1})[29]. The process was repeated three times for each sample.

Determination of total flavonoid contents

The quantification of total flavonoids was obtained by a colorimetric assay after reaction with aluminum chloride using a method described by Hongbin Zhu et al.[30] with slight modifications. To draw the standard curve, a standard solution (0.16 mg/mL) of rutin was prepared. Seven portions (of volumes: 1, 2, 3, 5, 7, 8, 10 mL) of the rutin standard solution were removed in seven 25-mL volumetric flasks. The following steps were, then, performed.

- 1.6 mL of 5% $NaNO_2$ solution were added to each flask, shaken up and then left to settle for 6 min.
- 1.25 mL of 10% $AlCl_3.6H_2O$ solution were added to each flask, shaken and left to settle for 6 min.
- 7.5 mL of 4.3% NaOH solution was added to each flask, followed by the addition of water to the scale.

The mixture was shaken, and left to settle for 15 min. The absorbance was read against the blank at 498 nm using UV/visible spectrophotometer. The concentration of flavonoids in each plant sample was calculated from the standard curve (y = 0.0109x + 0.0112, R^2 = 0.998) after adding 1 mL of the plant dry extract (dissolved in methanol 80%) to a volumetric flask (25 mL) with the same previous additions described in drawing rutin standard curve section. The concentrations were expressed as milligram rutin equivalent per gram dry extract (mg RE. g DE^1). The process was repeated three times for each sample.

RESULTS and DISCUSSION

Essential oil analysis

The hydro-distillation of the leaves of two plant species yielded light yellow-colored essential oils. The GC/MS analysis of those essential oils regarding the two different harvest seasons gave the following results. The analysis of essential oils of *I. viscosa* L. harvested in the summer showed the presence of twenty-four active components, representing 63.22% of *I. viscosa* L. essential oils, whereas for the autumn *I. viscosa* L. there were fifty active components representing 85.41% of *I. viscosa* L. essential oils. With regard to *I. graveolens* L., the corresponding figures were fifty-two and thirty-five active components, representing 96.46% and 58.26% of essential oils. The analysis revealed also the predominant presence of 3,5-di-tert-butyl-4-hydroxybenzaldehyde (10.48%), 2,6-di-tert-butyl-4-ethylphenol (7.76%), methyl-cyclopentane (7.01%) and α-selinene (5.61%) regarding *I. viscosa* L. that was harvested in the summer. As for the autumn harvest, the identified compounds were caryophyllene oxide (33.2%), heneicosane (6.29%), aromadendrene (5.6%) and dodecanoic acid (3.21%). The corresponding results for *I. graveolens* L. were borneol (35.21%), bornyl acetate (22.86%), anethol (5.57%) and adamantane (3.88%) for the summer harvest; and bornyl acetate (16.13%), β-cubebene (5.25%), adamantane (4.4%) and borneol (3.77%) for the autumn harvest.

Table 1. Compounds identified in the essential oils of *I. viscosa* L. and *I. graveolens* L. leaves using GC/MS in the order of their elution from the column

