ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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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 Istanbul University, Turkey.

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. Şeref DEMİRAYAK

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), Acknowledgement (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.

*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 has to be written in clear and concise English. Starting from 2016, the journal will be issued quarterly both in paper and on-line formates also publish special issues for national or international scientific meetings and activities in the coverage field.

1.2 Manuscript Categories

Manuscripts can be submitted as Research Articles and Reviews.

1.2.1 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.

1.2.2 Reviews integrate, correlate, and evaluate results from published literature on a particular subject. They expected to report new and up to date experimental findings. They have to have a well-defined theme, are usually critical, and may present novel theoretical interpretations. Up to date experimental procedures may be included. Reviews are usually submitted at the invitation of the Editors. However, experts are welcome to contact the Editors to ensure that a topic is suitable. Approval is recommended prior to submission.

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 as published documents.

1.5 Professional Ethics

Editors, reviewers, and authors are expected to adhere to internationally accepted criteria's 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 authors. 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 state-

ment 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 studies' Material and Methods.

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 the English, figures, or formatting in the manuscript before submission please contact to editorial office by e-mail for suggestions. Authors are required to subject their manuscript for

The responsibility for all aspects of manuscript preparation rests with the authors. 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". 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 of 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 Arabic number may be used. All code numbers in the text must have a citation to a publication or a patent on first appearance.

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

2.1.4 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. Title: 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, 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 summarized. 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.

Key words: 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 experi-

mental 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, ºC, 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.»

Acknowledgment: 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. Referencing style is given in our website in detail. Citations should be made as superscript arabic numbers. 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. 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. Vancouver style is used in the reference list. However, in-text citations should be given superscript numbers (e.g. 1) according to order in the manuscript. 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.

Journal article examples

Article with two authors example:

Tinworth, C. P., & Young, R. J. (2020). Facts, Patterns and Principles in Drug Discovery: appraising the Rule of 5 with measured physicochemical data. *Journal of Medicinal Chemistry*. doi:10.1021/acs.jmedchem.9b01596

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

Articles up to 6 authors should be given examples as above, and articles with more than 6 authors should be given as et al. after sixth author.

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.

3.1.1 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 assined as "Partially Submitted Manuscripts".

3.2.2 Institutions Authors should give their institutional information during submission.

3.2.3 Authors The authors' surnames, names, institutional information appear as entered order in the previous page. Filling all e-mail adresses 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.

3.2.4 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.

3.2.5 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 are accessible for arbitrators; so you should not add any information related to the institutions and authors in this summary part. Otherwise the article will 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.

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

3.2.7 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.

3.3.1 Adding Article This process consists 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.

3.3.2 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's appropriate or not.

3.3.3 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.

3.3.4 Page to Follow The Article The Main Page of Author ensures possibility

to follow the article. This page consists three different parts; some information and bridges related to the sent articles, revision required articles and the articles that are not completed to be sent.

3.3.4.1 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.

3.3.4.2 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.

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ORIGINAL ARTICLES

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Preparation and evaluation of ibuprofen loaded sodium alginate/sodium CMC mucoadhesive drug delivery system for sustained release

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ABSTRACT

In the present study, ibuprofen was formulated as Mucoadhesive microspheres by ionic gelation technique by using varying concentrations of polymers sodium alginate and sodium carboxy methyl cellulose. The mucoadhesive microspheres of Ibuprofen was characterized by drug content, particle size distribution, production yield, *in vitro* drug release, and entrapment efficiency. The XRD study suggested that there is a change in the physical behavior of drug from crystalline to amorphous within the formulation. SEM of optimized batch showed that particles were found to be spherical having a rough outer surface and was porous. The EE of microspheres ranged from about 28.69-68.51 %. The cumulative amount of drug released was found to be in the range of 43.72-84.39 %. The data obtained from the *in-vitro* drug release profiles of Ibuprofen determined that all the batches of mucoadhesive microspheres showed prolonged drug release. It could be concluded that the mucoadhesive microspheres of Ibuprofen showed prolonged release of the drug.

Keywords: Solubility, drug release, ibuprofen, drug entrapment efficiency.

INTRODUCTION

Microspheres may be described as solid, nearly globular particles size range from 1-1000 μm. Substances could be included inside microspheres in the solid or liquid state by synthesizing or consequently by absorption. Microspheres

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or microparticles are common terms that include both microcapsule & micro matrix. In micro-capsules, the entrapped substance is entirely enclosed by an individual capsule is hedged and in micromatrices, the entrapped substance is distributed all through the microsphere model. Microspheres comprise an essential section of an innovative drug delivery system because of their mini size and productive transporter measurements[1]. Microspheres with mucoadhesive properties can be developed for both targeted and controlled release drug delivery systems. Microspheres are commonly used for drug delivery to the systemic circulation and constitute a significant part of such novel drug delivery systems[2]. The mucosal membranes of organs such as gastrointestinal tract (GIT), ocular, buccal, vaginal, rectal and nasal are the various sites of drug absorption[3].

Ibuprofen is an Non-Steroidal anti-inflammatory drug (NSAID) taken orally to relieve inflammation, fever, and pain initiated by various disorders such as toothache, headache, menstrual cramps, back pain, minor injury and arthritis[4,5]. Ibuprofen is very effective for treating arthritis of joints (Rheumatoid Arthritis) and for treating osteoarthritis, high doses are required approximately 1800-3200 mg daily (or 200-800 mg every 4-6 h as a single dose). Although it has a short plasma half-life of 1-5 h following oral dosing which makes it a standard candidate for modified release formulation[6], it works by decreasing hormones. Ibuprofen is a monocarboxylic acid that is propionic acid derivative. Ibuprofen is biopharmaceutical classification system (BCS) class II drug (High permeability, low water solubility) and well absorbed from the gastrointestinal tract. It has low water solubility and is the limiting step for absorption and bioavailability. Exposure of stomach to high level of Ibuprofen can cause gastric irritation (ulceration or bleeding). Due to the narrow therapeutic index, 95% of the administered dose gets excreted from the body after 4 h of administration[7]. To overcome the problem of solubility and for achieving sustained/controlled drug delivery, a mucoadhesive drug delivery system (MDDS) containing mucoadhesive microspheres of ibuprofen has been formulated which would lead to less frequent dosing and therefore lower level of gastric irritation. Thus, the development of controlled-release dosage forms would clearly be useful. Investigators have formulated oral controlled-release products of ibuprofen by numerous methods [8-13]. MDDS utilize the property of bioadhesion of certain water soluble polymers that become adhesive to mucous membranes on hydration[14] and hence can be used for targeting a drug to a particular mucus tissue (e.g. gastrointestinal, buccal, nasal, etc.) for an extended period of time[15].

Sodium alginate, the sodium salt of alginic acid, is a natural hydrophilic polysaccharide containing two types of monomers, beta-D-mannuronic acid (M) and alpha-L-guluronic acid (G). Alginate forms 3-dimensional ionotropic hydrogel matrices, generally by the preferential interaction of calcium ions with the G moieties resulting in the formation of an inhomogeneous gel[16]. Sodium CMC was combined in formulation to improve viscosity and for the additive effect of mucoadhesive property. Sodium CMC was selected as a polymer instead of other polymers due to its better mucoadhesive capacity in comparison to that of other mucoadhesive polymers like poly (acrylic acid) (PAA), polycarbophils.

The objective of the present work was to develop mucoadhesive microspheres of ibuprofen to enhance its dissolution, bioavailability and control of drug release. To prepare the mucoadhesive microspheres of Ibuprofen, use two biocompatible polymers (Sodium alginate and Sodium CMC) in combination. To control/sustain the release of drug, decrease the frequency of dosing. To establish the relationship between formulation variable, select responses and characterization of mucoadhesive microspheres for improving the bioavailability of Ibuprofen at the target site of the mucosa. The optimized mucoadhesive microspheres batch were extensively characterized by SEM, FTIR, DSC, XRD and further evaluated for *in-vitro* drug release profile, and stability studies.

METHODOLOGY

Materials

Ibuprofen was purchased from Alkem Pharmaceuticals (P) Ltd., Baddi (india), Sodium alginate and Sodium CMC were obtained from Sisco research laboratory, India. Methanol, Calcium chloride, Ethanol, Dipotassium hydrogen phosphate, Sodium dihydrogen phosphate and Hydrochloric acid were obtained from High Purity Laboratory Chemicals (P) Ltd., Mumbai. Sodium chloride was supplied by Sigma-Aldrich, Mumbai, India. Unless otherwise stated, all chemicals were used as received without further purification.

Methods

Preparation of mucoadhesive alginate microspheres of ibuprofen

Microspheres of Ibuprofen were prepared by ionic-gelation technique or ionic cross-linking technique (Figure 1) using various ratios of Sodium Alginate and Sodium (CMC). Calcium chloride act as cross-linking agents to form the microspheres[17, 18]. Sodium alginate and sodium CMC were weighed accurately. The drug solution and the polymeric solution were prepared in a beaker by adding a small amount of distilled water followed by the addition of ethanol. The total

drug and polymers mixture was kept on magnetic stirrer for 1 h at 700 rpm to obtain a homogenous mixture of desired viscosity to pass easily through the syringe dropper. The total mixture was filled into a 5 ml syringe with gauge 21. Calcium chloride solution (10% w/v) was prepared separately and the solution was poured into this cross-linking solution dropwise with continuous stirring to form alginate microspheres. For strengthening, the beads formed were allowed to stir for 2 h. After completion of stirring, the beads were finally collected by filtration and were dried for 24 hrs in the oven at 40 °C and prepared microsphere is shown in figure 2. The composition of the different Microsphere batches prepared is shown in Table 1.

Table 1. Formulation parameters of mucoadhesive microspheres of ibuprofen

Formulation code	Drug (mg)	Sodium Alginate (mg)	Sodium CMC (mg)
F1	100	200	100
F2	100	200	200
F3	100	200	700
F4	100	100	400
F5	100	100	800
F6	100	100	100
F7	100	500	500
F8	100	600	600
F9	100	700	700

Figure 1. Ionic-gelation technique for preparation of sodium alginate/sodium CMC microspheres

Figure 2. Ibuprofen loaded mucoadhesive microspheres

Entrapment efficiency and production yield

For determination of the drug content, accurately weighed amount of ibuprofen loaded microspheres (50mg) of each formulation batch was crushed in mortar and pestle, the crushed microspheres were suspended in 50ml of 6.8 pH phosphate buffer solution for complete swelling at 37° C for overnight. The solution was filtered using Whatman filter paper, grade 40, the solution was centrifuged to remove polymeric debris. The clear supernatant solution was analyzed for drug content using *UV-visible* spectrophotometer at 228nm of wavelength[19]. The drug entrapment efficiency was calculated using the following equation

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Actual drug content in microsphere}}{\text{Theoretical drug content in microspheres}} \text{X100}$$

The production yield of microspheres of different batches after drying was calculated by using the weight of the final product as compared with the initial total weight of drug and polymers used for preparation. The formula used is given below:

Percentage yield (%) = $(M_1/M_2)*100$

Where M_1 &M_2 represents Practical mass (microspheres) and Theoretical mass (drug + polymers) respectively.

Particle size determination

Particle size of formulated microspheres was determined by laser diffraction analyzer (Mastersizer 2000 Version 5.61, UK Malvern Instruments). A well-dispersion of samples was formed, 2mg of each dispersion was weighed and dispersed in 10ml of distilled water and sonicated for 15 minutes. After that, the samples were analyzed for particle size determination[20-22].

Mucoadhesive property of microspheres

The mucoadhesive property of the microspheres was studied by an *in-vitro* adhesion method, also known as the wash-off test. Mucoadhesive microspheres (100) were spread onto the wet, rat intestinal tissue specimen on a glass slide which was further hung onto the grooves of USP tablet disintegrating test apparatus with the help of thread. The disintegrating apparatus was operated such that the tissue specimen was subjected to regular up and down movement in a beaker containing 0.1N HCl up to 1hr. The number of microspheres left on the tissue was counted[23]. Percentage mucoadhesion was calculated by the following formula:

Mucoadhesion (%) = N_1/N_2*100

Where N_1 & N_2 represents the number of adhered microspheres on mucosa and the number of applied microspheres on mucosa respectively.

Swelling index

Accurately weighed microspheres (W_o) were kept separately in a beaker containing phosphate buffer of pH 6.8. After a specified time, the microspheres were filtered; excess of water was removed from the microspheres and blotted with filter paper, weighed immediately on weighing balance. After 1 hr, the microspheres were reweighed (W_t)[24]. The percentage swelling index was calculated using the formula

Swelling Index = $(W_t - W_o)/W_o$*100

Characterization

Differential Scanning Calorimetry (DSC)

DSC was performed to study the thermal behavior of pure drug and the optimized batch of mucoadhesive microspheres[25]. The DSC was obtained using, DSC (Mettler Toledo, Switzerland). To do this analysis 3-8 mg of sample was secured in the aluminum pan and the temperature was increased up to 10°C/min. from 40-400° C.

X-ray Diffraction (XRD)

The effect of polymerization on the crystallinity of the drug, polymer and optimized batch can be studied by using an X-ray diffractometer (Miniflex 2, Rigaku, Japan), at room temperature and at 30kV. The scanning diffraction angle (2θ) ranging from 0° to 80°. X-ray diffractograms of pure drug, physical mixture and optimized formulation were recorded[26].

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was done to determine particle size distribution, shape, texture and surface morphology of the optimized batch. Dried Ibuprofen loaded mucoadhesive microspheres were placed on electron microscope brass stub, images of mucoadhesive microspheres were taken by random scanning of the stub[27].

***In-vitro* drug release studies**

(A) *In vitro* drug dissolution and release from ibuprofen loaded mucoadhesive microspheres were evaluated using a six vessels USP type II dissolution apparatus (Labindia, Bangalore), at 37± 0.5°C with constant stirring rate

of 50 rpm for $Rel_{24h.}$ A 200 mg drug equivalent sample of microsphere was placed in 900 ml of phosphate buffer (pH 6.8) at 37±0.5°C with constant stirring speed of 50 rpm. The powder was dispersed over the dissolution medium. Aliquots of sample (5ml) were withdrawn at different time intervals for 1 h and restored with an equal volume of the dissolution medium to keep sink conditions in the course of the experiment[28, 29]. The 0.45µm milipore filters was used for the sample filtration and the drug concentration in the samples was determined by measuring the absorbance of the samples at a wavelength of 228 nm using the *uv–vis* spectrophotometer followed by determination of mechanism of release by fitting the release rate data in various release kinetic models[30].

(B) The drug release studies were also carried out for a marketed tablet of Ibuprofen (Advil 200). The procedure and parameters used in the study were the same as above in the study.

Drug release study of optimized formulation

Model dependent methods

The kinetic model-dependent generally describe the dissolution profile. After choosing the selected function, the dissolution profiles were evaluated depending on the derived model perimeters. Zero-order, first-order, Higuchi, Korsmeyer-Peppas models are some approaches of dependent models. The following four were utilized to study the dissolution behavior in the present investigation.

Drug Release Kinetics

To know the mechanism and kinetics of drug release of the formulations, the results obtained from the *in vitro* drug release studies were analyzed by best fitted kinetic models.

1. Zero-order drug release: cumulative % drug release versus time.

2. First-order drug release: log cumulative % drug retained versus time.

3. Higuchi's model: cumulative % drug release versus square root of time.

4. Korsmeyer-Peppas model: log cumulative versus log time.

In these plots, the best fit model was chosen by looking at the R^2 values acquired[31, 32].

Zero-Order Model

To study the zero-order release rate kinetics the release rate data were fitted to the following equation:

$Q_t = Q_o + K_o T$

Where Q_t = amount of drug dissolved in time t,

Q_o = initial amount of drug in the solution,

K_o = Zero-order release rate constant.

First-Order Model

To study the first-order release rate kinetics the release rate data were fitted to the following equation:

$\text{Log } Q_t = \log Q_o + K_1 t/2.303$

Where Q_t = amount of drug released in time

Q_o = initial amount of the drug in the solution

K_1 = first-order release rate constant

Higuchi Model

The dissolution from a planer system having a uniform matrix follows the release rate pattern as per the equation:

$Q_t = K_H . t^{1/2}$

Where Q_t = amount of drug released in time t,

K_H = Higuchi dissolution constant.

Korsmeyer-Peppas model

The exponential relation of time with the fractional release of drug is predicted by this model. N is the exponent for the diffusion release mechanism. The equation is given below:

$M_t/M_\infty = K.t^n$

Where M_t/M_∞ = fraction of drug release,

K = release constant,

t = release time,

n = Diffusional exponent for the drug release.

RESULTS and DISCUSSION

Drug entrapment efficiency and production yield

The percentage entrapment efficiency of drug into the microspheres ranged from 28.69-68.51 % shown in figure 3(a). The percentage production yield of the drug in the mucoadhesive microspheres ranged from 35.19-68.65 % shown in figure 3 (b) which indicated that an increase in the amount of sodium alginate and sodium CMC also enhanced the amount of production yield (Table 2). Increasing the concentration of the sodium CMC in the formulation increased the bonds forming groups, thus increasing the mucoadhesicve force of the formulations[33]. Mucoadhesion behavior of alginate was due to the low surface tension (31.5 mN/m) of the alginate.

The drug content of drug in the mucoadhesive microspheres ranged from 28.67-72.54 %. The percentage entrapment efficiency and percentage production yield of batch 3 (F3) is highest among the all other formulations. So this formulation is selected for the optimized formulation.

Swelling index (%)

From the swelling study[34], it was showed that all prepared formulation of microspheres quickly swelled in phosphate buffer pH 6.8. The swelling index of alginate microspheres after a specified time lies within the range of 71.25-82.37 % (table 2) shown in figure 3 (c).

Table 2. Responses result of all batches

Batch	In vitro Wash-off Test (% Mucoadhesion After 1 hr)	Entrapment Efficiency (%)	Production yield (%)	Swelling index (%)
F1	68	32.24	51.76	71.25
F2	71	37.19	48.24	74.29
F3	84	68.51	73.25	82.37
F4	63	32.58	39.67	73.18
F5	82	58.37	68.65	80.13
F6	67	45.14	53.17	77.82
F7	72	28.69	24.19	79.18
F8	70	34.45	37.45	76.51
F9	75	39.86	35.19	72.41

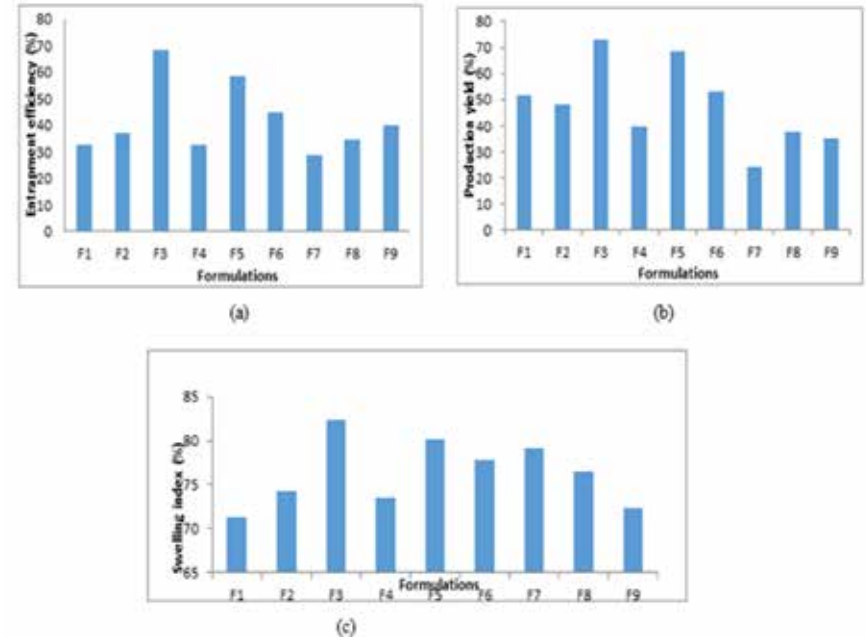

Figure 3. Drug entrapment efficiency (%) (a), production yield (%) (b) and Average swelling index (%) of microsphere

Mucoadhesive property of microspheres

The in vitro wash-off test for percentage mucoadhesion after 1 hour varied from 51 to 78%.

In-vitro drug release studies

The drug release studies were carried out on the prepared formulations as well as the marketed brand of Ibuprofen (Advil 200) for comparison of the drug release profile with optimized batch (F3). The cumulative amount of drug released from the marketed tablet was found to be about 74.45 % in 2 hours. The maximum amount of drug released from the microspheres formulations was 84.39. However, the drug release was found to be in the range of 35 to 77% approximately in 10 hours shown in figure 4. Moreover, it was observed that with an increase in the amount of polymers, the rate of drug release was retarded which was found to be in the range of about 43 % to 85 % at the end of the study period. The batch 3 (F3) was selected as the optimized batch due to highest entrapment efficiency and highest production yield.

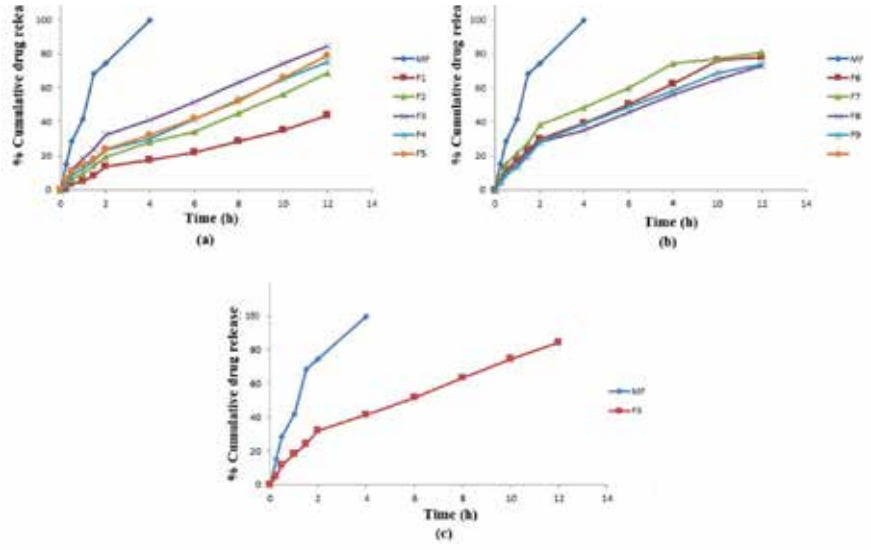

Figure 4. (a) Drug release profiles of batch F1-F5, (b) F6-F9 and (c) *In-vitro* drug release profile of optimized batch (F3) compared with marketed tablets (MF = Marketed Formulation).

Differential Scanning Calorimetry Analysis

DSC thermograms were recorded for pure ibuprofen and optimized batch (Figure 5). In both cases it was observed that the characteristic endotherm (corresponding to melt of the drug) did not shift appreciably, suggesting the lack of any interaction between the drug and excipients[35].

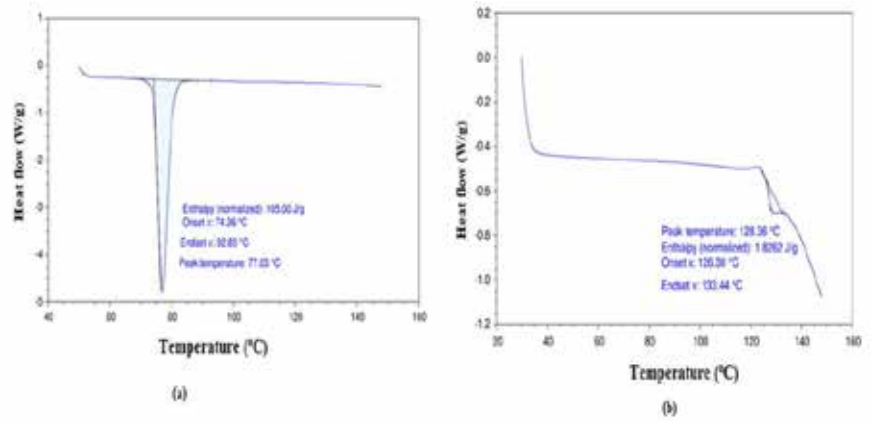

Figure 5. DSC thermogram of ibuprofen (a) and optimized batch (b). Scanning electron microscopy

The SEM study also revealed that there was no change in the morphology of drug loaded microspheres, and resulting microspheres were found to be discrete and spherical in shape and had nearly smooth surface as shown in the figure 6. During dissolution, the presence of drug particles on the surface of ibuprofen loaded microspheres may be responsible for an initial burst release of the drug[27].

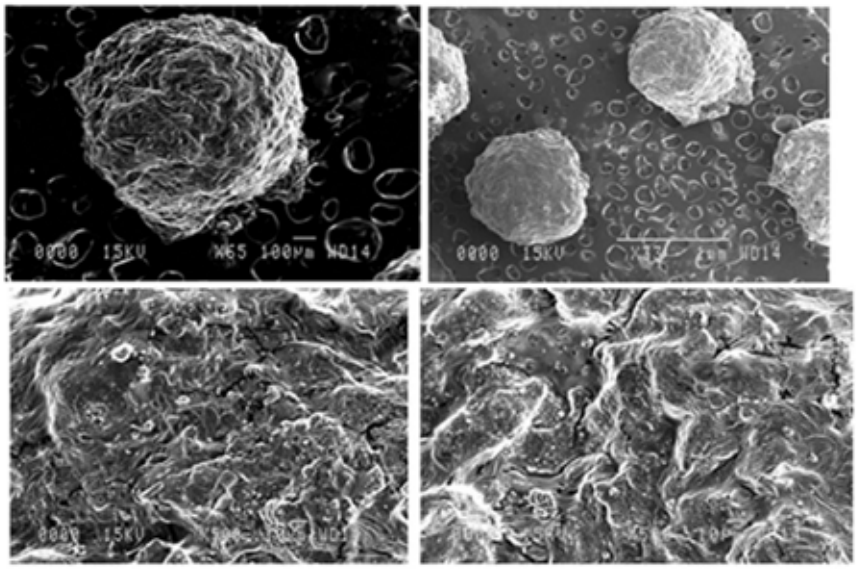

Figure 6. SEM images of mucoadhesive microspheres

X-Ray Powder Diffractometry (XRD)

The XRD patterns of drug, physical mixture (Sodium alginate, sodium CMC and drug) and Ibuprofen incorporated in mucoadhesive microspheres formulations are represented in Fig 7 (a-c). The presence of distinct characteristic peaks in the XRD pattern of Ibuprofen depicts its highly crystalline nature. Exploration of the XRD patterns of physical mixture shows a slight change in their intensity and optimized formulation shows less intense and wide diffraction peaks, which can be characterized by partial amorphous nature of Ibuprofen. XRD analysis does not exhibit any diffraction pattern of drug in optimized microspheres formulation, which reveals the significant reduction in the crystalline nature of the drug. XRD analysis of optimized formulation shows the presence of drugs as molecular dispersion in the optimized formulation of drug-loaded mucoadhesive microspheres.

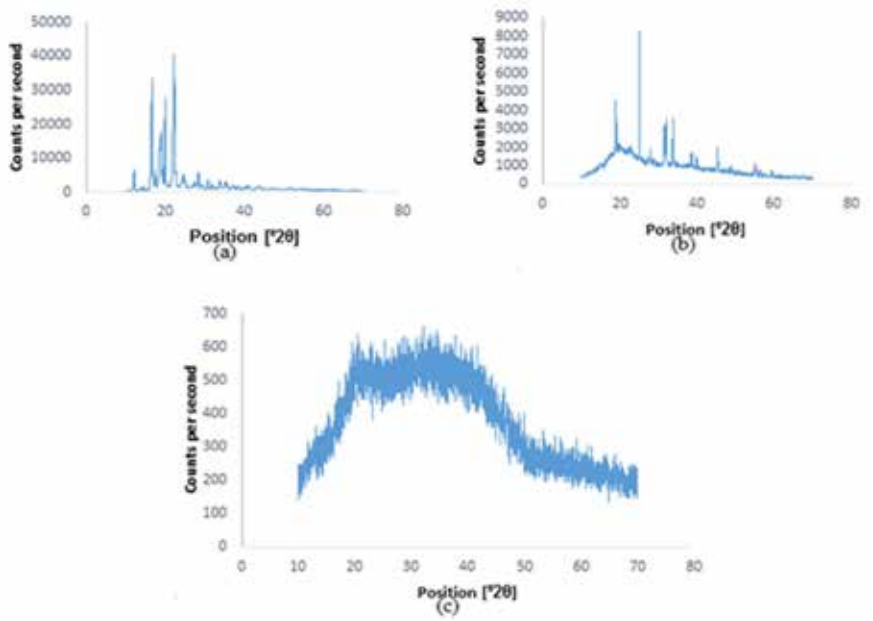

Figure 7. (a) XRD of pure drug (Ibuprofen), (b) physical mixture of polymers and (c) optimized batch of mucoadhesive microspheres

In-vitro drug release of mucoadhesive microspheres

The *in-Vitro* release data of drug were put into numerous models to determine the drug release kinetics. The batch 3 (F3) was found to fit in the Higuchi model as it showed the maximum value of the R^2. The Higuchi model showed that the drug discharge from the formulation by the Fickian diffusion mechanism. *In-vitro* drug release kinetics data of selected formulation (F3) is given in table 3.

Table 3. Various models with their R^2 values

Model	R^2 value
Zero-order	0.963
First-order	0.588
Higuchi	0.989
Korsmeyer-Peppas	0.603
Best Fit Model	**Higuchi model**

In the present study, ibuprofen was formulated as Mucoadhesive microspheres by ionic gelation technique (ionic cross-linking technique or drop extrusion method) by using varying concentrations of polymers sodium alginate and so-

dium CMC. The mucoadhesive microspheres of Ibuprofen were characterized by drug content, particle size distribution, production yield, *in-vitro* drug release, and entrapment efficiency. The optimized batch of microsphere (F3) was further evaluated by FT-IR, DSC, XRD, and SEM analysis. The data obtained from DSC studies confirmed no polymorphic change and chemical interaction with excipients in the drug-loaded microspheres. The XRD study suggested the change in the physical behavior of drug from crystalline to amorphous within the formulation. The SEM analysis shows that particles of all the formulated microspheres is spherical having a rough outer surface and is porous. The formulated batch F3 was chosen as optimized in terms of entrapment efficiency (68.51 %) and *in-Vitro* release of drug (84.39 %) in 12 hours. So from the result, it could be concluded that the concentration of polymers affected the various evaluation parameters. The Entrapment efficiency of the microspheres depends on variations in the concentration of polymers. The entrapment efficiency of microspheres ranged from about 28.69-68.51 %. The *in-vitro* drug release studies of each formulation was carried out for 12 hours in phosphate buffer pH 6.8. The cumulative amount of drug released was found to be in the range of 43.72-84.39 %. The data obtained from the *in-vitro* drug release profiles of Ibuprofen determined that all the batches of mucoadhesive microspheres showed prolonged drug release. The Higuchi model (R^2=0.9899) was found to be the best-fit model for the optimized batch (F3).

It could be concluded that the mucoadhesive microspheres of Ibuprofen showed prolonged release of the drug. The potential use of the formulations for a more effective management of inflammation and pain may be further explored with the help of long term pharmacokinetic and pharmacodynamic studies.

AUTHOR CONTRIBUTIONS

Kanika Sharma- Conceptualization, Writing – Original Draft Preparation; Sunita Devi- Conceptualization, Supervision.

DECLARATION OF INTEREST

The authors report no conflicts of interest.

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Haem Polymerization Inhibitory Activity and Cytotoxicity of Six Medicinal Plants Used in Cameroon for the Management of Malaria

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ABSTRACT

Malaria was successfully treated with both natural and synthetic products. However, recent progress in battling malaria has stalled due to drug resistance. Therefore, the search of novel antimalarials capable of reversing or evading resistance is much needed and this could be achieved through ethnomedicinal approaches. Six medicinal plants were screened for their antimalarial activity using the β-hematin inhibition (BHI) assay and their effect on the proliferation of three cancer cell lines (A549, MCF7 and PC3) was assessed by the MTT assay. Amongst the twenty-seven extracts screened, *Pseudospondias microcarpa* bark showed significant BHI activities with IC_{50} values of 2.5 ± 0.1 and 4.0 ± 0.2 μg/mL for DCM and MeOH extracts, respectively, while having no cytotoxic effect on A549, MCF7 and PC3. The current results support the ethnopharmacological use of *P. microcarpa* in the treatment of malaria, and it could constitute a useful source of potent antimalarial compounds.

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INTRODUCTION

Malaria caused by drug resistant *Plasmodium* can prove to be a fatal infection despite both prevention measures (e.g. bed nets) and drug treatment measures in endemic areas[1]. In 2019, there were an estimated 229 million cases claiming 409,000 lives compared to 228 million and 405,000 cases and deaths, respectively, in 2018[2]. Malaria is established in 91 countries and is ubiquitous in sub-Saharan Africa, where around 90% of global deaths have been documented, especially for the under-fives[3]. Global efforts to control and eliminate malaria have been further threatened since the emergence of drug resistance. However, this is not a new phenomenon; resistance to quinine was found in 1910 and to chloroquine in the 1970s[4,5]. One of the most effective recent introductions, artemisinin (and its semi-synthetic derivatives) have their efficacy also diminishing in the Greater Mankong Subregion and the zone of resistance is propagating through economic migration[6,7]. Among existing antimalarial drugs, resistance to quinine has been shown to develop slowly compared to other antimalarials. Therefore, quinine is still in use today and remains the drug of last resort for the treatment of multidrug resistant malaria infection[8,9]. Therefore, there is a continuing search for compounds that may be worthy of clinical development to fight the resistance phenomenon[10,11].

Quinoline antimalarials such as amodiaquine, chloroquine and quinine were found to act at the erythrocytic stage of the malaria parasite by inhibiting the polymerisation of haem to haemozoin[12]. The formation of haemozoin crystals, a critical process in malarial parasite detoxification has been identified as a suitable target for antimalarial drugs development[13]. Specifically, the parasite catabolises haemoglobin both for anabolic parasite replication to make room and for growth. However, in doing so it has the potential to release haem which can accumulates in its vacuoles up to toxic levels thereby killing the parasite[14,15].

In the present study, we evaluated the haem polymerization inhibitory activities of the crude extracts of six medicinal plants from the Cameroonian flora, in a cell free medium, as an indicator of their antimalarial potential. These include *Croton oligandrus* Pierre ex. Hutch, *Entandrophragma congoënse* (DeWild) A. chev., *Pseudospondias microcarpa* (A. Rich.) Engl., *Ruspolia hypocrateriformis* (Vatair feilding ephl) Milne-Redh, *Zanthoxylum lepreurii* Guill. & Perr. and *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler. They are used in

Cameroonian traditional medicine for the treatment of different ailments including malaria and malaria symptoms including anaemia and fever (Table 1)[16-22]. The cytotoxic effects of the plants were also evaluated against three cancer cell lines.

Figure 1. Selected medicinal plants studied: Croton oligandrus Pierre ex Hutch leaves a) and stem bark b); *Entandrophragma congoënse* A. Chev. stem bark c); *Ruspolia hypocrateriformis* Vahl Milne-Redh leaves d); *Pseudospondias microcarpa* (A. Rich.) Engl. stem bark e), leaves f) and fruits g); *Zanthoxylum lepreurii* Guill. and Perr. fruits h); *Zanthoxylum zanthoxyloides* (Lam.) Zepern. and Timler fruits i).

METHODOLOGY

Plant materials

Materials for extraction were collected from their natural habitat in three sites namely: S1) Mount Eloundem (3⁰49′1.794″N 11⁰25′59.412″E); S2) Melen (3⁰51′51.559″N 11⁰30′8.748″E) in Yaoundé, Centre-Cameroon and S3) Tsenfem (5⁰27′12.052″N 10⁰3′9.021″E) in Dschang, West-Cameroon. All collected plant parts (Figure 1) were identified and authenticated at the Cameroon National Herbarium Yaoundé by Mr Nana where their voucher specimens have also been deposited (Table 1).

Preparation of crudes extracts

Air-dried and ground plant material (200 g for each plant part collected) were extracted, successively, with *n*-hexane (HEX), dichloromethane (DCM) and methanol (MeOH), 800 mL using a Soxhlet extractor. Ten cycles were completed for each successive solvent extraction. The extracts were filtered and evaporated to dryness using a rotary evaporator at a temperature not exceeding 40 °C and subsequently kept at 4°C until required[22].

Evaluation of the antimalarial activity using *β*-hematin assay

The cell-free method described by Afshar et al[23] with some modifications, was used. Different concentrations of the extracts (10 μL, 0-250 μg/mL) prepared in DMSO were incubated with 100 μL of 3 mM of hematin, 10 μL of 10 mM oleic acid and 10 μL of 1 M HCl. Sodium acetate buffer 500 mM, pH 5 was used to adjust the volume to 1000 μL. All the solutions were pre-warmed at 40°C before initial mixing. The samples were incubated overnight at 37 °C with regular shaking. After incubation, samples were centrifuged (14,000 x g, 10 min, at 21 °C) to precipitate haemozoin pellets. This was then washed four times with sonication (30 min, at 21 °C; FS100 bath sonicator; Decon Ultrasonics Ltd.) using a solution of 2.5% (w/v) SDS in phosphate-buffered saline followed by a final wash in 0.1 M sodium bicarbonate, pH 9.0, until the supernatant was clear. After the final wash, the supernatant was removed, and the pellets were re-suspended in 0.5 mL of 0.1 M NaOH and transferred to a 96 well plate before determining the haemozoin content by measuring the absorbance at the Soret band (405 nm). The results were recorded as % inhibition (I%) of haem polymerization/crystallization using the following formula: I% = [(AB–AA)/AB] x 100, where AB: absorbance of blank (medium without sample containing 10 μL DMSO instead); AA: absorbance of test samples. Chloroquine diphosphate (prepared in distilled water) and medium without any extract were used as positive and negative controls, respectively.

In vitro cytotoxic assay

The cytotoxicity of the crude extracts against A549 (adenocarcinoma human alveolar basal epithelial cell line), MCF7 (human breast adenocarcinoma cell line) and PC3 (human prostate cancer cell line) was evaluated using the MTT assay.[24] The cells were grown in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% foetal bovine serum (FBS) and cultured at 37°C, 5% CO_2 and 95% humidity. For conducting the assay, the cells were seeded into 96 well plates (1.2×10^4/well) and incubated for 24 h. Cells were then treated with crude extract (0-250 μg/mL) for 24 h and the cell viability measured. Stock solutions (500 mg/mL) of crude extracts were prepared in DMSO and dilutions were made in cell culture media, with the final concentration of DMSO being below 0.1%. The resultant formazan crystals were dissolved in DMSO and optical density was read at 570 nm using a ClarioStar microplate reader (BMG Labtech, UK). The cytotoxicity was determined using the percentage absorbance compared to control cells [(absorbance of treated cells/absorbance of untreated cells) × 100].