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
						Iv-S	Iv-A	Ig-S	Ig-A
1.	**Methyl-Cyclopentane**	Cyclic alkane	520-636	523.07	1.503	**7.01**	-	-	**3.52**
2.	Toluene	Aromatic hydrocarbon	672-779	621.32	1.925	0.30	-	-	0.22
3.	6-Methyl-3,5-heptadiene-2-one	Enone	720-790	761.3	4.643	-	-	0.20	-
4.	1,3-Cycloheptadiene	Cyclic alkane	832-921	887.74	7.939	-	-	-	0.15
5.	Nerol oxide	Monoterpenoid Alcohol	1146-1154	775.9	8.557	-	-	0.17	-
6.	Camphene hydrate	Monoterpene	1144-1148	1022.87	8.763	-	-	0.27	-
7.	**Borneol**	Monoterpenoid Alcohol (Bicyclic monoterpenoid)	1152-1164	1077.1	9.495	-	0.11	**35.21**	**3.77**
8.	α-Terpineol	Monoterpenoid Alcohol	1178-1188	1026.68	9.724	-	0.10	0.11	-
9.	p-Mentha-1,5-dien-8-ol	Monoterpenoid Alcohol	1155-1164	1036.1	10.382	-	-	0.35	-
10.	Jasmolone	Monoterpenoid Alcohol	1095-1120	1075.05	11.658	-	0.09	-	-
11.	**Bornyl acetate**	Bicyclic monoterpenoid ester	1264-1283	1174.6	12.116	-	0.64	**22.86**	**16.13**
12.	Theaspirane	Norisoprenoid (Oxaspiro compound)	1190-1285	1213.27	12.276	-	0.07	-	-
13.	**Anethol**	Phenyl propene (monomethoxybenzene)	1273–1303	1383.41	13.375	0.52	1.92	**5.57**	0.61
14.	2,4-Di-tertbutylphenol	Alkylbenzene	1300-1400	1038.48	13.605	-	-	-	0.78
15.	Isobornyl propionate	Monoterpenoid ester	1405-1490	1472	13.981	-	0.16	1.01	-
16.	Caryophyllene	Bicyclic Sesquiterpene	1392–1426	1316.88	14.130	-	0.27	1.27	0.48
17.	**3,5-Di-tert-butyl-4-hydroxy-benzaldehyde**	Hydroxybenzaldehyde	1520-1600	1586.86	14.887	**10.48**	-	-	1.3
18.	**2,6-Di-tert-butyl-4-ethylphenol**	Alkylbenzene	1530-1596	1511	15.005	**7.76**	-	-	0.29
19.	**α-Selinene**	Sesquiterpene	1477–1510	1698.4	16.001	**5.61**	-	0.16	0.25

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
20.	**Guaia-10(14),11-diene**	Sesquiterpene	1560-1609	1511	16.018	-	**2.45**	-	-
21.	Spathulenol	Tricyclic Sesquiterpenoid alcohol	1562–1590	1556.3	16.156	1.60	-	0.30	-
22.	α-Curcumene	Sesquiterpene	1468–1494	1533.97	16.264	0.66	1.03	0.26	-
23.	α-Longipinene	Sesquiterpene	1337–1362	1552.3	16.476	-	0.19	-	-
24.	Apiole	Phenylpropene	1608–1634	1583.5	16.672	-	-	-	0.25
25.	β –Farnesene	Sesquiterpene	1438–1460	1227.7	16.711	-	0.36	-	-
26.	α-Copaen-11-ol	Tricyclic Sesquiterpenoid alcohol	1556–1594	1571.4	16.813	1.36	-	-	-
27.	γ-Muurolene	Sesquiterpene	1461–1487	1510	16.854	0.76	-	0.11	-
28.	Prenyl benzoate	Benzoic acid ester	1530-1589	1556.8	17.231	-	-	0.61	-
29.	Trans-β-Ionone	Sesquiterpene	1256-1308	1296	17.523	-	-	0.31	-
30.	6-Prop-2-enyl-1,3-benzodioxol-5-ol	Benzodioxole	1290-1356	1303	17.535	-	0.31	-	-
31.	Dodecanedioic acid	Dicarboxylic fatty acid	1030-1110	1081	17.923	-	-	-	0.38
32.	(+)-Nerolidol	Sesquiterpenoid alcohol	1225-1298	1241.4	18.015	1.45	1.30	-	-
33.	**2-Pentylpyridine**	Pyridine	1498-1556	1529	18.146	**5.42**	-	-	-
34.	Neryl propionate	Carboxylic ester	1024-1098	1069	18.473	-	-	0.71	-
35.	**α-Curjunene**	Sesquiterpene	1300-1398	1338	18.620	4.45	-	-	-
36.	Alloaromadendrene oxide	Sesquiterpene oxide	1543-1587	1571	18.862	-	-	0.17	-
37.	Caryophylladienol	Sesquiterpenoid alcohol	1456-1540	1528.75	18.960	-	-	-	0.50
38.	**β-Cubebene**	Sesquiterpene	1370–1393	1559.87	19.205	-	-	-	**5.25**
39.	2-Methoxy-3-methylpyrazine	pyrazines (Aromatic ether)	1572-1603	1590.26	19.371	-	0.48	-	-
40.	Thymol	Monoterpenoid phenol	1272–1304	603.5	19.389	-	-	0.63	1.34
41.	3-Carene	Monoterpene	1002–1025	1092.9	19.892	-	0.24	-	-