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± SEM (standard error of mean). IC_{50} values were calculated using the software Graphad Prism 7.02 (GraphPad Prism Software Inc., USA). Differences in means were estimated by means of repetitive measures followed by Mann Whitney test. Differences between means were regarded significant at $p<0.05$.

RESULTS and DISCUSSION

Plants are the almost exclusive source of healthcare for 80% of the world's population and particularly in the developing countries[25]. The ethnopharmacological properties of plants have been used as a primary foundation for drug discovery[26,27]. Natural products continue to provide a useful source of new drugs as evident from a four-decade review[28]. Fabricant & Farnsworth[29] demonstrated that 80% of the 94 medicinal plants (from which 122 compounds have been isolated and used as drugs) possess an ethnomedicine use indistinguishable (or related) to the current use of the active plant principle. The importance of ethnopharmacological studies involving medicinal plants remains important in drug discovery and the pharma sector has shown renewed interest especially for uncovering new prototypes to tackle drug resistance[30,31].

In the current investigation, the six plants from five families screened were selected on the basis of their use in traditional medicine in Cameroon for the treatment of malaria and associated symptoms, especially anaemia and fever.

A total of twenty-seven extracts were obtained from the different parts of the six medicinal plants collected. The extracting solvents used successively extracted the constituents of the plant gradually according to their affinity and increasing dielectric constant with each solvent[32]. The highest extraction yield was recorded for *Z. zanthoxyloides* fruits (13.7 %), while *P. microcarpa* stem bark provided the lowest extraction percentage yield of 3.3%(Table 1). Screening of the extracts for their haem polymerization inhibitory activity revealed three extracts that exhibited promising antimalarial activity with $IC_{50} \leq 30$ μg/mL. The results are shown in Table 2. The DCM and the MeOH stem bark extracts of *P. microcarpa* showed significant activity with IC_{50} values of 2.5 ± 1.5 and 4.0 ± 1.7 μg/mL, respectively. The fruit extracts of the same plant were inactive, whereas only the MeOH extract of the leaves showed better activity with IC_{50} of 13.0 ± 9.0 μg/mL. Interestingly, similar results were previously reported for the ethanol extracts of *P. microcarpa* stem bark and leaves IC_{50} 1.13± 0.16 and 26 ± 10 μg/mL, respectively, when tested *in vitro* against the multidrug resistant *Plasmodium falciparum* K1 and chloroquine-resistant FCM29

Cameroonian strains, respectively[33,34]. This suggests the haemozoin assay is a suitable assay to probe the antimalarial activity.

The twenty-seven extracts were also evaluated for *in vitro* cytotoxicity against A549, MCF7 and PC3. Doxorubicin was used as the reference drug. Most of the screened extracts exhibited low or no toxicity at the highest concentration tested against the assayed cell lines with IC_{50} values ≥50 μg/mL (Table 3). *E. congoënse* stem bark extracts were found to be the most cytotoxic against all the cell lines tested. A moderate cytotoxicity was observed for its *n*-hexane extracts against A549 and MCF7, and DCM extract against PC3 with IC_{50} = 28.1 ± 6.3, 40.3 ± 5.2 and 32.2± 6.1 μg/mL, respectively, while significant toxicity against MCF7 (IC_{50} = 21.6 ± 0.9 μg/mL) was exhibited by the DCM extract. Extracts of *P. microcarpa,* which demonstrated significant haemozoin inhibitory activity, were found to have no cytotoxic effect on the tested cell lines (IC_{50} values >250 μg/mL). This suggests that extracts that inhibits haemozoin crystallisation may share similar targets and/ or mechanisms of action as acridines

Table 1. Medicinal plants studied, place of collection, Voucher number, ethnomedical uses and yield of extraction

Plant name (Family)	Voucher Nº	Place	Ethnomedical uses	Chemical contents	Plant Part	Weight of extract (g)			Yield (%w/w)
Solvent						**HEX**	**DCM**	**MeOH**	
C. *oligandrus* (Euphorbiaceae)	6687/SFR	S1	Anaemia, cancer, pneumonia, splenomegaly	Diterpenes	Leaves	4.91	5.7	7.07	8.8
					Stem Bark	2.24	1.27	4.48	4.0
E. *congoense* (Meliaceae)	43234/SFR	S1	Gastric ulcer and malaria	Limonoids, steroids, tirucallane triterpenes	Stem Bark	4.82	2.38	11.95	9.6
P. *microcarpa* (Anacardiaceae)	41437/SFR	S1	Anaemia, malaria, helminthiasis	Flavonoids	Fruits	3.14	0.73	5.43	4.6
					Leaves	6.13	1.24	5.72	6.5
					Stem Bark	1.6	0.8	4.27	3.3
R. *hypocrateriformis* (Acanthaceae)	37822/SFR	S2	Anaemia, diarrhoea,	Flavonoids	Leaves	6.05	4.54	18.58	14.6
Z. *lepreurii* (Rutaceae)	106669/ SFR	S3	Anaemia, malaria	Benzophenantrine, acridone and aporphine alkaloids	Fruits	11.97	7.72	19.87	19.8
Z. *zanthoxyloides* (Rutaceae)	21793/SFR	S3	Anaemia, cancer, fungi infection and malaria	Acridone alkaloids, coumarins, amides, lignans	Fruits	34.05	3.38	10.43	23.9

S1: Mount Eloundem; S2: Melen; S3: Tsenfem; HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol. Yield of extraction is calculated as (the sum extracts weight for a given plant) x 100/ weight of powder (e.g. mepacrine) and 4-aminoquinlines (e.g. chloroquine, amodiaquine) which continue to play a role in the search for drugs that can evade parasite resistance.[4] The inhibition of haemozoin formation in parasites continues to be an attractive target for the development of new antimalarial drugs from medicinal plants[35]. This is due in part, to the idea that the formation of haemozoin in the parasite vacuole is essential for its survival[36]. Consequently, screening plant extracts that can inhibit haemozoin formation can allow rational mechanism-based discovery, for screening ethnomedical plant prototypes.

Our study provides some evidence on the haemozoin inhibitory-antimalarial mode of action as well as safety *in vitro* of *P. microcarpa*, thereby supporting its traditional use for the treatment of malaria. Notably, given that Adongo et al[37] showed that an ethanolic extract of *P. microcarpa* dosed to rats (*per oral*) proved safe. In the light of the afore mentioned results, we therefore, plan to isolate the active component and measure the antimalarial activity of the plant *in vivo* using animal models infected with strains of *Plasmodium* to determine its suitability for further development.

Table 2. Inhibition of β-hematin formation assay (IC_{50}) of the different extracts

Plants	**IC_{50} (µg/mL)**		
Solvents	**HEX**	**DCM**	**MeOH**
C. oligandrus L (COL)	> 250	> 250	> 250
C. oligandrus SB (COBSB)	180.0 ± 6.0	164.8 ± 53.0	> 250
E. congoënse SB (ECSB)	> 250	> 250	> 250
P. microcarpa F (PMF)	> 250	> 250	> 250
P. microcarpa L (PML)	> 250	> 250	13.0 ± 9.0 $^{\beta}$
P. microcarpa SB (PMSB)	73.9 ± 25.8*	2.5 ± 1.5 $^{\beta}$	4.0 ± 1.7 $^{\beta}$
R. hypocrateriformis L (RHL)	206.7 ± 52.^{0}	> 250	170.3 ± 77.9*
Z. leprieurii F (ZLF)	> 250	45.8 ± 25.0 *	> 250
Z. zanthoxyloides F (ZZF)	> 250	> 250	> 250
Chloroquine	0.43 ± 0.08 $^{\beta}$		

F: fruits; L: leaves; SB: stem bark. HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol.

IC_{50}: concentration of extract needed to produce 50% of the *β*-hematin formation inhibition, Values are presented as mean ± SEM (n=3). *p<0.05, $^{\beta}$p<0.001 vs control using Mann-Whitney test.

Table 3. Cell growth inhibitory activities (IC_{50}) of the different extracts against A549, MCF7 and PC3 cancer cells

Plants	IC_{50} (µg/mL)								
Cell lines	A549			MCF7			PC3		
Solvents	HEX	DCM	MeOH	HEX	DCM	MeOH	HEX	DCM	MeOH
COL	> 250	206.1 ± 14.4*	217.2 ± 39.5*	51.4 ± 11.0 $^{\beta}$	84.4 ± 18.4 $^{\beta}$	> 250	45.5 ± 62.4 $^{\beta}$	123.6± 1.8*	50.0± 2.6 $^{\beta}$
COBSB	> 250	> 250	> 250	31.6 ± 9.0*	> 250	> 250	71.7 ± 1.5 $^{\beta}$	59.8 ± 3.0 $^{\beta}$	> 250
ECSB	28.1± 6.3*	111.0 ± 20.5*	> 250	40.3 ± 5.2 $^{\beta}$	21.6 ± 0.9*	> 250	204.7 ± 17.7*	32.2 ± 6.1 $^{\beta}$	> 250
PMF	32.5 ± 8.5 $^{\beta}$	53.0 ± 8.8 $^{\beta}$	> 250	185.8 ± 5.5*	61.1 ± 2.9 $^{\beta}$	> 250	139.6± 21.2*	66.2 ± 2.0 $^{\beta}$	> 250
PML	> 250	> 250	> 250	> 250	146.2 ± 9.8*	> 250	> 250	147.5 ± 3.7*	> 250
PMSB	> 250	177.1± 612.5*	> 250	89.0 ± 2.9 $^{\gamma}$	> 250	> 250	63.0 ± 2.5 $^{\beta}$	217.3± 8.6*	> 250
RHL	127.8 ± 38.6*	113.8 ± 3.5*	> 250	> 250	40.3 ± 6.6 $^{\beta}$	> 250	> 250	64.4 ± 4.3 $^{\beta}$	> 250
ZLF	55.3 ± 6.8 $^{\beta}$	67.3 ± 9.2 $^{\beta}$	> 250	84.7 ± 5.8 $^{\gamma}$	174.0 ± 18.2*	> 250	49.5 ± 9.0 $^{\beta}$	58.1 ± 5.1 $^{\beta}$	> 250
ZZF	105.0 ± 6.8 $^{\gamma}$	206.6 ± 11.5*	> 250	43.6± 6.5 $^{\beta}$	54.4 ± 8.5 $^{\gamma}$	> 250	10.6 ± 1.0*	93.2 ± 4.4 $^{\gamma}$	> 250
Doxo	1.3 ± 0.3*			0.7 ± 0.1*			16.4 ± 2.9*		

F: fruits; L: leaves; SB: stem bark. HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol. Doxo = Doxorubicin. IC_{50}: concentration of extract causing 50% of cells death. Values are presented as mean ± SEM (n=3). *$p<0.05$, $^{\alpha}p<0.01$, $^{\beta}p<0.001$, $^{\gamma}p<0.0001$ vs control using Mann-Whitney test.

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STATEMENT OF ETHICS

None needed

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Stephanie T. Guetchueng investigation, writing-original draft, writing-reviewing and editing, data curation; Lutfun Nahar supervision, writing-original draft, data curation, writing-reviewing and editing; Kenneth James Ritchie supervision, writing-reviewing and editing, data curation; Fyaz M.D. Ismail supervision, writing-reviewing and editing, data curation; Andrew R. Evans writing-reviewing and editing, data curation; Alembert T. Tchinda writing-original draft; writing-reviewing and editing; Arrey P. Tarkang writing-original draft; writing-reviewing and editing, data curation; Emmanuel N. Nnanga writing-reviewing and editing; Satyajit D. Sarker supervision, writing-original draft, writing-reviewing and editing, data curation

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Phytochemistry and Antibacterial Efficacy of Northeastern Pakistani *Artemisia rutifolia* Stephan ex Spreng. Extracts against Some Clinical and Phyto-pathogenic Bacterial strains

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ABSTRACT

Recently, most researches have focused on the biological activities of the extracts obtained from different *Artemisia* species due to the presence of essential compounds with strong activity against some gram-negative and gram-positive bacteria. In this study, five extracts of *Artemisia rutifolia* Stephan ex Spreng. from the northeastern Gilgit-Baltistan region of Pakistan were analyzed for total flavonoid and total phenolic contents and their antibacterial activities against some clinical and phyto-pathogenic bacterial strains were assessed with agar disk diffusion method. Results indicated that the methanol, ethanol, chloroform, ethyl acetate and *n*-hexane extracts of *A. rutifolia* are rich in flavonoids and phenols and all the tested extracts showed the broad spectrum growth inhibition of the tested gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*). Overall, methanol and ethyl acetate extracts showed better activities even at lower concentrations (5 mg/

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ml) where *B. subtilis* and *P. aeruginosa* were the most susceptible strains. Hence, the MICs of these two effective extracts (methanol and ethyl acetate extracts) were tested against most susceptible bacterial strains (*B. subtilis* and *P. aeruginosa*) at 1-4 mg/ml conc. Results of MICs showed that both the methanol and ethyle acetat extracts were effective against *B. subtilis* and *P. aeruginosa* at 3 and 4 mg/ml concentrations where ethyl acetate extract exhibited higher inhibitory effect than the methanol extract. Therefore, extracts of *A. rutifolia* could be used as operational sources against pathogenic bacterial diseases.

Keywords: *Artemisia rutifolia*, TFC, TPC, antibacterial activity, minimum inhibition concentrations.

INTRODUCTION

Artemisia L. is a noteworthy member of the Asteraceae family, which is a polymorphic genus and is important from both economic and therapeutic point of view. Species of this genus are mostly found in the northern hemisphere especially in the temperate zones, but few taxa are also present and reported from the southern hemisphere of the world[1]. There are ~500 species in the *Artemisia* genus including both shrubs and herbs[2] which are considered as a diverse genus from the Asteraceae family of the Anthemideae tribe[3]. In plants, there exist some organic and inorganic compounds and also individual elements are present which gives therapeutic effects against various infections.

For many years, the utilization of *Artemisia* species as medicine is a common exercise in traditional medicine and it is still continued in many communities. The extracts and essential oils from different *Artemisia* species are extensively used for a variety of medicinal purposes due to their pharmacological significance producing most of the medicinally significant secondary metabolites[4,5] with a sequence of biological activities including antioxidant and antimicrobial activities[6].

Artemisia rutifolia Stephan ex Spreng from the genus *Artemisia* is a shrub native to the northern Pakistan and called vernacular name is *Afsanteen*. It reaches the height of 20 to 80 cm[7] and is used traditionally in the North Pakistan for the treatment of asthma, cough, fever, inflammation, abdominal pain, cancer, and other ailments[8,9]. It has been showed that the essential oil from *A. rutifolia* possess compounds like thujone, germacranolide, eudesmanolide sesquiterpenoids and guaianolide[10] mainly responsible for the therapeutic effects against diseases.

Bacteria and viruses are the pathogens responsible for many health problems in humans and the occurrence and expansion of antibiotic resistance, as well

as the evolution of new disease causing bacterial and fungal strains are of great concern to the global health community. In this regard, the screening of antimicrobial potentials from plant extracts could be more helpful in monitoring phytopathogens and clinical uses as natural antimicrobials.

Frequently used medicinal plants of our community especially *Artemisia* plants are excellent drug sources to cope with problems posed by drug resistant microbes. While in the recent past, much focus has been given towards the pharmacological activities of *Asteraceae* plants[12,13]. There exist a knowledge gap about the antibacterial activity of some *Artemisia* species including *A. rutifolia* and the literature search also indicated no or very limited reported data availability on the antibacterial activity of this plant. Therefore, the present study aimed to report the TFC, TPC and the potential antibacterial activity of methanol, ethanol, ethyl acetate, chloroform and *n*-hexane extracts of *A. rutifolia* from the Northeast Gilgit-Baltistan region of Pakistan.

METHODOLOGY

The present study was conducted in the Biotechnology laboratory, Department of Biotechnology, University of Okara, Pakistan and Applied Microbiology and Biotechnology laboratory (AMBL), International Islamic University Islamabad Pakistan. *A. rutifolia* (Figure 1) was collected (Collectors, Adil hussain and Mujtaba Hassan), from the natural environment in the Ataabad Hunza-Nagar district of Gilgit-Baltistan region of Pakistan (Table 1). The study area (Gilgit-Baltistan) is situated in the northeast of Pakistan with diverse climate and the area is very much popular for its immense plants biodiversity[14]. It is situated in between the longitude latitude 35° to 37° east and 72° to 75° north having 7 major districts including Astore, Diamer, Baltistan, Ganche, Gilgit, Ghizar and Hunza-Nagar. The collected sample of *A. rutifolia* was first pressed with a wooden presser, dried up then mounted and labeled on the herbarium sheet (Figure 2). The prepared herbarium was submitted to the herbarium of Pakistan Museum of Natural History (PMNH) Islamabad, Pakistan to obtain herbarium specimen number[15] for future reference. The details collection, source and GPS locality details of *A. rutifolia* specimen are given in Table 1. The collected specimen was identified by assessing various morphological characteristics and by relating those characters with the already available herbarium specimen prior to the assessment of phytochemicals and antibacterial activity.

Solvent Extraction

Before the extraction with organic solvents, the plant specimen was cleaned with deionized water and then shade dried for almost a week. The dried leaves

and aerial parts were grinded to fine powder with the help of mortar and pestle and the powder was filtered using gauss cloth. The powdered sample was stored in air tight containers at 4°C for further use. Five organic solvents like methanol, ethanol, chloroform, *n*-hexane and ethyl acetate were used to obtain extracts from the plants using soxhlet extraction procedures. Briefly, 10 grams of the powered samples were taken in the muslin cloth for continuous extraction process using soxhlet apparatus at a temperature below the boiling temperature of all solvents. A portion of the powdered samples of plants were soaked in the solvent in a conical flask, wrapped with aluminum foil and placed in shaker for 48 hrs at 120-130 rpm. After 48 hrs, the obtained extracts were filtered using whattman filter paper No: 1. Evaporation of the solvent from extract was done and the residue containing extract was dissolved in sterile dimethylsulfoxide (DMSO, 9:1) in 50 mg/ml concentration. The extract was then filtered with 0.22 micro filters (Type GV- Millipore) and then kept at 4°C for further study.

Total flavonoid content (TFC) in *A. rutifolia* extracts

The quantitative determination of total flavonoids content (TFC) was performed using the aluminum chloride colorimetric technique[16] with little modifications. Briefly, 20 µl test samples were taken from each stock solution, with the addition of 10 µl of aluminum chloride in 90 µl of water (w/v). 160 µl of water was added in 96 well plates along with 0.1 % of 10 µl potassium acetate. The solution was incubated for 30 minutes at ambient temperature. The absorbance was measured at 415 nm. The total flavonoids content was determined by using a microplate reader. The experiment was repeated thrice and results were expressed with unit µg QE/mg DW (micrograms equivalent to quercetin milligram dry weight).

Total phenolic content (TPC) in *A. rutifolia* extracts

The total phenolic content of *A. rutifolia* crude extract was estimated by using folin's ciocalteu's reagent[17]. 20 µl extract was taken and mixed with 90 µl of folin ciocalteu reagants (v/v) in 96 well plates. The solution was incubated for 5 minutes, and 90 µl of sodium carbonate solution was added. The assay plate reader absorbance was set at 630 nm, and the absorbance of 96 well plates was measured using a microplate reader. A calibration curve (R2= 0.98) was obtained by using gallic acid as a positive standard. The experiment was repeated thrice and results were noted, the expression of the result is mentioned with unit µg GAE/mg DW (as gallic acid equivalent milligram dry weight)[16].

Antibacterial activity of *A. rutifolia* extracts

For the antimicrobial activity of *A. rutifolia* extracts, both gram-positive and gram-negative pathogenic bacterial strains were used. The strains were *S. aureus, B. subtilis, E.coli*, and *P. aeruginosa* obtained from the Microbiology laboratory of Mirpur University of Science and Technology (MUST) AJK Pakistan. The stock cultures of the strains were maintained in nutrient agar slant at 4°C and were subcultured on monthly basis. Microscopic identification of the bacterial strains was done prior to the assessment of antibacterial activity of the plant extracts. For the antimicrobial activity of extracts, agar disk diffusion method was used[18]. Briefly, the plant extract residues (40 mg) were dissolved in the solvent which was sterilized with Millipore filter (0.22 mm) then loaded over sterile filter paper discs (8 mm in diameter) to get final concentration of 10 mg/disc. About 10 ml of Mueller-Hilton agar (MHA) medium was poured into sterile petri dishes as a basal layer followed with 15 ml of seeded medium previously inoculated with bacterial suspension (100 ml of medium/1 ml of 107 CFU) to attain CFU/ml of medium. Plant extract concentrations were loaded in sterile filter paper discs and were placed on the top of MHA plates. The standard antibiotic levofloxacin was used as a positive control and DMSO was used as negative control. The plates were kept in the fridge at 5°C for 2 hrs to allow diffusion of extracts then incubated at 35°C for 24 hrs. The measurement of inhibition zones was done by vernier caliper or zone reader scale and was considered as the indication for antibacterial activity.

Minimum inhibitory concentrations (MIC's) of *A. rutifolia* extracts

After assessing the susceptibility of the bacterial strains, the most effective extracts of *A. rutifolia* with strong antibacterial activity at 5 mg/ml were further assessed for MIC's against most susceptible bacterial strains at lower concentrations using disk diffusion method[18]. Different concentrations of the effective plant extracts (1-4 mg/ml) were arranged separately by dissolving 40 mg in 2 ml of the solvent. The standard antibiotic levofloxacin and DMSO were used as positive and negative controls. Inhibition zones were measured with a vernier caliper or zone reader scale for each concentration of the effective plant extracts.

Statistical analysis

Accuracy in measurement was obtained using the SPSS program (SPSS Inc. Chicago IL version 12.0). All readings were taken three times and 95% was the confidence interval for mean. Level of significance was ($P<0.05$).

Table 1. Collection details of *A. rutifolia* from the Gilgit-Baltistan region of Pakistan with voucher specimen number

Artemisia Sp.	**Location**	**Latitude**	**Longitude**	**Altitude (m a.s.l.)**	**Herbarium specimen no**	**Collectors**
Artemisia rutifolia Stephan ex Spreng.	Ata abad Hunza-Nagar	N-36'20.35	E-74'52.15	2419	PMNH-00046359	Adil Hussain and Mujtaba Hassan

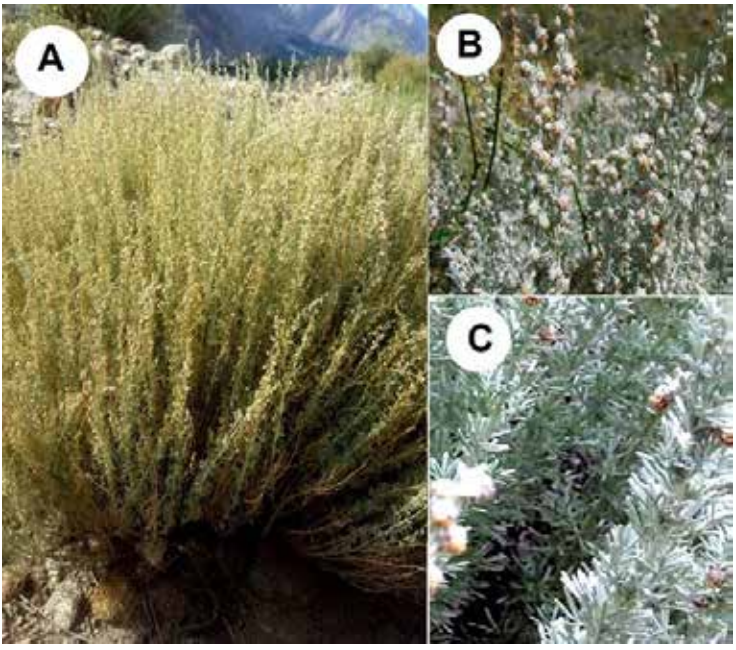

Figure 1. Morphology of *A. rutifolia* collected from Gilgit-Baltistan Pakistan A) Habit, B) Aerial part with synflorescence, C) Middle cauline leaves

Figure 2. Herbarium specimen (PMNH-00046359) of *A. rutifolia* deposited in the Pakistan Museum of Natural History (PMNH) Islamabad, Pakistan

RESULTS and DISCUSSION

Plants extraction yield (%)

The percentage yields of plant extract obtained from *A. rutifolia* using different solvents are given in Table 2. The extract from 40 g dried plant material with methanol yielded plant extract residue of 3.83 g (9.58 %), ethanol yielded plant extract residue of 4.12 g (10.31%), ethyl acetate yielded 1.73 g (4.32 %), chloroform yielded 1.56 g (3.92 %) and *n*-hexane yielded 0.50 g (1.25 %) respectively.

Table 2. Percentage yield (w/v) of *A. rutifolia* extracts obtained using different solvents

Sr. No	Solvent	Plant biomass	Extract obtained	% Yield (w/v)
1	Methanol	40g	3.83g	9.58%
2	Ethanol	40g	4.12g	10.31%
3	Ethyl Acetate	40g	1.73g	4.32%
4	Chloroform	40g	1.56g	3.92%
5	n-Hexane	40g	0.50g	1.25%

TPC and TFC of *A. rutifolia* extracts

The quantitative estimation of TFC and TPC of the *A. rutifolia* confirmed higher phenol and flavonoid contents in its extracts. The maximum amount of phenols and flavonoids were recorded for ethanol extract and in comparison, ethyl acetate, chloroform, *n*-hexane, and methanol exhibited slightly lower TPC and TFC values respectively (Figure 3 and 4). The amount of TPC for *A. rutifolia* extracts was in the range between 31 μgGAE/mg to 57 μgGAE/mg (Figure 3). Ethanol extract showed a greater extent of TPC (57 μgGAE/mg) and *n*-hexane displayed minimum TPC values (31 μg GAE/mg).

TFC of *A. rutifolia* extracts recorded were in the range between 57.21μgQE/mg to 93.75μgQE/mg (Figure 4) where the ethanol extract showed maximum TFC (93.75 μgQE/mg) and *n*-hexane displayed minimum TFC (57.21 μgQE/mg). The overall pattern of the amount of flavonoids and phenols recorded in *A. rutifolia* extracts from highest to lowest is as follow: Ethanol > methanol > ethyl acetate > chloroform > *n*-hexane.

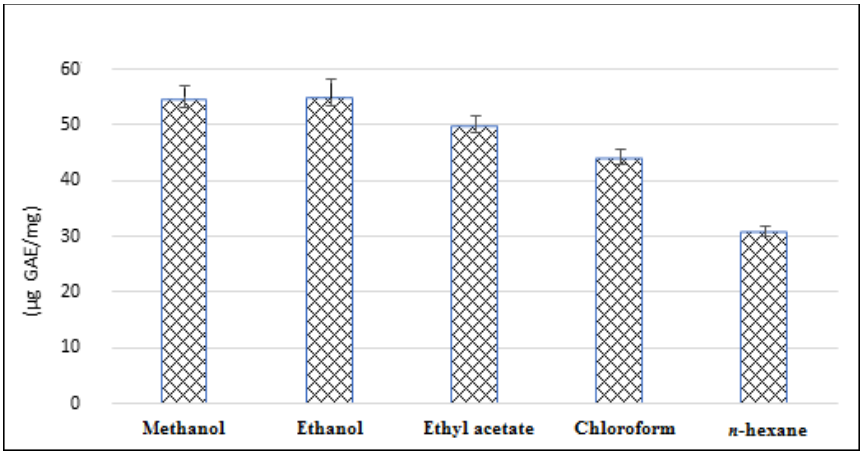

Figure 3. Total phenolic content (TPC) in different *A. rutifolia* extracts

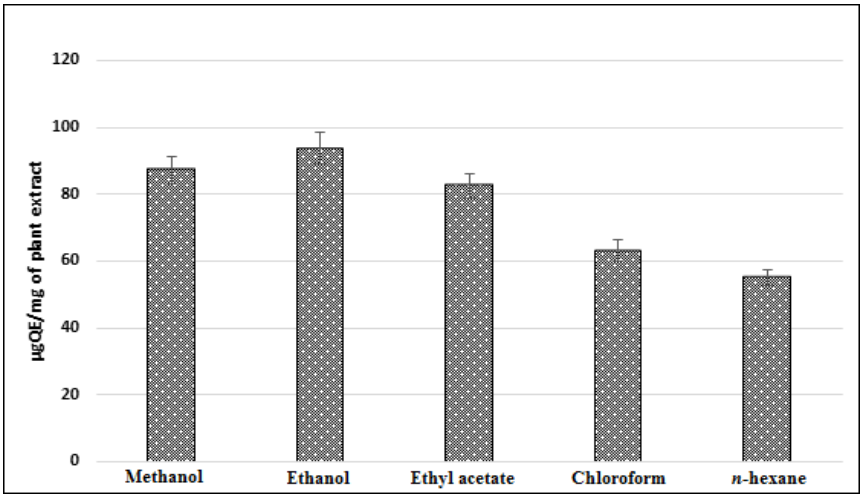

Figure 4. Total flavonoid content (TFC) in different *A. rutifolia* extracts

Antimicrobial activity of *A. rutifolia* extracts

Antibacterial activity of *A. rutifolia* extracts against two strains of gram-positive bacteria (*B. subtilis* and *S. aureus*) and gram negative bacteria (*E. coli,* and *P. aeruginosa*) using disc diffusion method displayed a very noteworthy outcomes. The antibacterial activity of organic solvent extracts displayed changing magnitudes of inhibition configurations with standard positive and negative controls depending on the tested strains susceptibility. Growth of all the tested bacterial strains was inhibited by all extracts of *A. rutifolia*. The mean inhibi-

tory zones of extracts against tested bacterial strains are summarized in Table 3 and illustrated in Figures 5-9. All extracts of *A. rutifolia* maximally retarded the microbial growth at the concentrations of 50, 25 mg/ml while slightly lower growth inhibition was recorded at 10 and 5 mg/ml concentrations for all extracts (Tables 3).

The methanol extract of *A. rutifolia* exhibited inhibitory effects (zones of inhibition) against the pathogenic strains at different concentrations ranges from 10.11 to 19.21 mm as shown in Table 3 and illustrated in Figure 5. At 50 mg/ml concentration, highest inhibitory effect of methanol extract was recorded against *B. subtilis* (19.21 mm), these are followed by *S. aureus* (15.23 mm). While minimum inhibitory effect of 14.05 mm was observed in *P. aeruginosa* and *E. coli*. At 25 mg/ml, the methanol extract displayed higher inhibitory effect against *B. subtilis* (18.73 mm) and minimum effects were noted for *S. aureus* (13.76 mm), *P. aeruginosa* (12.18 mm) and *E. coli* (12.33 mm). At 10 mg/ml, methanol extract displayed higher inhibitory effect against *B. subtilis* (15.21 mm) and minimum effects at 10 mg/ml methanol extract were noticed against *S. aureus* (10.01 mm), *E. coli* (11.44 mm) and *P. aeruginosa* (12.33 mm). At 5 mg/ml concentration, maximum inhibitory effect was shown against *B. subtilis* (14.45 mm) and lower effects were recorded for *E. coli* (10.11 mm) and *P. aeruginosa* (10.56 mm). Overall, the *S. aureus* strain was the most resistant to the methanol extract of *A. rutifolia,* at 5 mg/ml concentration, while other tested strains showed more susceptibility to the methanol extract at different concentrations respectively.

A. rutifolia ethanol extract demonstrated zones of inhibition range from 9 to 17 mm against the tested bacterial strains at different concentrations as shown in Table 3 and illustrated in Figure 6. The ethanol extract when taken 50 mg/ml, displayed maximum inhibitory effects against *P. aeruginosa* (17 mm), *B. subtilis* (16 mm) and *S. aureus* (16 mm) while lower effect (14 mm) was observed for the *E. coli* strain. At 25 mg/ml concentration, ethanol extract displayed maximum inhibitory effects against *P. aeruginosa* (16 mm) and *S. aureus* (15 mm) and slightly lower effects were observed for *B. subtilis* (13 mm) and *E. coli* (11 mm). At 10 mg/ml concentration, higher inhibitory effects (13 mm) were noticed against *S. aureus* and *P. aeruginosa* and low inhibitory effects were recorded against *B. subtilis* (12 mm) and *E. coli* (11 mm). When 5 mg/ml concentration of ethanol extract used, a greater inhibitory effect was observed against *P. aeruginosa* (11 mm) and *B. subtilis* (10 mm) and lower effect was noticed against *E. coli* (9 mm). Overall at 5 mg/ml concentration of *A. rutifolia* ethanol extract*, S. aureus* was the most resistant strain while all tested bacterial strains were most susceptible to the ethanol extract at different concentrations.

A. rutifolia ethyle acetate extract exhibited inhibitory effects against the pathogenic strains at different concentrations ranges from 10 to 19 mm as shown in Table 3 and illustrated in Figure 7. At 50 mg/ml, highest inhibitory effect of *A. rutifolia* ethyle acetate extract was noticed against *B. subtilis* (19 mm) and *P. aeruginosa* (18 mm) and minimum (16 mm and 15 mm) for *E. coli* and *S. aureus* were observed. At 25 mg/ml concentration, ethyle acetate extract displayed higher effects against *B. subtilis* (17 mm) and *P. aeruginosa* (16 mm) and low inhibitory effects at 25 mg/ml were perceived for *B. aureus* (15 mm) and *E. coli* (15 mm). At 10 mg/ml concentration, ethyle acetate extract exhibited higher inhibitory effects (16 mm) against *B. subtilis* and *P. aeruginosa* (15 mm), while lower inhibitory effects at this concentration were seen for *E. coli* (11 mm) and *S. aureus* (13) mm). At 5 mg/ml, higher inhibitory effects of 14 mm against *P. aeruginosa* and *B. subtilis* and lower effects against *E. coli* (10 mm) and *S. aureus* (11) were recorded for the ethyle acetate extract. When 5 mg/ml concentration of ethyle acetate extract used, none of the tested bacterial strains displayed resistance but all were most susceptible.

A. rutifolia chloroform extract displayed inhibitory effects against the pathogenic strains at different concentrations ranges from 7 to 19 mm (Table 3, Figure 8). At 50 mg/ml, maximum inhibitory effects of chloroform extract were perceived against *P. aeruginosa* (18 mm), *B. subtilis* (16 mm) and *S. aureus* (14) while lower effect (9 mm) was perceived for *E. coli*. At a concentration of 25 mg/ml, chloroform extract displayed greater inhibitory effects against *P. aeruginosa* (19 mm) and *B. subtilis* (14 mm) while lower inhibitory effect was shownby *S. aureus* (12 mm) and *E. coli* (7 mm). At 10 mg/ml concentration, maximum inhibitory effects were detected against *P. aeruginosa* (16 mm) and *B. subtilis* (13 mm), while minimum effects of the chloroform extract were observed against *S. aureus* (12 mm). The chloroform extract of *A. rutifolia* showed that *E.coli* was the most resistant strain at 10 mg/ml concentration with no zone of inhibition. At 5 mg/ml concentration chloroform extract showed maximum inhibitory effects of 15 mm against *P. aeruginosa* and 13 mm against *B. subtilis* while lower was noticed against *S. aureus* (11). At 5 mg/ml concentration, *A. rutifolia* chloroform extract displayed that *E. coli* was the most resistant strain while the rest of the tested strains were susceptible to the chloroform extract of *A. rutifolia* at different concentrations.

A. rutifolia n-hexane extract also executed inhibitory effects for the tested strains at different concentrations with zones of inhibition range from 11 to 19 mm (Table 3, Figure 9). At 50 mg/ml, maximum growth inhibitions by *A. rutifolia n*-hexane extract were noted for *P. aeruginosa* (19 mm), *B. subtilis* (15 mm) and *E. coli* (14) and minimum inhibition (13 mm) was observed for *S. au-*

reus. *n*-hexane extract at 25 mg/ml concentration, displayed higher inhibitions against *P. aeruginosa* (17 mm) and *B. subtilis* (14 mm) while lower retardation in growth at 25 mg/ml concentration were discerned against *E. coli* (13 mm) and *S. aureus* (12.5 mm). At 10 mg/ml concentration, *n*-hexane extract indicated higher growth inhibition (15 mm) for *P. aeruginosa* and *B. subtilis* (14 mm), while lower retardations in microbial growth at 10 mg/ml were perceived for *S. aureus* (12 mm) and *E. coli* (11 mm). At 5 mg/ml concentration n-hexane extract of *A. rutifolia* showed greater growth inhibition (14 mm) for *P. aeruginosa* and while lower inhibition (11 mm) was noticed for *B. subtilis* as shown in Table 3 and illustrated in Figure 9. At 5 mg/ml concentration of *A. rutifolia* *n*-hexane extract, *E. coli* and *S. aureus* were the most resistant strains with no zones of inhibition, while other strains were most susceptible to the *n*-hexane extract.