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
42.	γ-Elemene	Sesquiterpene	1327–1344	1027.34	19.931	0.73	-	-	-
43.	γ-Caryophyllene	Sesquiterpene	1405–1440	1525.68	20.035	-	-	0.68	-
44.	(+)-γ-Cadinene	Sesquiterpene	1498–1531	1561.3	20.339	-	-	0.28	0.21
45.	**Aromadendrene**	Sesquiterpene	1419–1465	1549.5	20.533	1.27	**5.6**	0.21	0.67
46.	**(+)-Epi-bicyclosesquiphel-landrene**	Sesquiterpene	1564-1599	1571.56	20.733	-	-	**2.66**	-
47.	Geranyl acetate	Monoterpenoid ester	1358–1388	1556	20.813	-	-	0.80	0.23
48.	Terpinylisobutyrate	Monoterpenoid ester	1256-1298	1278	20.876	-	0.65	-	-
49.	**Adamantane**	Polycyclic alkane	1376-1443	1406	20.894	**3.07**	**2.7**	**3.88**	**4.4**
50.	α-Cadinol	Sesquiterpenoid alcohol	1635–1664	1070	21.197	-	-	0.25	-
51.	(E)-Stilbene	Diarylethene	1530-1579	1543.22	21.330	-	-	-	0.46
52.	**Menthol**	Monoterpenoid Alcohol	1169–1194	1500	21.506	-	**2.78**	-	-
53.	**(+)-Beta-selinene**	Sesquiterpene	1473–1496	1000	21.506	-	-	**2.65**	-
54.	2-Isopropyl-tricy-clo[4.3.1.1(2,5)]undec-3-en-10-ol	Fatty acid Alcohol	1540-1590	1588.15	21.632	-	0.42	-	-
55.	Isoaromadendrene epoxide	Sesquiterpenoid	1398-1434	1409.3	21.649	1.56	0.48	0.94	-
56.	α-Farnesene	Sesquiterpene	1486–1497	1557.6	21.815	1.75	-	0.54	-
57.	Batyl-alcohol (Batilol)	Alkylglycerol	1550-1587	1573.7	21.938	-	-	-	0.94
58.	3-Isopropyltricyclo[4.3.1.1(2,5)] undec-3-en-10-ol	Alcohol	2120-2189	2139.7	21.982	0.59	-	-	-
59.	10s,11s-Himachala-3(12),4-diene	Sesquiterpenoid	1390-1450	1416.92	22.049	-	-	0.35	-
60.	Geranyl butyrate	Monoterpenoid ester	1550-1598	1578.8	22.381	-	-	0.32	-
61.	Dodecane	Alkane	1420-1487	1447.7	22.471	-	-	-	1.94
62.	(+)-Ar-tumerone	Sesquiterpenoid ketone	1203-1280	1236.67	22.570	-	-	0.57	-
63.	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-	Monoterpenoid ketone	1530-1599	1575.2	22.742	-	-	0.24	-

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
64.	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	Alkane	1498-1546	1527.76	22.799	-	0.55	-	-
65.	2-Pentadecanone, 6,10,14-trimethyl	Ketone	1548-1606	1586.67	22.948	-	-	0.33	-
66.	2-Undecanone, 6,10-dimethyl-	Dialkyl Ketone	1779-1850	1815.58	22.971	-	0.74	-	-
67.	β -Patchoulene	Tricyclic sesquiterpene	1450–1464	1362.6	23.096	-	0.24	-	-
68.	Longifolenaldehyde	Sesquiterpenoid Aldehyde	1500-1590	1548.47	23.171	-	-	0.18	-
69.	α-Humulene	Monocyclic sesquiterpene	1435–1470	1514.46	23.251	-	0.59	-	-
70.	Benzoic acid	Aromatic carboxylic acid	1510-1614	1574.87	23.730	1.81	-	-	-
71.	Hexahydrofarnesylacetone	Sesquiterpenoid lactone	1831–1855	1776.6	23.893	0.59	-	-	-
72.	3-Phenylpropyl isovalerate	Fatty acid ester	1820-1870	1835.28	23.943	-	-	0.57	-
73.	Tetradecane	Alkane	1404-1467	1438.3	24.035	-	0.90	-	0.90
74.	1-Heptatriacotanol	Fatty alcohol	1400-1470	1438.5	24.184		0.48	-	-
75.	Aromadendrene oxide-(1)	Sesquiterpenoid	3750-3790	3785.78	24.424	-	-	0.41	-
76.	**α –Guaiene**	Sesquiterpene	1424–1454	1511.18	24.464	-	**2.71**	-	-
77.	Methyl decanoate	Fatty acid methyl ester	1323–1329	1566.74	24.784	-	0.52	-	-
78.	Linalool	Monoterpenoid alcohol	1088–1109	1150.32	24.945	0.80	-	-	-
79.	Z-7-Pentadecenol	Fatty acid alcohol	1000-1096	1029.37	25.105	-	0.65	-	-
80.	Farnesol	Sesquiterpenoid alcohol	1678–1700	1591.6	25.494	-	-	0.29	-
81.	Methyl 14-methyl pentadecanoate	Fatty acid methyl ester	1478-1548	1509.17	25.530	-	-	-	1.74
82.	Propyl cinnamate	Alkyl cinnamate (Phenylpropanoid)	1750-1809	1798.63	25.889	-	-	0.26	-
83.	Hexadecane	Alkane	1198-1230	1202.83	25.894	-	0.82	-	0.90
84.	Palmitic Acid	Saturated long-chain fatty acid	1660-1690	1675.19	26.066	-	-	1.21	-

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
85.	Octathiocane	Homomono-cyclooc-tasulfur	1623-1689	1640	26.123	0.57	-	-	0.35
86.	**Geranylgeraniol**	Diterpenoid Alcohol	1809-1850	1818.24	26.209	-	**2.68**	0.13	-
87.	**Dodecanoic acid**	Fatty acid	1557–1587	2085.04	26.598	-	**3.21**	-	-
88.	Retene	polycyclic aromatic hydrocarbon	1200-1250	1213.8	26.667	-	-	0.35	-
89.	**Heptadecane**	Alkane	1840-1906	1890.31	27.101	-	0.15	-	**3.36**
90.	Diisobutyl phthalate	Phthalate ester	1700-1790	1719.57	27.148	-	-	0.85	-
91.	**Caryophyllene oxide**	Sesquiterpenoid oxide	1563–1595	1691.54	27.342	**3.10**	**33.2**	**3.51**	1.53
92.	Heptanoic-acid	Carboxylic acid	1499-1549	1504.17	27.360	-	-	-	1.24
93.	3-Phenylpropyl isobutyrate	Cinnamyl phenylpropyl	700-789	761.1	27.777	-	-	1.86	-
94.	2-Methylbenzyl benzoate	Benzoate ester	1350-1396	1375.9	28.046	-	0.30	-	-
95.	Benzyl salicylate	Benzoate ester / phenol	1857–1881	1532.1	28.132	-	-	0.12	-
96.	Phytol	Acyclic diterpene alcohol	2104–2136	1449.36	28.315	-	0.97	-	-
97.	1-Hexadecene	Alkene	2000-2080	2036.14	28.504	-	0.42	-	-
98.	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	Alcohol	1612-1678	1646	28.841	-	0.39	-	-
99.	Phthalic acid, ethyl isopropyl ester	Phthalic acid esters	1761-1790	1784	29.242	-	0.27	-	-
100.	**Octadecane**	Alkane	1343-1379	1362.65	29.319	-	**2.76**	-	**2.49**
101.	1,7-Octadien-3-one, 2-methyl-6-methylene-	Ketone	1800-1870	1813.56	29.365	-	-	0.34	-
102.	9-Nonadecene	Alkene	1045-1079	1066.76	29.660	-	1.26	0.45	-
103.	Phenylethyl salicylate	Benzoate ester / phenol	1932-1967	1951.9	29.808	-	-	0.27	-
104.	Pyrene, hexadecahydro-	Pyrene	1512-1567	1540	29.946	-	0.40	-	-
105.	Longipinene epoxide	Sesquiterpenoid	1602-1688	1619.1	30.157	-	0.50	-	-
106.	Nonadecane	Alkane	1510-1550	1516	31.067	-	1.05	-	0.56