Table 3. Antibacterial activity of *A. rutifolia* extracts with different solvents against pathogenic bacterial strains

Sr. No	Solvents	Concentration (mg/ml)	Zone of inhibition (mm) for bacterial strains			
			E. coli (Mean ± S.D)	*B. subtilis* (Mean ± S.D)	*S. aureus* (Mean ± S.D)	*P. aeruginosa* (Mean ± S.D)
1	Methanol	5	10.11±0.88	14.45±1.43	0±0.00	10.56±0.22
		10	11.44±1.81	15.21±3.12	10.01±1.11	12.33±1.97
		25	12.23±1.95	18.73±4.97	13.76±2.15	12.18±1.10
		50	14.04±3.39	19.21±5.77	15.23±2.87	14.19±3.12
2	Ethanol	5	9.44±0.50	10.33±0.78	0±0.00	11.17±0.87
		10	11.67±1.11	12.06±1.90	13.15±2.19	13.56±2.21
		25	11.70±1.20	13.11±2.03	15.45±2.66	16.88±3.55
		50	14.07±2.21	16.12±4.05	16.32±4.15	17.05±4.96
3	Ethyl acetate	5	10.91±1.09	14.56±3.65	11.67±1.68	14.12±3.24
		10	13.45±2.16	16.22±3.87	13.54±2.24	15.11±3.33
		25	15.14±3.40	17.03±5.12	15.08±3.66	16.22±4.08
		50	15.76±3.98	19.83±5.34	16.66±4.72	18.78±5.41
4	Chloroform	5	0±0.00	13.08±1.98	11.34±1.23	15.56±3.51
		10	0±0.00	13.11±2.05	12.11±1.43	16.32±4.55
		25	7.12±0.15	14.32±3.08	12.45±1.67	19.11±5.60
		50	9.49±0.56	16.65±3.88	14.11±4.11	18.02±5.10
5	n-Hexane	5	0±0.00	11.78±1.15	0±0.00	14.39±2.19
		10	11.44±1.70	14.41±3.44	12.21±1.63	15.11±4.21
		25	13.21±2.79	14.65±3.48	12.55±1.70	17.12±5.67
		50	14.34±3.11	15.12±2.50	13.01±2.01	19.19±5.61

Values are the average of at least three readings (±SD)

Minimum inhibitory concentrations (MIC's) of the effective extracts of *A. rutifolia*

Results of antimicrobial activity of the *A. rutifolia* extracts corroborated that at 5 mg/ml concentration, few strains were resistant, while most of the strains were susceptible at all concentrations (5, 10, 25 and 50 mg/ml) respectively (Table 4). Moreover, in the methanol and ethyl acetate extracts, all the tested bacterial strains were susceptible and these two extracts showed a strong activity against the tested strains even at lowest concentration of 5 mg/ml. Among the strains, *B. subtilis* and *P. aeruginosa* bacterial strains were most susceptible at low concentration of 5 mg/ml of all extracts. Hence, experiments were conducted to check the MIC's of the most effective plant extracts (methanol and ethyl acetate) against the most susceptible bacterial strains (*B. subtilis* and *P. aeruginosa)* at lower concentrations (1-4 mg/ml). The results of MICs are given in Table 4 (Figures not shown). The MIC effect of *A. rutifolia* methanol extract started at 3 mg/ml with inhibition zones of 4 mm and 5 mm against *B. subtilis* and *P. aeruginosa* and the inhibitory effects of ethyl acetate extract also started at 3 mg/ml with inhibition zones of 5 mm and 6 mm against *B. subtilis* and *P. aeruginosa*. Overall, both the methanol and ethyl acetate extracts of *A. rutifolia* displayed higher inhibitory effects against *P. aeruginosa* as compared to *B. subtilis* at lower concentrations.

Table 4. MIC's of the most effective extracts of *A. rutifolia* against the most susceptible bacterial strains

Solvent used	Conc. mg/ml	Inhibition zones (mm)	
		Gram + *B. subtilis* (Mean ± S.D)	Gram - *P. aeruginosa* (Mean ± S.D)
Methanol	1	0±0.00	0±0.00
	2	0±0.00	0±0.00
	3	4.83±0.65	5.66±0.85
	4	7.33±1.55	9.67±2.31
Ethyl acetate	1	0±0.00	0±0.00
	2	0±0.00	0±0.00
	3	5.83±0.67	6.66±1.02
	4	9.12±2.23	10.18±3.01

Values are the average of at least three readings (±SD)

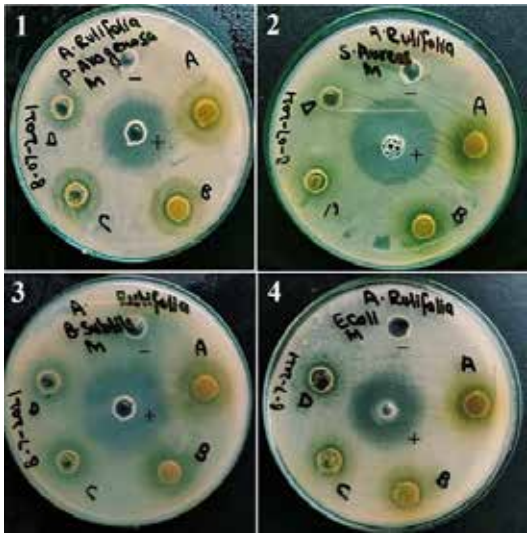

Figure 5. Growth inhibition of pathogenic bacteria by methanolic extract of *A. rutifolia. 1= P. aeruginosa, 2= S. aureus, 3= B. subtilis, 4= E. coli.* A-D are the extract concentrations used against the tested bacterial strains, A= 50 mg/ml, B = 25 mg/ml, C = 10 mg/ml, D = 5 mg/ml, - = Negative control (DMSO), + = Positive control (Levofloxacin)

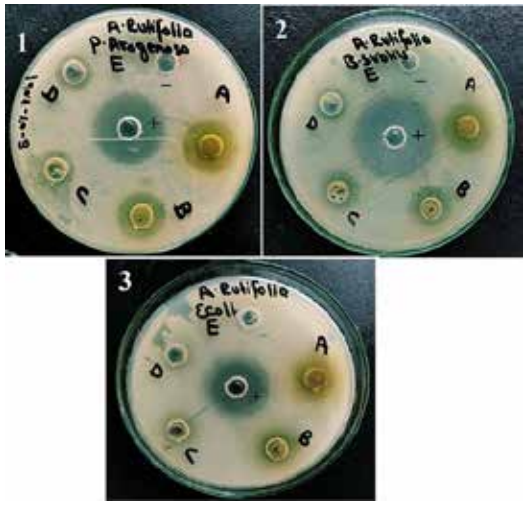

Figure 6. Growth inhibition of pathogenic bacteria by ethanol extract of *A. rutifolia. 1= P. aeruginosa, 2= B. subtilis, 3= E. coli.* A-D are the extract concentrations used against the tested bacterial strains, A= 50 mg/ml, B = 25 mg/ml, C = 10 mg/ml, D = 5 mg/ml, - = Negative control (DMSO), + = Positive control (Levofloxacin)

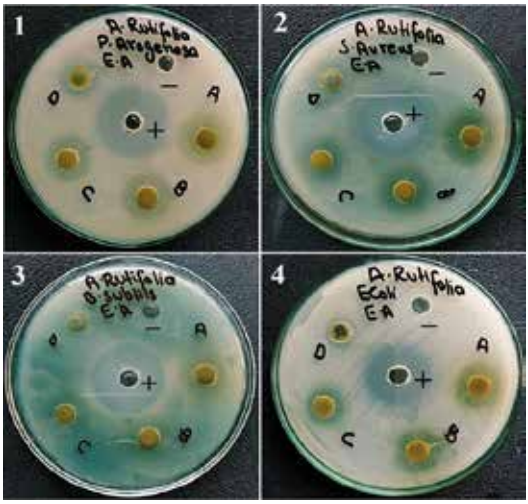

Figure 7. Growth inhibition of pathogenic bacteria by the ethyl acetate extract of *A. rutifolia. 1= P. aeruginosa, 2= S. aureus, 3= B. subtilis, 4= E. coli.* A-D are the extract concentrations used against the tested strains, A= 50 mg/ml, B = 25 mg/ml, C = 10 mg/ml, D = 5 mg/ml, - = Negative control (DMSO), + = Positive control (Levofloxacin)

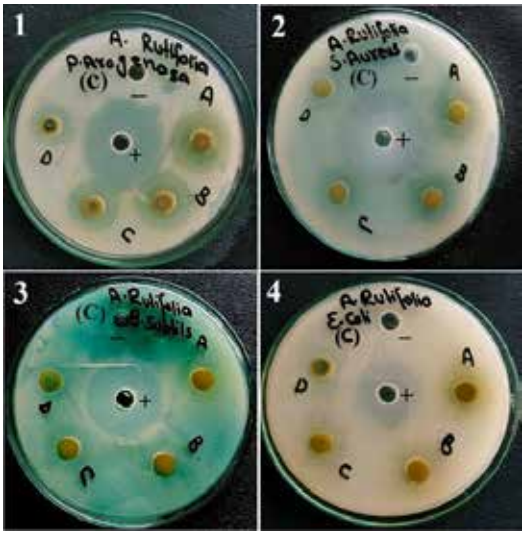

Figure 8. Growth inhibition of pathogenic bacteria by chloroform extract of *A. rutifolia. 1 P. aeruginosa, 2= S. aureus, 3= B. subtilis, 4= E. coli.* A-D are the extract concentrations used against the tested strains, A= 50 mg/ml, B = 25 mg/ml, C = 10 mg/ml, D = 5 mg/ml, - = Negative control (DMSO), + = Positive control (Levofloxacin)

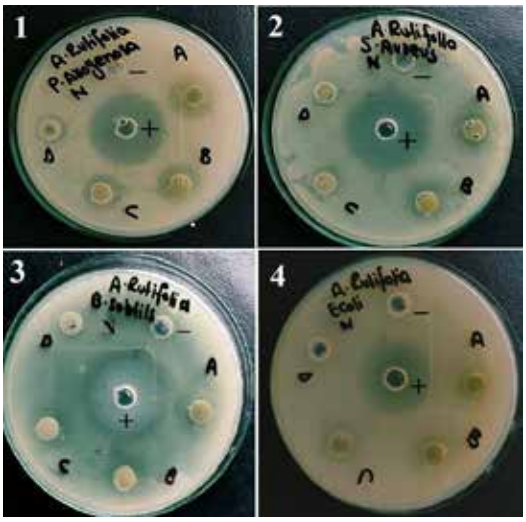

Figure 9. Growth inhibition of pathogenic bacteria by n-hexane extract of *A. rutifolia. 1= P. aeruginosa, 2= S. aureus, 3= B. subtilis, 4= E. coli.* A-D are the extract concentrations used against the tested strains, A= 50 mg/ml, B = 25 mg/ml, C = 10 mg/ml, D = 5 mg/ml, - = Negative control (DMSO), + = Positive control (Levofloxacin)

In this study, the antibacterial activities of A. rutifolia extracts were assessed against clinical and phytopathogens initiating human diseases and damaging most important crops. We adapted two approaches before selecting A. rutifolia plant for its TFC, TPC and potential antimicrobial activity. Firstly, we selected *A. rutifolia* on the basis of its local occurrence and its extensive folk traditional uses in the studied area. Secondly, a very scarce availability of data on the phytochemistry and biological activates of *A. rutifolia*. The findings regarding TPC and TFC of *A. rutifolia* confirmed the presence of phenols and flavonoids in its extracts. The maximum amount of phenols and flavonoids were recorded for ethanol extract and minimum for *n*-hexane extract as shown in Figures 3 and 4. Plants are rich in significant phytochemicals and their utilization could be very significant in enhancing the therapeutic approaches to cure pathogenic as well as genetic diseases**.** This milestone could be easily achieved if the phytochemical profile of plant species is well understood. A lot of studies globally reported the presence of significant phytochemicals in the extracts of different *Artemisia* species[19-39] proposing *Artemisia* species a very rich source of essential chemical constituents with potential biological activities including antioxidant,[40-42] antimicrobial,[40,41,43-47] antiviral,[48-53] antimalarial,[54-58] anticancerous,[59-62] antidiabetic/hypoglycemic,[63-68] anti-inflammatory,[61,69,70] and anthelmintic activities[71-73].

Here, all the *A. rutifolia* extracts showed effective growth retardation against two gram positive (*B. subtilis* and *S. aureus*) and two gram negative bacterial strains (*E. coli,* and *P. aeruginosa*) at concentrations of 50 and 25 mg/ml while low growth retardation was observed against the tested strains at 10 and 5 mg/ml concentrations in all extracts of *A. rutifolia*. Among the 5 tested extracts of *A. rutifolia,* the methanol and ethyle acetate exhibited better antibacterial activity even at lowest concentration of 5 mg/ml where *B. subtilis* and *P. aeruginosa* were the most susceptible strains. It is assumed that the *Artemisia* species possess significant secondary metabolites which give therapeutic effect5 against diseases and a lot of studies on antimicrobial and antioxidant activities of *Artemisia* species around the world have been reported[22,41,46,74-84].

In a study, antimicrobial activity of methanolic extracts of the aerial parts of *A. oliveriana, A. diffusa*, *A. turanica* and *A. scoparia* against *S. aureus, B. subtilis, E. coli, C. albicans* and *P. aeruginosa* were documented[77] against pathogenic bacteria.

Suresh *et al.*[46] studied antimicrobial activity of ethanolic extracts of *A. pallens* and *A. abrotanum* that showed maximum activity at 30 mg/ml against *B. stearothermophilus* and *P. cepacia*. Two flavones from *A. giraldii* were found to be effective against *S. lutea, S. aureus, E. coli, Proteus* sp.*, P. aeruginosa, T. viride* and *A. flavus*[75].

Ahameethunisa and Hopper[22] showed six organic solvent extracts of *A. nilagirica* from India with inhibitory effect against gram-positive and gram-negative bacteria except for *E. faecalis, K. pneumonia* and *S.aureus*.

Akrout *et al.*[80] investigated the antiradical and antimicrobial activities of *A. campestris* essential oil from Tunisia where its essential oil displayed a strong inhibitory effect on *E. coli* bacterial strain. The methanol extracts of *A. campestris* were also scrutinized for antibacterial activity by Naili *et al.*[81] and the extract was found to have a sturdy inhibitory effect on *B. subtilis* and *S. aureus* strains. The essential oils and ethanolic extracts of *A. santonicum* from Tekirdağ and *A. absinthium*, *A. scoparia* and *A. vulgaris* from Erzurum were evaluated for antimicrobial activity against 4 bacteria and *C. albicans*. Only *A. scoparia* oil was reported to have an inhibitory effect against *C. albicans* and *E. col*[74].

In another study, *A. scoparia* was also reported with antimicrobial activity against 15 oral bacteria using the minimum inhibitory concentration (MIC) method by Cha *et al.*[78]. Dulger *et al.*[85] investigated *A. absinthium* extracts and showed inhibitory effect against *Salmonella* and *Bacillus* strains.

In a study, *A. arborescen, A. absinthium, A. scoparia, A. campestris, A. vulgaris* and *A. santonicum* from Turkey were examined for their antimicrobial activity against eight bacterial and one fungal strain where the studied *Artemisia* species displayed a better antimicrobial activity[41]. In another study, antibacterial activity of methanol extracts of aerial part of *A. sieberi* against *E. cloacae P. aeruginosa, E.coli* and *P. mirabilis* were found to have better inhibitory action[82].

The essential oil and compounds of *A. annua* flowering part were tested against *S. Enteritidis, E. coli* O157, *S. Typhi, L. monocytogenes* and *Y. enterocolitica,* where all the extracts showed great effect against foodborne pathogens83. Study of Javid *et al.*[84] showed chloroform, ethyl acetate and butanol extracts of *A. indica* with high inhibitory effect between 15-20 mm against *S. aureus, P. aeruginosa* and *E. coli.*

It is believed that these reported antimicrobial activities of different species related to Asteraceae including the species of genus *Artemisia* are primarily accredited to its most active ingredients like the alkaloids and polyphenols[86,87]. Other crucial group of compounds like flavonoids from plant extracts has been found to possess antioxidants and antimicrobial actions[88-90]. Antibacterial results of the current investigation validate that *A. rutifolia* extracts screened are proven to be operative antimicrobials which might be due to the presence of phenols and flavonoids which are validated to be conceivably active in controlling disease causing bacteria.

Conclusively, all the extracts (Methanolic, ethanolic, chloroform, ethyl acetate and *n*-hexane) of *A. rutifolia* are rich in flavonoids and phenols and exhibited potential antimicrobial activity against the tested pathogenic bacterial strains at different concentrations (5 mg/ml, 10 mg/ml, 25 mg/ml and 50 mg/ml). MICs results showed that the methanol and ethyl acetate extracts are effective against *B. subtilis* and *P. aeruginosa* with low concentrations of 3 and 4 mg/ml and the ethyl acetate extract possess a higher 392 inhibition activity against *P. aeruginosa* and *B. subtilis* as compared to the methanol extract. Hence, the isolation and purification of therapeutic phenols and flavonoids from *A. rutifolia* extracts could be used as an operational source against human and plant bacterial infections. It is recommended that, more detailed phytochemical and pharmacological studies are needed on *A. rutifolia* extracts in order to find out active compounds against clinical and phyto-pathogenic bacterial strains.

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CONFLICT OF INTEREST

Nothing to declare

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Analysis of data: Hassam Rasheed, Muhammad Aslam Khan, Adil Hussain (these authors contributed equally)

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Critical revision of the manuscript: Adil Hussain, Muhammad Sajid

Statistically analysis: Adil Hussain

Technical and Financial Support: Adil Hussain, Syed Ali Imran Bokhari (these authors contributed equally)

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Efficacy of Liquid-Liquid Extraction and Protein Precipitation Methods in Serum Sample Preparation for Quantification of Fexofenadine in Human Serum

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ABSTRACT

Biological samples contain many intrinsic and extrinsic compounds in different concentrations which makes it very challenging to analyze it and cannot normally be injected directly into the analyzing system without sample preparation. Two different sample extraction procedures liquid-liquid extraction and protein precipitation have been employed in this study to quantify fexofenadine in human serum and the recovery rates have been compared. Protein precipitation by methanol has an advantage over liquid-liquid extraction with recovery rates of more than 90% but has limitations due to rise of column back pressure. Due to better recovery rate and quick sample preparation technique the protein precipitation method has been chosen for extraction of drug from serum sample. The developed HPLC method was validated and found to be accurate, precise and specific within the linearity range of 0.8- 4.0 μg/mL. Lower limits of detection and quantification were established as 0.6 and 0.8 μg/mL respectively.

Keywords*:* Liquid-liquid extraction, protein precipitation, bioanalysis, sensitivity

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INTRODUCTION

In the pharmaceutical industry pharmacokinetics, toxicokinetics and bioequivalence studies are immensely facilitated by bioanalysis as it provides quantitative measure of the active drug and its metabolite. Sample preparation is an important aspect of bioanalytical estimation because biological samples are extremely complex matrices composed of many components like proteins, which can lead to protein binding of the analyte that can interfere with good separation and detection. Thus biological samples cannot normally be injected directly into the analyzing system without sample preparation[1]. Numerous sample preparation techniques have been developed for bioanalytical purposes. Solid phase extraction, liquid – liquid extraction and protein precipitation are a few of them.

Fexofenadine, a selective peripheral H1 blocker is a second generation antihistamine. Chemically it is (±)-4[1 hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-butyl]-α, α-dimethyl benzeneacetic acid hydrochloride (Figure 1).

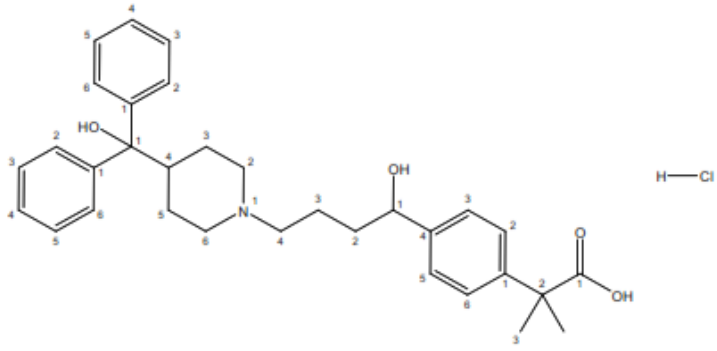

Figure 1. Fexofenadine hydrochloride

Unlike the first generation antihistamines it does not cause sedation. It can exist in zwitter ionic form that prevents it to pass the blood-brain barrier and therefore it is non- sedative[2]. Fexofenadine is widely prescribed for seasonal allergic rhinitis, hay-fever, sneezing, rhinorrhea, itchy nose/palate/throat, itchy/watery/red eyes etc.[3]. After oral application it takes about one hour for its action and two to three hours to reach maximum plasma concentration[4]. Steady state plasma concentration of fexofenadine remains in the range of 0.058-4.677μg/mL[5].

It has been a subject of interest among researchers worldwide for quite a few years now and several chromatographic methods have been reported for determination of Fexofenadine in human plasma. Nevertheless it can be quite

challenging to determine Fexofenadine in biological samples such as serum, plasma or whole blood owing to a number of interferences that may affect the analysis. So prior to analysis biological samples need purification to remove unwanted components and also sometimes the compound of interest needs to be concentrated. Different kinds of extraction techniques like solid phase extraction, liquid- liquid extraction, protein precipitation have been employed to purify these samples[6]. Previous work on fexofenadine quantification in biological samples using different extraction techniques are presented here in a tabular format [7-20] (Table 1).

Table 1. Previous work done on biological quantification of fexofenadine.

References	Extraction techniques	Solvents/ Cartridge	Instrumentation	Recovery
Miura et al., 2007	Solid phase extraction	Methanol, Oasis HLB cartridge	HPLC coupled with UV	Around 67-71.5%
Nigori et al., 2007	Solid phase extraction	Methanol, Oasis HLB cartridge	LC/MS	58.2±1.5%
Yamane et al., 2007	Solid phase extraction	Methanol, ammonium acetate, acetic acid, Oasis HLB cartridge	LC-MS/MS	92.5%
Bharathiet al., 2008	Solid phase extraction	Methanol, ammonia, Oasis HLB cartridge	LC-MS/MS	91.5%
Fu et al., 2004	Solid phase extraction	Methanol, acetate buffer 96-well plate	LC-MS/MS	>70%
Uno et al., 2004	Solid phase extraction	SPE C_{18} minicolumn	HPLC with fluorescence	72.8-76.7%
Hofmann etal.,2002	Solid phase extraction	Methanol, acetate buffer, triethylamine. C_{18} SPE cartridge	LC-MS/MS	88.96±2.9%
Coutant et al., 1991	Solid phase extraction	Analytichem C_{18} minicolumn	HPLC with fluorescence	59.6-66.5%
Isleyen et al., 2007	Liquid-Liquid extraction	Formic acid, DCM:EA:DEE(30:40:30)	LC/MS	52-55%
Stanton et al., 2010	Protein precipitation	Acetonitrile	LC-MS/MS	95.3±10.3%
Pathak et al., 2008	Protein precipitation	Acetonitrile, trichloroacetic acid	HPLC with fluorescence	81.79%-85.23%
Guo et al., 2010	Protein precipitation	Methanol	LC-MS/MS	87.6-93.6%
Flyne et al., 2011	Protein precipitation	Ammonium formate, methanol, acetonitrile	LC-MS/MS	87.6-93.6%
Helmy et al., 2015	Liquid-liquid extraction	Diethylether	HPLC coupled with UV	95.4%

Most of the reported methods used solid phase extraction techniques for sample preparation. Though solid phase extraction is a very selective method and yields high recoveries and is highly reproducible it is a very complicated, lengthy and costly method[21].

Compared to solid phase extraction, relatively few methods are reported where liquid-liquid extraction or protein precipitation has been used to determine fexofenadine in biological samples. Isleyen et al., 2007 and Helmy et al., 2015; both used liquid-liquid extraction (LLE) technique in their attempt to eliminate the need of solid phase extraction. These methods were sensitive, precise and accurate with the absolute recoveries 52-55% and 95.4% respectively. This sample preparation procedure is efficient and cost-effective but it is labor intensive, difficult to automate and require a large amount of organic solvent[22].

Protein precipitation (PP) with miscible organic solvents (usually acetonitrile or methanol) is the simplest approach that requires minimal method development and removes the majority of the protein from the sample[23]. Pathak et al., 2008; Guo et al., 2010; Flyne et al., 2011; all used protein precipitation techniques for sample preparation in their attempt to quantify fexofenadine in biological matrices. PP is the most commonly used sample preparation method because of its ability to remove the unwanted plasma proteins from samples prior to analysis with minimal method development requirements and low cost[24]. The only drawback it has is it may increase the back pressure of the HPLC system and may affect the column performance[25].

In bioanalysis the recovery rate is directly related to the extraction procedure. Moreover, proper sample pretreatment procedure should be developed to ensure sufficient sensitivity and selectivity, whereas the run time should be kept to a minimum in order to obtain adequate speed.

The aim of this study was to find out the optimum extraction method for determining fexofenadine in human serum by RP-HPLC with UV detector from liquid- liquid extraction and protein precipitation. The recovery rates were compared to see which better fits routine laboratory applications. We also validated the developed HPLC method using the protein precipitation extraction procedure with greater recovery rate.

METHODOLOGY

Drugs, Chemicals and study products

Fexofenadine and Cetirizine (Internal standard, IS) were obtained as a gift sample from Beximco pharmaceutical limited, Bangladesh. High purity deionized water was obtained from Millipore, Milli-Q (Merck KGaA, Darmstadt, Germany) water purification system and used throughout the process, HPLC grade acetonitrile, methanol was purchased from active fine chemicals, Bangladesh. Ammonium acetate, trifluoroacetic acid and acetic acid were purchased from Merck, Germany. Dichloromethane, ethyl acetate, diethyl ether were analytical grade and also purchased from active fine chemicals.

Instrumentation and chromatographic system

A high performance liquid chromatographic system was used from Hitachi High-tech Science Corporation, Tokyo, Japan comprising of Hitachi Chromaster 5110 quaternary pump for constant flow and constant pressure delivery, a column oven (Chromaster 5310 Column Oven), Chromaster 5210 auto sampler and Photodiode array detector (Chromaster 5430 detector). Data was integrated using Agilent Open Lab control panel CDS software running on a personal computer. The chromatographic analysis was performed on a C18 column (250 mm x 4.6 mm i.d., 5µm particle size), LaChrom, Hitachi, Japan with C18 guard column (23mm × 4 mm; 3 µm), LaChrom, Hitachi, Japan. Different mobile phase compositions were considered for successful separation of the analyte from different matrix interferences.

Mobile phase optimization

The separation efficiency along with the system suitability parameters like retention time (RT), Tailing factor, and number of theoretical plates were checked for the mobile phase as in Table 2.

Table 2. Mobile phase choosen for Fexofenadine bioanalysis

Mobile Phase Composition	Buffer	Buffer: Methanol: Acetonitrile
MP1	5mM ammonium acetate, pH adjusted to 4.0 with 0.03% trifluoroacetic acid	50:30:20
MP2	5mM ammonium acetate, pH adjusted to 4.3 with acetic acid	55:10:35
MP3		55:15:30
MP4		57:10:33

Mobile phases were filtered through 0.2µm Nylon 66 membrane filters and degassed before use to remove particulate matter. The mobile phase was pumped isocratically at a flow rate of 1.0 mL/min during analysis at ambient temperature. The volume of injection was 20 µL. Eluent was detected at 220 nm.

Collection of serum sample

The human serum was collected from healthy volunteers after obtaining approval from the National Research Ethics Committee. After collection it was allowed to stand for 30 min and then centrifuged at 3000 rpm for 10 minutes and serum was collected. Then the serum sample was stored in the freezer at -20˚C for further use.

Preparation of standard stock solution

10 mg of Fexofenadine working standard was accurately weighed and transferred into 50 mL clean dry volumetric flask and about 20 mL of diluent (methanol: water 70:30, v/v) was added to dissolve it completely by shaking. Finally the volume was made up to 50 mL with same solvent to make a solution of 200 µg/mL. The stock solution of IS was prepared by dissolving 10.0 mg of Cetirizine in 50 mL of diluents and further diluted to make the final concentration of 100 µg/mL. Working solutions of Fexofenadine and Cetirizine were stored in 4°C.

Preparation of serum sample

To prepare a serum sample, three samples at a concentration of 8, 16, 24 and 32 µg/mL were prepared from stock solution. Serum samples were prepared by spiking 30 µL of Fexofenadine solution from each dilution and 30 µL of cetirizine solution in to 270 µL of blank serum to produce serum samples with the concentration of 0.8, 1.6, 2.4 and 3.2 µg/mL.

Extraction technique

Liquid-liquid extraction (LLE)

For liquid-liquid extraction an extraction solution was prepared using HPLC grade dichloromethane: ethyl acetate: diethyl ether in the ratio of 30:40:30 (% v/v/v). In this procedure 270 µL of serum was taken into a centrifuge tube where 30 µL of fexofenadine solution and 30 µL cetirizine solution was added and vortexed for 5 second for adequate mixing. After vortexing, 150 µL of formic acid solution was added and again vortexed for 5 seconds. 5 mL of extraction mixture was then added into the centrifuge tube and vortexed again for 40 seconds. This mixture was then centrifuged at 5500 rpm for 5 min. The organic layer was separated and evaporated to dryness. The solution was reconstituted into 500 µL diluent and vortexed. Finally it was poured into clean and dried HPLC vial for injection.

Protein precipitation (PPT)

Methanol extraction

Samples of spiked serum with fexofenadine (300 µL) were transferred to 2 mL eppendorf tubes where 30 µL of cetirizine solution was added and vortexed for 30 seconds. Then 870 µL of methanol was added to precipitate the protein. The eppendorf tubes were vortexed for 30 seconds and kept static for settling down. After 15-20 minutes samples were centrifuged for 12 min at 12,000 rpm.

The supernatant was collected, transferred to autosampler vials through a 0.22 syringe filter and directly injected into HPLC.

Acetonitrile extraction

Samples of spiked serum with fexofenadine (300 µL) were transferred to 2 mL eppendorf tubes where 30 µL of cetirizine solution was added and vortexed for 30 seconds. The protein was precipitated with 870 µL of Acetonitrile. The eppendorf tubes were vortexed again for 30 seconds, left aside for approximately 15- 20 minutes and then centrifuged for 12 min at 12,000 rpm. The supernatant was collected, syringe filtered to autosampler vials and directly injected into HPLC. This whole procedure is summarized in Figure 1.

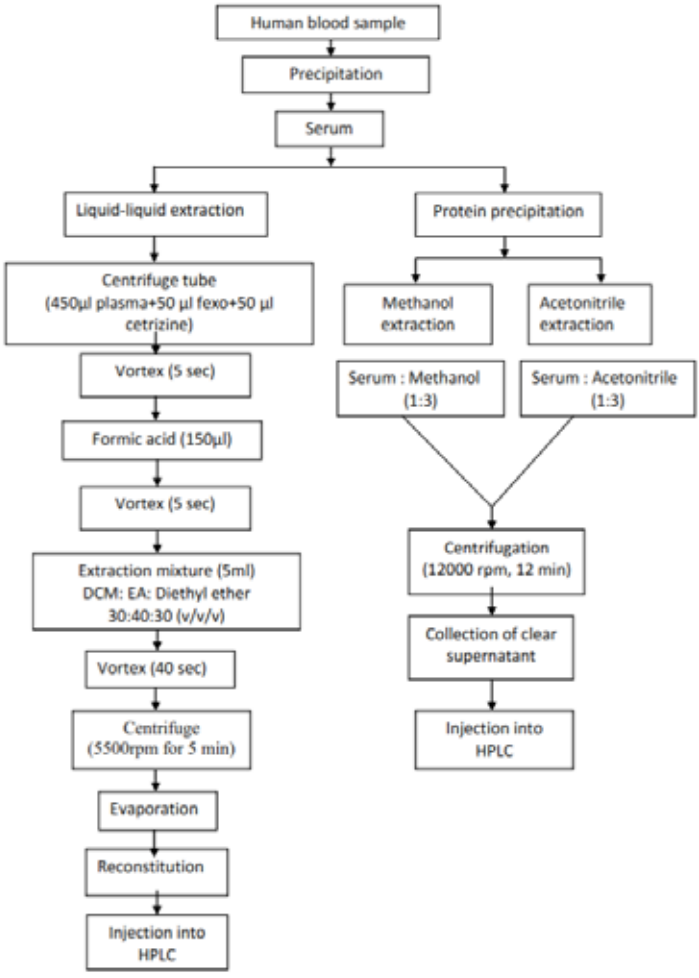

Figure 2. Sample preparation for bioanalysis

Method validation

After evaluating the recovery rates of fexofenadine from serum samples by liquid-liquid extraction and protein precipitation the bioanalytical method was validated using the extraction procedure that gave greater recovery rates. The method was validated according to ICH guidelines for validation of bioanalytical procedures in terms of linearity, specificity, limit of detection (LOD), lower limit of quantitation (LLOQ), recovery, accuracy and precision[26].

Linearity, LOD and LLOQ

Linearity was tested for the concentration range of 0.8–4.0 μg/mL. For the determination of linearity, standard calibration curves of six points (0.8, 1.0, 1.5, 2.0, 3.0 and 4.0) were used along with zero concentration blank serum sample to confirm the absence of interferences. The acceptance criterion for correlation coefficient is 0.99 or more, otherwise the calibration curve would be rejected. Three replicate analyses were performed for each concentration.

The LLOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable accuracy (percent error <20%) and precision (coefficient of variation <20%). The analyte response at the LLOQ level should be at least five times greater than the analyte response of the zero calibrator. The lower limit of detection (LOD) was the minimum concentration that can be detected by detector response with analyte response, which should be equal to or greater than three times of the analyte response of the zero calibrator. LLOQ was measured by five replicate analyses of the analyte.

Specificity

The specificity was evaluated by analyzing the chromatogram of the human drug-free serum from different volunteers (n=6) in triplicate to check for the matrix interference. The retention time of fexofenadine in the HPLC chromatogram of spiked serum and blank samples were compared to define any endogenous materials and/or degradation peaks appearing at the same retention time as fexofenadine or cetirizine.

Recovery and matrix effect

The recovery rate was determined by comparing the peak area ratios (fexofenadine/IS) of serum samples that have gone through the sample preparation and extraction procedures with the serum free samples directly injected in the mobile phase. This procedure was repeated for the four different serum concentrations of fexofenadine such as 0.8, 1.6, 2.4 and 3.2 μg/mL with three replicate analyses for every concentration.

Precision and accuracy

To determine the precision of the assay, replicate analysis of four concentration levels of fexofenadine were used (0.8, 1.6, 2.4 and 3.2 µg/mL). Intra-day precision and accuracy were determined by repeated analysis of the group of standards on one day. Inter-day precision and accuracy were determined by repeated analysis on three consecutive days. The concentration of each sample was determined using a standard curve prepared and analyzed on the same day.

RESULTS and DISCUSSION

The sample preparation step before the HPLC analysis is intended to facilitate the determination of components of the drug candidate that involve pharmacokinetics and metabolic stability. Two extraction procedures, liquid-liquid extraction and protein precipitation, have been employed to extract fexofenadine from human serum and the recovery rates for both procedures have been considered. Chromatogram of fexofenadine and cetirizine has been observed at the wavelength of 220 nm (Figure 2).

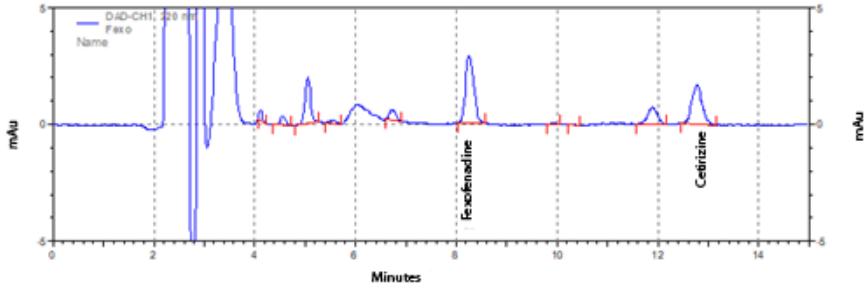

Figure 3. Chromatogram of serum sample spiked with fexofenadine and cetirizine at 220.0 nm

Method optimization

Fexofenadine is amphoteric in nature as it has both a carboxylic acid end and a tertiary amine end in its structure. Therefore it would remain in an ionized form regardless of acidic or basic pH conditions. Peak tailing, worsening of peak shapes, long retention time, proper chromatographic separation to avoid overlapping of sample peak with the serum's interfering peaks- all these factors need to be closely monitored while developing the method. Moreover aging of analytical column makes the optimization of the mobile phase with the right balance of pH and organic content more crucial. A solution of 5mM ammonium acetate has been chosen as the buffer solution. The pH of the buffer was first adjusted with 0.03% trifluoroacetic acid (TFA) considering TFA's effect in improving peak shape and resolution by solubilizing interfering proteins in

the serum sample[27]. Also it has an impression of facilitating chromatographic separation of zwitterionic compounds like fexofenadine. But with time it was observed that the pH of the buffer is not stable and the baseline keeps fluctuating. So we replaced the mobile phase additive and instead of TFA we adjusted the buffer's pH at 4.3 with acetic acid. This gave us a stable pH condition as well as steady baseline. Another challenge during method development was figuring out the right combination of organics and buffer. Higher portion of the buffer resulted in longer retention time. But more organic compounds can coagulate proteins that are left in the serum sample and block the column. Considering all these factors different ratios of buffer, methanol and acetonitrile have been tested and the observation has been listed in table no 3. A mobile phase consisting of 5mM ammonium acetate (pH~4.3), methanol and acetonitrile in the ratio of 57:10:33 has been finalized for further method validation.

Table 3. Mobile phase optimization

Mobile Phase Composition	Buffer: Methanol: Acetonitrile	Retention time		Remark
		Fexofenadine	Cetirizine	
MP1	50:30:20	8.32 min	14.26 min	Fluctuation of baseline makes this system unstable
MP2	55:10:35	7.69 min	13.28 min	Peaks overlapping with serum's interfering peaks
MP3	55:15:30	12 min	25 min	Too long retention time for IS
MP4	57:10:33	11.63 min	18.62 min	Well separated peaks

Recovery rates after liquid-liquid extraction

The absolute recoveries and extraction efficiency were determined by the HPLC analysis of fexofenadine for three different concentrations 0.06 µg/mL, 1 µg/mL and 2 µg/mL in serum and compared with the peak areas ratios with those obtained from direct injection of the same amount of fexofenadine dissolved in the diluent. Recovery rates are given in table no 4. The value of recovery rates was approximately 33- 42%, which is really low.

A representative chromatogram (Figure 3) was generated to show that other components, which could be present in the sample matrix, are resolved from the parent analyte. No significant changes in retention times of the drugs clearly indicated the specificity of the method.