Peak	Compound	Chemical Group	RI* Range[31]	RI	Rt	Area %			
107.	Eicosane	Alkane	1912-1990	1988.7	36.299	-	-	0.09	0.35
108.	**Heneicosane**	Alkane	2020-2074	2050.25	37.024	-	**6.29**	0.42	0.43
109.	Tetracosane	Alkane	2156-2190	2186.76	37.756	-	0.31	0.17	0.16
110.	Hexacosane	Alkane	2402-2440	2412.5	37.869	-	-	-	0.18
111.	1-Heptacosanol	Fatty alcohol	2610-2649	2617.8	38.671	-	0.10	-	-
112.	Octacosane	Alkane	2750-2799	2791.37	42.608	-	0.6	-	-
Number of active compounds						24	50	52	35
Main chemical groups in each essential oil		**Total Monoterpenes %**				**0.80**	**4.77**	**61.97**	**21.47**
		Hydrocarbon Monoterpenes %				-	0.24	0.27	-
		Oxygenated Monoterpenes %				0.80	4.53	61.7	21.47
		Total Sesquiterpenes %				**24.89**	**48.92**	**16.1**	**8.89**
		Hydrocarbon Sesquiterpenes %				15.23	13.44	9.13	6.86
		Oxygenated Sesquiterpenes %				9.66	35.48	6.97	2.03
		Oxygenated Diterpenes %				**-**	**3.65**	**0.13**	**-**
		Phenylpropanoids %				**0.52**	**1.92**	**7.69**	**0.86**
		Others %				37.01	26.15	10.57	27.04
Total %						63.22	85.41	96.46	58.26

RI: (retention index or Kovats index) calculated with respect to n-alkanes (C6-C24). %: Relative percentage obtained from the area of the peak and electronic integration measurements using a selective mass detector. Iv-S: I. viscosa L./Summer, Iv-A: I. viscosa L./Autumn, Ig-S: I. graveolens L./Summer, Ig-A: I. graveolens L./Autumn

Hydro-distillation is a simple and fast method for extracting the essential oils from plants. It has a good yield, good recovery of essential oil constituents and less labor-intensive[32].

According to the GC/MS analysis of the essential oil samples, as presented in Table 1, 3,5-di-tert-butyl-4-hydroxybenzaldehyde (10.48%) (hydroxybenzaldehyde) and caryophyllene oxide (33.2%) (Oxygenated sesquiterpene) were the main compounds in *I. viscosa* L. essential oils of the summer and autumn harvests respectively. 3,5-di-tert-butyl-4-hydroxybenzaldehyde has been used

as an intermediate compound for synthesizing pharmaceutical products that have a strong anti-oxidant properties[33]. In addition, caryophyllene oxide has strong anticancer activities against breast cancer cell lines (MCF7 and T47D)[34], colon cancer (HCT 116 and HT29), pancreatic cancer (PANC-1), as well as having antioxidant, antimicrobial and antifungal properties[35].

I. graveolens L. essential oils were found to have a large amount of oxygenated monoterpenes (35.21% of borneol and 22.86% of bornyl acetate, in the summer harvest, and 16.13% bornyl acetate in the autumn harvest). Essential oils of plants that contain bornyl acetate and borneol were reported to have antimicrobial, antioxidant and insecticidal properties[20].

Studies similar to this one were carried out on the same plant species. In those studies the main constituents of the essential oils varied depending on plant parts, harvest regions and extraction methods[36]. In this study, the composition of *I. viscosa* L. essential oils from the autumn harvest showed some similarities to its counterpart in other studies in Syria[37], Algeria[15], Jordan[38] and Morocco[20], particularly concerning the content of caryophyllene oxide. However, *I. viscosa* L. essential oil compositions from the summer harvest did not show any similarities to the other studies. On the other hand, the composition of *I. graveolens* L. essential oils from the autumn and summer harvest showed some similarities with studies from Turkey[22,39], Algeria[40], Iran[23], Tunisia[26], and Jordan[38], particularly concerning the content of bornyl acetate and borneol. The amounts of compounds may increase, decrease or even disappear altogether depending of the harvest season. For example, in the essential oils of *I. graveolens* L., the largest amount of bornyl acetate, a main compound, was recorded in July while it decreased in January. Furthermore, β-selinene was only found in July, whereas s-cadinol was found in October and, then, dramatically decreased in January[26].