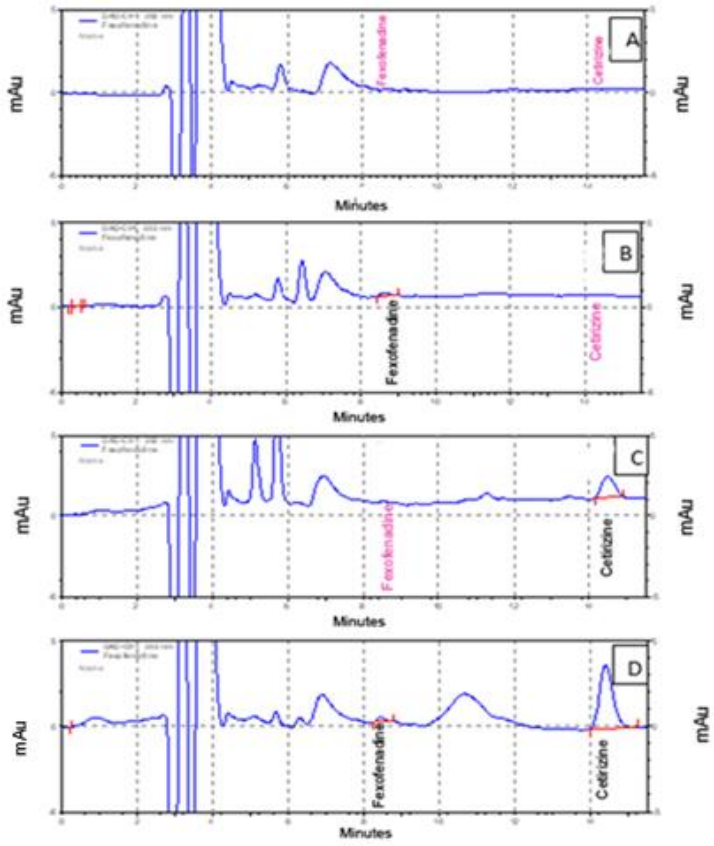

Figure 4. Representative chromatogram of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D) after liquid- liquid extraction.

Recovery rates for protein precipitation

In case of acetonitrile precipitation the peaks were all splitted. It may be due to sample solvent incompatibility with mobile phase. The solubility of investigational compounds is an important factor in method selection and in this study the investigational compounds are methanol soluble hence methanol was selected as the most suitable serum precipitation method. (Figure 4).

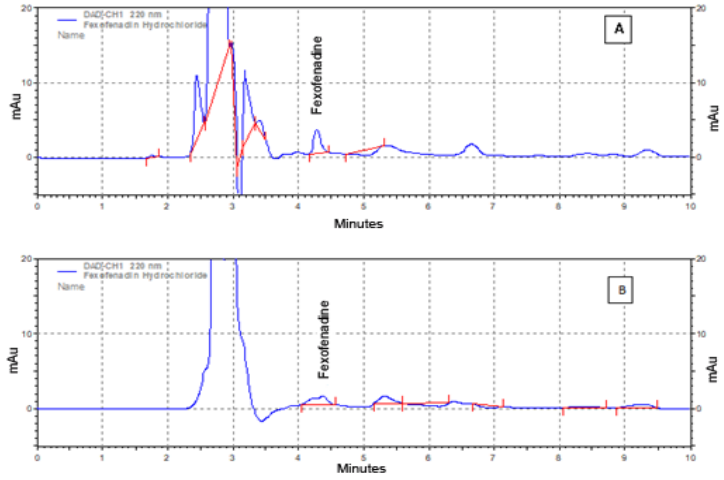

Figure 5. Representative chromatograms of fexofenadine after protein precipitation with methanol (A) and Acetonitrile (B).

Mean drug recovery was calculated by comparing the peak area ratios of extracted serum samples with those obtained from non extracted calibrators with the same amount of drug. The recovery rate for 0.06 µg/mL concentration was 74.26%, for 1 µg/mL 93.20% and 95.20% for 2 µg/mL concentration. A representative chromatogram (Figure 5) was generated to show that other components, which could be present in the sample matrix, are resolved from the parent analyte that ensures specificity of the method.

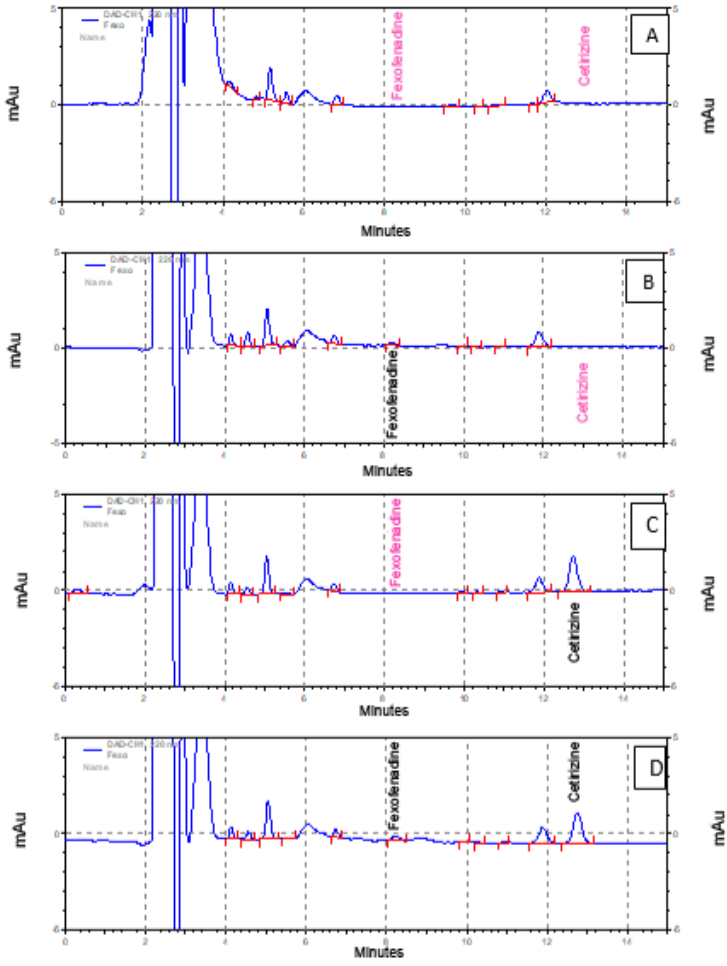

Figure 6. Representative chromatogram of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D) after protein precipitation.

It was found that the recovery rates after protein precipitation was higher than the recovery rates after liquid- liquid extraction (Table 4).

Table 4. Recovery of Fexofenadine from Serum by liquid-liquid extraction and protein precipitation

Added concentration (µg/mL)	% Recovery	
	Liquid-liquid extraction	Protein precipitation
0.06 µg/mL	33.87	74.26
1 µg/mL	40.23	93.20
2 µg/mL	42.22	95.20

The recovery rate for 0.06 µg/mL concentration is very low, which is why a higher concentration of 0.8 µg/mL has been chosen as the lower limit of quantification.

Method validation

Compared to liquid-liquid extraction, protein precipitation gave higher recovery rates. A method has been validated using protein precipitation for sample preparation to quantify fexofenadine in human serum.

System suitability

To ensure the instrument performance, system suitability parameters such as peak asymmetry, peak capacity factor, peak purity, and theoretical plate number were monitored. The retention time for fexofenadine and cetirizine was 11.13 minute and 19.51 minute respectively. Peak asymmetry was less than 2 and the peak purity value was greater than 0.9 with an RSD value 1.36%. The value of peak theoretical plates is more than 2000 for both fexofenadine and cetirizine, which is in acceptable range.

Selectivity

The selectivity of the method was investigated by comparing the chromatograms of blank serum, serum sample spiked with only fexofenadine, serum sample spiked with only internal standard cetirizine and serum sample spiked with both fexofenadine and cetirizine. The method was found to be specific and selective. The retention times were 11–12 min and 18–19 min for fexofenadine and cetirizine respectively. There were no interfering peaks from endogenous substances at the elution time of fexofenadine and cetirizine as shown in Figure 6.

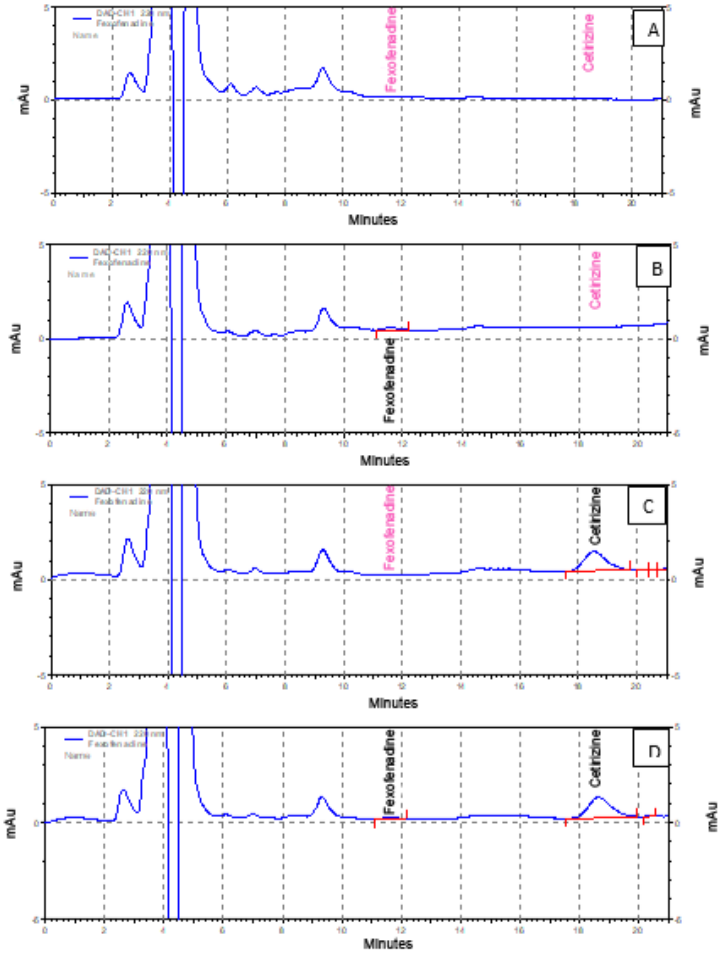

Figure 7. Representative chromatograms of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D)

Linearity, LOD and LLOQ

The method shows linearity over the concentration range of 0.8– 4.0 μg/mL, with a coefficient of correlation (R^2) 0.9986 (Figure 7).

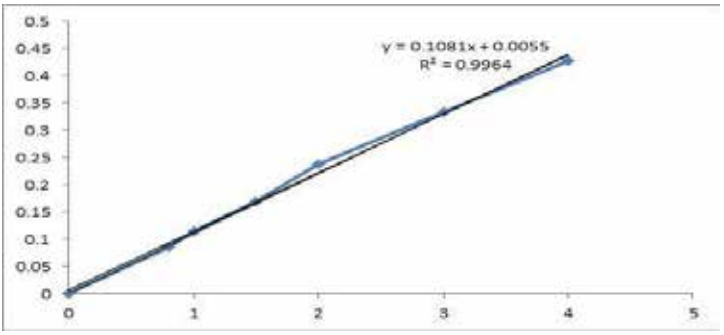

Figure 8. Calibration curve

The LLOD and LLOQ was found to be 0.6 µg/mL and 0.8 µg/mL respectively with accepted accuracy (percent error <20%) and precision (coefficient of variation <20%). Results are presented in Table 5.

Table 5. Linearity, lower limit of detection and quantification.

Parameters	Values
Linearity range (µg/mL)	0.8-4.0
Correlation coefficient(R^2)	0.9964
Regression equation	y = 0.1081x + 0.0055
LOD (µg/mL)	0.6
LLOQ (µg/mL)	0.8

Recovery and matrix effect

The extraction recoveries of fexofenadine were determined at four different concentrations (0.8, 1.6, 2.4, and 3.2 µg/mL). The method showed good efficiency in terms of recovery as the average recovery for fexofenadine ranges from 97.89 to 102.93% (Table 6).

Table 6. Recovery rates of fexofenadine.

Added concentration	% Recovery (mean±SD)	%RSD
0.8	102.93±1.94	1.88
1.6	99.678±0.43	0.43
2.4	97.897±1.32	1.35
3.2	100.32±1.54	1.46

The recovery of cetirizine was consistent and precise. Extraction method was simple and faster as there was no drying step present in the sample preparation process.

Accuracy and precision

The intra-day precision of 0.08, 0.24, 2.40, and 4.80 μg/mL was in the range of 0.49–1.80 % and inter-day precision was 0.41– 1.98%. The accuracy was in the range of 108.21– 92.75% and 93.45%- 104.67% for intra- day and inter- day respectively. Results are presented in table 7.

Table 7. Intra- day and inter- day accuracy and precision.

Within day (intra-day)				Between day (inter-day)		
Nominal conc. (μg/mL)	Conc. Found (μg/mL; mean±SD)	Precision (%)	Accuracy (%)	Conc. Found (μg/mL; mean±SD)	Precision (%)	Accuracy (%)
0.8	0.75± 1.9	1.80	93.23%	0.81± 0.87	1.56	93.45%
1.6	1.4± 0.4	0.49	92.75%	1.56 ± 1.9	0.41	95.56%
2.4	2.6± 1.3	1.30	108.99%	2.8 ± 1.7	1.87	104.67%
3.2	3.4± 1.5	1.47	106.21%	3.6 ± 1.4	1.98	102.55%

Sample preparation prior to chromatographic separation is a crucial part of the bioanalytical method development process. It is performed to dissolve or dilute the analyte in a suitable solvent, removing the interfering compounds and pre-concentrating the analyte. Sensitivity and selectivity of a method is highly dependent on it. In this study of fexofenadine liquid-liquid extraction provided low recovery rates compared to protein precipitation technique, which gave protein precipitation an edge over liquid-liquid extraction for quantification of fexofenadine in human serum. The low recovery rate can be a result of solvent-mobile phase incompatibility or selection of an extraction mixture. As fexofenadine is methanol soluble, methanol worked just fine as a protein precipitating agent. Besides these protein precipitation technique is a very quick process for sample preparation as there is no drying step and it does not need any extra instrumental set up, but it causes increased column back pressure which is harmful for analytical columns. Every day after running the serum samples the column needs to be washed properly for around 35 to 45 minutes

to maintain column quality and peak shape. The developed method is simple, reproducible, accurate and precise.

In most of the bioanalytical procedures sample preparation takes about half to three quarter of the total time of analysis yet most technical innovations of the recent years are related to separation and detection rather than sample preparation or extraction. It is a work of great importance but has not been enough emphasized. There is great scope for further innovative sample preparation techniques to quantify drugs in biological samples.

STATEMENT OF ETHICS

An approval from the National Research Ethics Committee of Bangladesh Medical Research Council has been obtained (registration number 06924082017).

CONFLICT OF INTEREST

There are no conflicts of interest.

AUTHOR CONTRIBUTION

All authors have contributed significantly in the work presented here. Author MHS formulated the presented research project and supervised all the work along with SMM. Author MHS and FM designed the research work. The data acquisition, data analysis and statistical analysis was done by authors FA [1,2] and FM. Author FA [1,2] performed the experimental work. Drafting of the manuscript was done by FA[1,2] and SRR which was corrected by FM, SS and FA[1]. Authors FM, FA[1], and MHS finalized the final drafting of the manuscript. All authors read and approved the final manuscript.

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Potent colon cancer cell line toxicity of the fruit extracts of *Heptaptera triquetra* (Vent.) Tutin

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ABSTRACT

Cytotoxic activities of the fruit extracts of *Heptaptera triquetra* (Vent.) Tutin were investigated on the colon cancer COLO205 and KM12 cell lines. The dichloromethane extract of the fruits of *H. triquetra* showed the highest cytotoxic activities with IC_{50} values of 9.3 and 5.3 ug/mL on the COLO205 and KM12 cell lines, respectively. Whereas, the ethyl acetate extract of the fruits showed moderate cytotoxic activity with IC_{50} values of 22.3 ug/mL against the KM12 cell lines.

Keywords: Cytotoxic activity, *Heptaptera triquetra*, Apiaceae.

INTRODUCTION

Cancer is a major public health problem worldwide and is the second leading cause of death. Cancers of the colon and rectum are the third estimated new cancer cases and deaths among adult Americans in 2022[1]. Natural products continue to play a major role in the drug discovery of new anticancer drugs[2]. As part of our continuing studies on the genus *Heptaptera* (Apiaceae), we report here the cytotoxic activity of *Heptaptera triquetra* fruits on colon cancer cells.

The genus *Heptaptera* Marg. & Reut. (Apiaceae) is represented by 11 species worldwide, four of them; *H. cilicica* (Boiss. & Balansa) Tutin, *H. anisoptera* (DC.) Tutin, *H. anatolica* (Boiss.) Tutin and *H. triquetra* (Vent.) Tutin are growing in Turkey[3,4]. *H. triquetra* is only found in the European section of Tur-

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key[4]. *Heptaptera* species are known to contain sesquiterpene coumarin derivatives[5-11], these compounds have various biological activities such as; cytotoxicity, P-glycoprotein inhibitory, cancer chemopreventive, anti-inflammatory, antibacterial, antileishmanial, antiviral, antidiabetic, cholinesterase inhibitory etc.[10-17].

METHODOLOGY

Plant Material

The fruits of *Heptaptera triquetra* were collected in the vicinity of Tekirdağ in July 2013 and identified by Prof. A. Duran. A voucher specimen (A. Duran 9704) was deposited in the Herbarium of Selçuk University, Faculty of Sciences, Department of Biology (KONYA).

Extraction

Pulverized fruits (50 g) of the plant were sequentially extracted by maceration with dichloromethane (CH_2Cl_2) and methanol. The extracts were individually concentrated in a rotary evaporator under reduced pressure to yield crude extracts. Dichloromethane and methanol extracts of the fruits were 4.24 g (8.48 %) and 4.05 g (8.10 %), respectively. The methanol extract was redissolved in a mixture of methanol/water (10:90) and then partitioned with ethyl acetate (EtOAc), the resulting extracts were separately concentrated in vacuo to dryness. Ethyl acetate and aqueous-methanol extracts of the fruits were 1.23 g (2.46 %) and 2.81 g (5.62%), respectively.

Cytotoxicity Assay on Colon Cancer Cells

The assay used for this study was a two-day, two cell line XTT bioassay[18], an in vitro antitumor colorimetric assay developed by the MTL Assay Development and Screening Section. Colon cancer cell lines used were COLO205 and KM12. Sanguinarine was used as a positive control. The assay was performed as described previously[11].

RESULTS and DISCUSSION

This is the first report on the cytotoxic activity of the fruits of *H. triquetra*. The dichloromethane extracts of the fruits exhibited strong inhibitory activity on the colon cancer COLO205 and KM12 cell lines. The ethyl acetate extract of the fruits exhibited moderate inhibitory activity on the KM12 cell lines. The cytotoxic activities observed with these extracts are shown in Table 1.

Table 1. Cytotoxic activities of the extracts

Extracts	Cytotoxic activity (IC_{50} values in ug/mL)	
	COLO205	**KM12**
1	9.3	5.3
2	> 50	22.3
3	> 50	> 50

1: CH_2Cl_2 extract of the fruits; 2: EtOAc extract of the fruits; 3: aqueous-methanol extract of the fruits

The dichloromethane extract of the fruits of *H. triquetra* showed the highest cytotoxic activities with IC_{50} values of 9.3 and 5.3 ug/mL on the COLO205 and KM12 cell lines, respectively. The ethyl acetate extract of the fruits showed moderate cytotoxic activity with an IC_{50} values of 22.3 ug/mL on the KM12 cell line and a weak cytotoxic activity against the COLO205 cell line with an IC_{50} value greater than 50 ug/mL. Previously, researchers reported some sesquiterpene coumarin derivatives from the chloroform extracts of the fruits of *H. anatolica, H. anisoptera*[8] and *H. cilicica*[19]. Cytotoxic activities of the certain sesquiterpene coumarins were described earlier[10-12], thus, the cytotoxic compound(s) of the fruits of *H. triquetra* may be the similar type of compound(s). Currently, the bioactivity guided fractionation of the dichloromethane extract of *H. triquetra* fruits is in progress.