The major compounds of the essential oils of *I. viscosa* L. and *I. graveolens* L. from different areas in Syria and abroad are shown in Table 2 and Table 3.

According to the results in Table 2 and Table 3, it can be concluded that the chemical compositions of the plants vary according to various factors. Some of these factors include genetic factors, environmental factors, seasonal change, plant parts, developmental stage and extraction methods[26,31]. Therefore, the chemical composition of *I. viscosa* and *I. graveolens* from different periods is an important factor in determining their bioactivity.

Table 2. Major components found in the essential oils of *I. viscosa* L. obtained from different areas

Major compounds of *I. viscosa* L. essential oil	Area % Syria (AL-Qardaha region)		Area % Syria (Al-Qadmous region)[36]	Area % Algeria (Sidi Rezine village, South)[8]	Area% Algeria (Sidi Rezine village, South)[8]	Area% Algeria (Northwest)[15]	Area % Jordan (Irbid)[38]	Area % Morocco (Fez)[20]
	PP: L EM:HD HS:S	PP: L EM:HD HS:A	PP: L EM:HD	PP: L EM:HD	PP: L EM:SD	PP: L EM:HD	PP: AP EM:HD	PP: L EM:HD
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	10.48	-	-	-	-	-	-	-
2,6-Di-tert-butyl-4-ethylphenol	7.76	-	-	-	-	-	-	-
Methyl-Cyclopentane	7.01	-	-	-	-	-	-	-
Bornyl acetate	-	-	-	-	-	-	-	41
Borneol	-	-	-	-	-	-	-	9.3
12-Carboxyeudesma-3,11 (13) diene	-	-	-	28.88	56.81	-	-	-
Linolenic acid	-	-	-	7.80	-	-	-	-
Pentacosane	-	-	4.36	5.43	2.31	-	-	-
Heneicosane	-	6.29	-	-	-	-	-	-
n-Hexadecanoic acid	-	-	-	5.38	1.91	-	-	-
Caryophyllene oxide	-	33.2	7.83	-	-	10.4	2.57	5.7
Heptacosane	-	-	-	4.82	2.09	-	-	-
Butyl hydroxy toluene	-	-	-	4.11	2.63	-	-	-
Fokienol	-	-	-	3.37	1.89	9.6	20.87	-
2,3-Didehydrocostic acid	-	-	-	-	3.25	-	-	-
α-Eudesmol	-	-	-	-	-	7.6	2.68	-
Trans-Nerolidol	-	-	13.64	-	-	7.0	19.75	-
γ-Eudesmol	-	-	-	-	-	6.2	-	-
β–Eudesm-6-en-4α-ol	-	-	-	-	-	-	5.64	-

Major compounds of *I. viscosa* L. essential oil	Area % Syria (AL-Qardaha region)		Area % Syria (Al-Qadmous region)[36]	Area % Algeria (Sidi Rezine village, South)[8]	Area% Algeria (Sidi Rezine village, South)[8]	Area% Algeria (Northwest)[15]	Area % Jordan (Irbid)[38]	Area % Morocco (Fez)[20]
α-Vetivone	-	-	-	-	-	-	3.60	-
Selin-11-en-4α-ol	-	-	-	-	-	-	2.18	-
Selina-6-en-4-ol	-	-	4.46	-	-	-	-	-
α-Selinene	5.61	-	-	-	-	-	-	-
8-Cedren-13-ol	-	-	3.10	-	-	-	-	-
Cedren-14-olacteate	-	-	-	-	-	-	2.0	-
Dodecanoic acid	-	3.21	-	-	-	-	-	-
Khusimol	-	-	-	-	-	-	1.80	-
E-Farnesene epoxide	-	-	16.55	-	-	-	-	-
Alpha. copaene-11-ol	-	-	3.03	-	-	-	-	-
α-amorphene	-	-	-	-	-	-	-	6.6
Aromadendrene	-	5.6	-	-	-	-	-	-