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Could the increase in oxidative stress be the reason for the increased polyamine levels in diabetic obese and non-diabetic obese patients?

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ABSTRACT

Putresin, spermine, and spermidine are important polyamines found in all living organisms. In this study, as a first in the literature, we aimed to investigate polyamines levels and their relationship with oxidative stress in obese adults. The study was carried out with 85 obese patients and 29 healthy controls. Glucose, HbA1c, urea, uric acid, CRP, Total antioxidant status and Total oxidant status putrescine, spermine and spermidine levels were analysed. The study found putrescine and spermidine levels in obesity (0.25 ±0.13) (2.29 ±0.79) were found to be significantly lower, respectively, than the control group (0.38± 0.08) (1.80 ± 0.68) ($p<0.05$). It was observed that both OSI and TOS values in the diabetic obese group were statistically higher than both the control group and the non-diabetic obese group. As a result; although polyamine levels are low in obesity, increased oxidative stress in the diabetic obese group caused an increase in polyamine levels.

Keywords: Polyamines, obesity, diabetes mellitus, oxidative stress

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INTRODUCTION

The World Health Organization (WHO) has defined obesity as "excessive fat accumulation that impairs health" [1]. Obesity is a complex and multifactorial disease which affects health negatively. At the same time, it increases the risk of developing metabolic diseases such as Type 2 Diabetes mellitus (T2DM), fatty liver, as well as cardiovascular diseases such as hypertension, myocardial infarction, stroke, and also various cancers. These diseases, which occur on the basis of obesity, constitute 70% of early deaths [1, 2]. According to 2016 data, 39% of adults aged 18 and over worldwide are overweight and 13% are obese. The prevalence of obesity has increased 3 times in the world since 1975 [1].

Obesity is the most important risk factor for T2DM. This is due to the fact that in most cases diabetes occurs on the background of obesity. Common obesity and T2DM are increasingly defined as "diabesity" [3].

Putrescine, spermine, and spermidine are important polyamines found in all living organisms. They can be synthesized endogenously in cells or taken exogenously with food. The precursor molecule is arginine in the urea cycle. Ornithine is synthesized from arginine. Putrescine, formed by decarboxylation from ornithine, is the precursor compound of spermine and spermidine [4] (Figure 1).

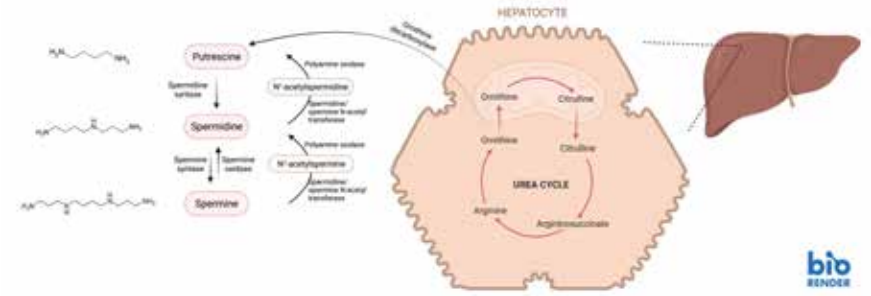

Figure 1. Formation of spermidine and spermine from putrescine.
Figure created with BioRender.com

Polyamines are important for cell growth and differentiation. They are involved in functions such as DNA synthesis and stability, regulation of transcription, ion channel regulation, and protein phosphorylation [5]. Polyamines, which are known to have antioxidant properties, also affect the stabilization of lipids, brain development, nerve growth and regeneration [5, 6].Since polyamines are positively charged at physiological pH, they tend to bind to negatively charged biomolecules such as DNA, RNA, proteins, and phospholipids, acting as polycations. Many studies showed that polyamines perform their functions by binding to biomolecules [7,8]. It was found that polyamines play a role in proinsulin

biosynthesis and insulin secretion [9]. The role of sperm and spermidine in insulin production is provided by stimulating insulin secretion and participating in the proliferation of island cells. In a study conducted in 2003, it was found that spermine and spermidine at physiological concentrations inhibit glucose from reacting with proteins non-enzymatically (glycation) [10].

Obesity is also defined as an increased chronic oxidative stress state. Oxidative stress occurs as a result of the disruption of the balance between oxidants and antioxidants. In obesity, excess free fatty acid induces oxidative stress by causing lipid peroxidation [11, 12].

In this study, we aimed to investigate the level of polyamines in obesity and its relationship with oxidative stress. Our study is the first in the literature to measure putrescine, spermine and spermidine levels in obese adults and to investigate their relationship with oxidative stress.

METHODOLOGY

Ethics committee approval was obtained from the Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (Decision No: 205). Informed consent was obtained from the patients who applied to Medipol Mega University Hospital for routine examination. The study was carried out with 85 obese patients with a mean age of 18-70 years and 29 healthy controls. According to body mass index (BMI) values, between 18.5 and 24.9 kg/m^2 formed the control group, and values of 24.9 kg/m^2 and above formed the obese group. The individuals, which constituted the study group, were divided into subgroups as diabetic obese (n:29) and non-diabetic obese (n:56) according to the criteria of the American Diabetes Association (ADA) [13].

Being younger than 18 years old or older than 70 years old, smoking, have kidney function disorders, hypertension, heart disease, osteoarthrosis, cancer, polycystic ovarian disease, inflammatory and infectious diseases were adopted as exclusion criteria.

The blood taken from the whole study group was centrifuged at 900 rpm for 15 minutes. If the separated sera could not be studied on the same day, they were aliquoted and brought up to -80°C. Glucose, glycosylated hemoglobin (HbA1c), urea, uric acid, C-Reactive Protein (CRP) levels were measured quantitatively in Cobas-Roche 6000 autoanalyzer using immuno chemiluminescence method. Hemogram test was performed by flow cytometric method on Symex 2000i device.

Total antioxidant status (TAS) and Total oxidant status (TOS) were measured spectrophotometrically [14,15].

Analysis of Total antioxidant assay

ABTS [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)] (Sigma-Aldrich, Taufkirchen,Germany) reagent is radicalized by hydrogen peroxide (Sigma-Aldrich, Taufkirchen, Germany). When serum is added, antioxidant-sin the serum neutralize existing ABTS radicals. The absorbance is measured at 658 nm.

Analysis of Total oxidant assay

Fe_2SO_4 dissolves in water, releasing Fe^{2+}. Oxidants found in serum enable Fe $^{2+}$ to Fe^{3+} oxidation. TheX-orange (Sigma-Aldrich, Taufkirchen, Germany) reagent used givesa colored complex with Fe^{3+}. The absorbance is measured at 658 nm.

Oxidative stress index(OSI) was calculated using the TOS/TAS x100 formula. Putrescine, spermine and spermidine levels were studied by High performance liquid chromatography (HPLC) method.

Analysis of Putrescine, Spermine and Spermidine

For the measurement of putrescine, spermine and spermidine concentration, firstly, 200 µl of patient serum was centrifuged at 15000xg at 4°C for 10 minutes. 100µl of supernatant was taken and added with 100µl of cold 1.5 M $HClO_4$, and then was stirred at 25°C for 1 minute. Then, 50µl of cold 2 M K_2CO_3 was added and mixed for 10 seconds. It was evaporated with CO_2 gas under vacuum device and then stirred at room temperature for 1 minute. It was centrifuged at 15000xg for 10 minutes at 4°C and 100 µl of supernatant was collected. It was diluted by adding 150 µl of H_2O on it. For HPLC analysis, 700µl of H_2O, 50µl of 1.2% (w/v) benzoic acid and 50µl of sample was added into the vials, respectively. Two injections were made from each sample and their averages were taken. Measurements were performed using an HPLC system (Waters, Milford, MA) equipped with a reverse-phase column (Nova-Pak C18; 3.9 150 mm; 4 m particle size; Waters) and precolumn (Nova-Pak C18; 3.9 20 mm; 4 m particle size; Waters). Fluorescence detection was set to 340 and 450 nm excitation and emission wavelengths, respectively [16].

Statistical Analysis

Statistical analyzes were performed with SPSS (version 17, Chicago, IL, USA) program and Graphpad prism 8.0(San Diego, California, USA). Group distributions for TAS, TOS, glucose, CRP, hemogram, and HbA1c were evaluated using the Kolmogorov-Smirnov test. Student's t test was used for the parameters with normal distribution in the group, and Mann-Whitney U test was used for

the parameters that did not fit the normal distribution.

In subgroup analysis, parametric t-test and one-way ANOVA were used for groups with normal distribution. Mann-Whitney-U and non-parametric analysis of variance Kruskall-Wallis test were used for the variables that did not fit the normal distribution. Pearson correlation analysis test was used for correlation analysis between groups. Statistical significance level was accepted as $p \leq 0.05$.

RESULTS and DISCUSSION

The prevalence of obesity has increased to pandemic dimensions in the last 50 years [2]. Obesity is the second most important cause of preventable death after smoking (sitting is the new smoking)[1,2]. The fact that obesity increases very rapidly, causes many diseases, especially cardiovascular and endocrine, and can be prevented, increases the importance of obesity studies [2].

Oxidative stress arises from an imbalance between free radicals and the antioxidant defense system of the cell. Free radicals, which increase in obesity, have an effect on hypothalamic neurons in the control of hunger and satiety and, accordingly, in the control of body weight [17]. It is known that oxidative stress increases in obesity. In the study of Catoi et al., TAS and TOS examinations were performed in 23 obese and healthy control groups each, and it was observed that TAS levels were lower and TOS values were higher in obese patients compared to the normal-weight healthy group [11]. Serum TAS levels were evaluated in a population-based study in 3042 adult obese adults. In a study conducted with randomly selected 1514 men and 1528 women, it was revealed that obese male participants had 10% lower TAS concentrations than normal-weight men, and female obese participants had 6% lower TAS concentrations than normal-weight ones [11].

In our study, when the obese group and the control group were compared, no statistical difference was observed in TAS values. OSI values were numerically higher in the obese group, while TOS values were statistically higher (Table 1). High OSI values revealed that oxidative stress was increased in our obese study group, which was consistent with the literature.

Table 1. Biochemical parameters of control and obese groups

	Control Group n=29	**Obese Group n=85**	**p**
Putrescine (nmol/mL)	0,39±0,08	0,25±0,13	<0,05
Spermin (nmol/mL)	6,8±1,87	6,47±1,84	>0,05
Spermidine (nmol/mL)	2,29±0,79	1,80±0,68	<0,05
TAS (Trolox Eqv./L)	1,01±0,13	1,03±0,21	>0,05
TOS (mM H_2O_2 Eqv./L)	19,65±2	22,24±5,46	<0,05
OSI (AU)	1,97±0,3	2,25±0,77	>0,05
Glucose (mg/dl)	93,23±9,31	129,3±69,52	<0,05
HgA1c (%)	4,78±0,45	6,24±1,87	<0,05
CRP (mg/L)	1,75±1,73	4,04±3,92	<0,05
Urea (mg/dl)	27,60±7,68	30,87±9,12	>0,05
Uric acid (mg/dl)	4,55±1,56	5,54±1,52	<0,05
Leukocyte	6,84±1,57	7,52±1,57	<0,05
Erythrocyte	4,72±0,44	4,94±0,54	>0,05
Hemoglobin	13,26±1,14	13,78±1,69	>0,05
Lymphocyte	2,1±0,65	2,53±0,71	<0,05

Abbreviations: BMI: Body mass index; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; CRP: C reactive protein; HgA1c: Hemoglobine A1c. $p<0,05$ was considered statistically significant.

When subgroup analyzes were performed (Table 2), it was observed that both OSI and TOS values in the diabetic obese group were statistically higher than both the control group and the non-diabetic obese group Similar to TOS, glucose and HbA1c OSI was found to be correlated with glucose and HbA1c (Table 3). It was thought that oxidative stress increased in parallel with the increase in blood glucose level and increased oxidative stress increased polyamine levels.

Table 2. Biochemical parameters of control, non-diabetic obese and diabetic obese groups

	Control Group n=29	Obese Group		p	Intergroup Significance[a]
		Non-Diabetic Obese n=56	Diabetic Obese n=29		
BMI (kg/m^2)	22,43 ± 1,79	30,04 ± 4,14	30,92 ± 5,90	<0,05	1-2 1-3
Putrescine (nmol/mL)	0,39±0,08	0,21±0,1	0,33±0,14	<0,05	1-2 2-3
Spermin (nmol/mL)	6,80±1,87	6,23±1,35	6,93±2,49	>0,05	
Spermidine (nmol/mL)	2,29±0,79	1,54±0,52	2,31±0,7	<0,05	1-2 2-3
TAS (Trolox Eqv./L)	1,01±0,13	1,06±0,2	0,98±0,22	>0,05	
TOS (mM H_2O_2 Eqv./L)	19,65±2,0	20,59±2,89	25,41±7,56	<0,05	1-3 2-3
OSI (AU)	1,97±0,36	2,02±0,55	2,69±0,94	<0,05	1-3 2-3
Glucose(mg/dl)	93,23±9,31	99,6±8,98	186,7±95,72	<0,05	1-2 1-3 2-3
HbA1c (%)	4,78±0,45	5,28±0,44	8,11±2,17	<0,05	1-2 1-3 2-3
CRP (mg/L)	1,75±1,73	3,25±2,94	5,56±5,05	<0,05	1-2 1-3
Urea (mg/dl)	27,60±7,70	30,21±8,64	32,16±10,02	>0,05	
Uric acid (mg/dl)	4,55±1,56	5,69±1,54	5,25±1,47	<0,05	1-2
Leukocyte	6,84±1,57	7,69±1,62	7,21±1,45	>0,05	
Erythrocyte	4,72±0,44	4,95±0,55	4,92±0,53	>0,05	
Hemoglobin	13,26±1,15	13,90±1,77	13,55±1,54	>0,05	
Lymphocyte	2,10±0,65	2,55±0,74	2,48±0,67	<0,05	1-2

(Abbreviations: BMI: Body mass index; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; CRP: C reactive protein; HgA1c: Hemoglobine A1c. $p<0,05$ was considered statistically significantp<0,05 was considered statistically significant. [a]Groups (Groups 1–3) of statistical difference are stated)

Table 3. Correlation matrix

	BMI	Putrescine	Spermidine	Spermin	TAS	TOS	OSI	Glucose	HbA1C	Uric acid	Urea	CRP
BMI	1,00											
Putrescine	-0,24*	1,00										
Spermidine	-0,22*	0,41*	1,00									
Spermin	-0,08	0,05	0,29*	1,00								
TAS	0,18	-0,03	-0,13	0,00	1,00							
TOS	0,19*	0,13	0,07	-0,05	-0,11	1,00						
OSI	0,06	0,08	0,10	-0,06	-0,70*	0,76*	1,00					
Glucose	0,17	0,14	0,18	0,16	0,04	0,45*	0,26*	1,00				
HbA1C	0,25*	0,12	0,16	0,12	0,01	0,44*	0,28*	0,91*	1,00			
Uric acid	0,17	-0,15	-0,25*	-0,02	-0,03	-0,02	0,04	-0,09	-0,08	1,00		
Urea	0,10	0,05	-0,08	0,17	0,05	0,00	0,00	0,06	0,07	0,40*	1,00	
CRP	0,36*	-0,17	-0,05	0,05	-0,04	0,08	0,10	0,37*	0,37*	0,06	0,00	1,00
	BMI	**Putrescine**	**Spermidine**	**Spermin**	**TAS**	**TOS**	**OSI**	**Glucose**	**HbA1C**	**Uric acid**	**Urea**	**CRP**
BMI	1,00											
Putrescine	-0,24*	1,00										
Spermidine	-0,22*	0,41*	1,00									
Spermin	-0,08	0,05	0,29*	1,00								
TAS	0,18	-0,03	-0,13	0,00	1,00							
TOS	0,19*	0,13	0,07	-0,05	-0,11	1,00						
OSI	0,06	0,08	0,10	-0,06	-0,70*	0,76*	1,00					
Glucose	0,17	0,14	0,18	0,16	0,04	0,45*	0,26*	1,00				

(Abbreviations: BMI: Body mass index; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; CRP: C reactive protein; HgA1c: Hemoglobine A1c. $p<0,05$ was considered statistically significant)

In this study, the levels of putrescine, spermine and spermidine, known as polyamines, were found to be lower in the obese group. Putrescine and spermidine levels in obesity) were found to be significantly lower, respectively, than the control group (Table 1). Spermin levels were found to be lower in the obese group compared to the control group (although it was not statistically significant (Table 1). When the correlation analyzes were examined, a negative correlation was found between BMI and Putrescine and Spermidine (Table 3). Our study revealed that in addition to the decrease in polyamine levels in obesity, as obesity level increases, polyamine levels decrease (Table 1).

When the subgroup analyzes were performed, the putrescine and spermidine levels were found statistically lower in the non-diabetic obese group compared to the control group (Table 2). A striking finding was that the putrescine and spermidine levels, in the non-diabetic obese group, were found to be lower when compared to those of the diabetic obese group (Table 2). Spermine levels in the diabetic obese group were also numerically higher than in the non-diabetic obese group (Table 2).

A study in patients with metabolic syndrome with and without type 2 diabetes showed that serum putrescine level was significantly elevated in patients with T2DM compared to those without T2DM and significantly correlated with HbA1c levels [18]. Unlike this study, which associated high polyamine level with hyperglycemia, oxidative stress was found to be correlated with hyperglycemia in our study. It was thought that polyamine levels, which showed antioxidant properties, increased to compensate for the increased oxidative stress. The increase of uric acid, which has antioxidant properties, supports this idea (Table 2)

There are opinions that polyamines prevent oxidative stress by inhibiting the auto-oxidation of metals or by acting as a direct antioxidant. Many studies showed that polyamines greatly modulate the homeostasis of reactive oxygen species. Homeostatis performs this function directly or indirectly by regulating antioxidant systems or by suppressing the production of reactive oxygen species [19].

When HbA1c levels were examined, statistically significantly higher values were found in the non-diabetic obese group compared to the control group. At the same time, statistically significantly lower polyamine levels were found in the non-diabetic obese group (Table 2). This finding suggested that the increase in polyamine levels was due to oxidative stress, not hyperglycemia. Otherwise, we should have seen high polyamine levels in the non-diabetic obese group.

In a study, it was observed that spermine or spermidine, or both were able to protect cells from ROS at normal or supraphysiological concentrations, but spermine was stronger in this regard 20. In our study, it was observed that the increase in spermidine was parallel with the increase in ROS.

In another study conducted on children, it was reported that serum polyamine levels were significantly higher in obese children [21]. The increase in polyamine levels in obese children may be associated with increased oxidative stress with obesity, suggesting that polyamine levels may be different from adults due to physiological growth.

In conclusion, our study is the first in the literature to measure putrescine, spermine and spermidine levels in obese adults. Putrescine, spermine and spermidine levels were found to be lower in non-diabetic obese compared to the control group, but it was observed that polyamines increased with increasing oxidative stress in diabetic obese.

ETHICAL STATEMENT

Our study was approved by Medipol University local ethics committee (Decision No: 205). There are no ethical issues with human or animal subjects.

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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.

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AUTHOR CONTRIBUTIONS

These authors contributed equally.

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