PP: Plant Part, L: Leaves, AP: Arial Parts, EM: Extraction Method, HD: Hydro-Distillation, SD: Steam DistillatioN, HS: Harvest Season, S: Summer, A: Autumn

Table 3. Major components of essential oils of *I. graveolens* L. obtained from different areas

Major components of I.graveolens L. essential oil	Area % Syria (AL-Qardaha region)		Area % Turkey (Bingol University Campus)[38]	Area % Turkey (Gaziantep/ Karatas highway (steppe land)[22]	Area% Algeria (Sidi Rezine village, South)[8]	Area% Iran (Shush)[23]	Area % Tunisia (Chebba salt marsh)[26]	Area % Jordan (Al-Jubeiha region)[33]
	PP: L EM:HD HS:S	PP: L EM:HD HS:A	PP: AP EM:HD	PP: L EM:HD	PP: AP EM:SD	PP: AP EM:HD	PP: AP EM:HD	PP: AP EM:HD
Bornyl acetate	22.86	16.13	-	68.5	-	-	45.34	70.58
Borneol	35.21	3.77	20.4	7.7	18.3	5.44	37.29	-
Isobornylacetate	-	-	-	-	50.8	-	-	-
Anethol	5.57	-	-	-	-	-	-	-
Thymol	-	-	-	-	-	-	4.62	-
Camphene	-	-	-	4.6	-	-	3.20	1.97
Adamantane	3.88	4.4	-	-	-	-	-	-
β-Cubebene	-	5.25	-	-	-	-	-	-
1,8-Cineole	-	-	22.4	-	-	54.89	-	-
α-Cadinol	-	-	11.8	-	-	-	-	-
P-Cymen	-	-	-	-	-	16.2	-	-
β-Pinene	-	-	-	-	-	6.94	-	-
ι-Cadinol	-	-	-	-	6.2	-	6.09	-
α-Terpineol	-	-	-	-	-	-	1.71	-
(2E,6E)-Farnesol	-	-	-	-	-	-	1.37	-
Caryophyllene oxide	-	-	-	-	-	-	1.30	1.82
β-Caryophyllene	-	-	-	-	-	-	1.22	-
Epi-a-cadinol	-	-	-	4.0	-	-	-	-
Eicosane	-	-	-	3.2	-	-	-	-

PP: Plant Part, L: Leaves, AP: Arial Parts, EM: Extraction Method, HD: Hydro-Distillation, SD: Steam Distillation
HS: Harvest Season, S: Summer, A: Autumn

Total phenolic and flavonoid contents

Based on the equation curves, the total amounts of phenols and flavonoids in the dry extracts of the inula plant leaves were calculated. The results showed that *I. viscosa* L. contained greater amounts of phenols and flavonoids when compared to *I. graveolens* L. (before and after blooming). The amounts of phenols and flavonoids in *I. viscosa* L. before blooming is greater than those after blooming. However, the amounts of phenols and flavonoids in *I. graveolens* L. after blooming is greater than those before blooming (Table 4).

Table 4. The total amounts of phenols and flavonoids in *I. viscosa* L. and *I. graveolens* L. dry extracts

Plant	n	Phenols content		Flavonoids contents	
		Yield(mean) ± SD mg Gallic acid. E / g D.E	CV%	Yield(mean) ± SD mg Rutin.E / g D.E	CV%
I. viscosa L. (S)	3	920 ± 1.573	0.002	42.587 ± 0.4343	0.0102
I. viscosa L. (A)	3	724.565 ± 2.627	0.0036	37.390 ± 0.442	0.012
I. graveolens L. (S)	3	516 ± 0.563	0.0011	24.532 ± 0.515	0.021
I. graveolens L. (A)	3	678.04 ± 1.766	0.0026	30.419 ± 0.363	0.012

n: number of repetitions, SD: standard deviation, CV%: the coefficient of variation in percent, S: for summer season, A: for autumn season

Phenols and flavonoids, the most common groups of secondary metabolites, are important in plants for normal growth development and defense against infections. Flavonoids are phenolic compounds and are very important pigments for flower coloration as they produce yellow or red/blue pigmentation in petals. They also protect plants from attacks by microbes and insects. They also show anti-allergic, anti-inflammatory, anti-microbial, anticancer, antioxidant[30] and free radical scavenging properties[41] as well as antidiabetic and weight loss effects[42].

Among different spectrophotometric techniques, The ultraviolet-visible (UV/Vis) spectrophotometry appears to be suitable for the quantification of phenolic and flavonoid contents in the plants extracts[3] due to its operational simplicity, speed, low cost of implementation, and wide availability in control laboratories[43]. Therefore, this technique is more accessible methods than analytical chromatography techniques, such as high performance liquid chromatography (HPLC)[3].

The present study revealed the phenolic contents in the extracts of *I. viscosa* L. and *I. graveolens* L. dry leaves. The amounts of phenolic compounds were found to be 920 and 724.56 mg gallic acid equivalent/g dry extracts in *I. viscosa* L. regarding the summer and autumn harvest respectively; the corresponding amounts for *I. graveolens* L. were 516 and 678 mg gallic acid equivalent/g dry extract. As for the flavonoids contents, they were found to be 42.6 and 37.4 mg rutin equivalent/g dry extract in *I. viscosa* L. in summer and autumn harvest respectively; the corresponding amounts for *I. graveolens* L. were 24.53 and 30.42 mg rutin equivalent/g dry extract.

Different studies have reported identifying many phenolic and flavonoid compounds in *I. viscosa* L. Examples of such compounds include: chlorogenic acid, hyperoside, protocathuic acid, apigenin, 7-o-methylaromadendrin, inuviscolide[6,44,45], hispidulin hexoside, patuletin, spinacetin[46] and kaempferol[47]. Gökbulut et al. from Turkey, identified the phenolic contents in methanolic extract of the whole *I. viscosa* L. plant as 176.9 ± 7.8 mg gallic acid equivalent / g extract[48]. In addition, an Algerian study by Amrouche et al. reported that the methanolic extracts of *I. viscosa* L. leaves had high content of polyphenols (106.34 ± 1.49 mg gallic acid equivalent.gdw^{1} and 125.73 µg quercetin equivalent.gdw^{-1})[49]. In a second Algerian study, Mouas et al. reported that the ethanolic extracts of aerial parts of the plant had 138.30 ± 0.00 mg EGA/mg DE and 34.57 ± 0.04 mg QE/mg DE of polyphenols and flavonoids respectively[7]. Another study from Turkey, performed by Bayar and Genc, reported that the total phenolic and flavonoid contents of organic extracts (hexane, ethyl acetate and methanol extracts) of the whole *I. viscosa* L. plant were determined to be in the ranges of 11.38-136.18 mg GAE / g extract and 14.38-28.83 mg QE/g extract[44] respectively.

Large amounts of phenolic compounds and flavonoids (1.63%, calculated as gallic acid, for phenols and 0.52% calculated as quercetin, for flavonoids, equivalents per 100 g of dry mass) were identified in the methanolic extracts of *I. graveolens* L. grown in Iraq[50]. Chlorogenic acid, quinic acid, hyperoside, protocatechuic acid and quercetin were the major phenolic compounds found in the methanolic extracts of *I. graveolens* L. leaves grown in Turkey, with 845 ± 41 µg/kg of gallic acid and 51 ± 3 µg/kg of rutin[51].

This study provides information about the major constituents of essential oils of *I. viscosa* L. and *I. graveolens* L. from Wadi Al-Janayin region in Syria. It confirms that the methanolic extracts of *I. viscosa* L. and *I. graveolens* L. have the largest phenolics and flavonoids contents when compared to the above-mentioned studies. This contrasts with some of the findings reported in the literature regarding both species (for both essential oils and extracts) in other

countries, which may be attributed to the differences in soil contents and the environment.

STATEMENT OF ETHICS

Ethical approval was not required to perform this study as no human participants or experimental animals were involved.

CONFLICT OF INTEREST STATEMENT

All authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

All authors contributed to data analysis and interpretation as well as revising the article. They also gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

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