ACTA PHARMACEUTICA SCIENCIA

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Nurşen Başaran (Hacettepe University,

Address
İstanbul Medipol Üniversitesi
Kavacık Güney Kampüsü
Göztepe Mah. Atatürk Cad.
No: 40 34810 Beykoz/İSTANBUL
Tel: 0216 681 51 00
E-mail
editor@actapharmsci.com
secretary@actapharmsci.com
Web site
http://www.actapharmsci.com
Agency Communication
Mert Sarsağ
Graphic-Design
Sertan Vural - Medicomia
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Kavacık Mah. Ekinciler Cad. No:19
Medipol Üniversitesi Kuzey Yerleşkesi
Tel: (0216) 681 53 72

Contents

Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia (Acta Pharm. Sci.) is a quarterly multidisciplinary research journal published by İstanbul Medipol University.The journal publishes articles in related disciplines as well as in all fields of pharmacy (Pharmaceutical Technology, Pharmacognosy, Pharmaceutical Chemistry, Clinical Pharmacy, Pharmacology, Toxicology, etc.). The language of the journal is English and it is open to all nationalities. The journal accepts articles of the type specified in the instruction for publication after the editor and referees control.

History of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is the oldest scientific journal in the field of pharmacy in Turkey and 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, 12 font size, and all kinds of articles must be double-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. Years of the references should be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication. References should conform to APA style and be numbered consecutively in the order in which they are cited in the text.

*Obligatory files are manuscript main document, title page and copyright form for submission. If exist, supplementary files should also be added.

1. Scope and Editorial Policy

1.1 Scope of the Journal

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

The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part.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

All manuscripts 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 statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the 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 fonts "Times" and "Symbol." 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 experimental descriptions. General reaction, isolation, preparation conditions should be given only once. The title of an experiment should include the chemical name and a bold Arabic identifier number; subsequently, only the bold Arabic number should be used. Experiments should be listed in numerical order. Molar equivalents of all reactants and percentage yields of products should be included. A general introductory section should include general procedures, standard techniques, and instruments employed (e.g., determination of purity, chromatography, NMR spectra, mass spectra, names of equipment) in the synthesis and characterization of compounds, isolates and preparations described subsequently in this section. Special attention should be called to hazardous reactions or toxic compounds. Provide analysis for known classes of assay interference compounds.

The preferred forms for some of the more commonly used abbrevations are mp, bp, ^{o}C, K, min, h, mL, μL, g, mg, μg, cm, mm, nm, mol, mmol, μmol, ppm, TLC, GC, NMR, UV, and IR. Units are abbreviated in table column heads and when used with numbers, not otherwise. (See section 4 for more abbreviations)

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

2.2.6 Ancillary Information. Include pertinent information in the order listed immediately before the references.

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

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

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

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

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

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. APA style is used in the reference list and citations. 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. APA 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. *Bioorganic & Medicinal Chemistry*, 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.

APA Style examples:

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.

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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.

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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.

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

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

ORIGINAL ARTICLES

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Evaluation of Probiotic Use of The Students of Istanbul Medipol University

Neda Taner[1], Eşref Tatlıpınar[2*], Nihal Büyükuslu[1]

1 Istanbul Medipol University, School of Nutrition and Dietetics, Istanbul, Turkey
2 Istanbul Medipol University, School of Pharmacy, Department of Pharmaceutical Toxicology, Istanbul, Turkey

ABSTRACT

This research; is planned to determine the level of knowledge about probiotic nutritions and, the consumption of them by university students. The date range of the study was 2018. A total of 100 students were surveyed at the İstanbul Medipol University, 47 men and 53 women aged 18-30 years. General information and demographic status of participants were questioned. Also, height and weight data were recorded by their written notifications. In parallel with the ever-increasing work on the positive effects of probiotics on health; increased consumption of probiotics and increased knowledge.

Microorganisms can directly or indirectly cause the formation of many diseases. 20% of the students consume these nutrients on the recommendation; and 24% of them did not consume it because they think did not need it. Although probiotic dairy products are mostly used for symptoms of constipation, there is not enough information in the context of other diseases.

Keywords: Microflora, probiotic nutrient, fermentation

INTRODUCTION

Probiotics are selectable viable microorganisms used as nutritional supplements that contribute to maintaining human health or have potential benefits for disease prevention. [1] On the other hand, prebiotics, are defined as food components that stimulate the reproduction and activity of beneficial bacteria in the colon, thereby indirectly cause benefit to the host. [2] Symbiotics are also non-digestible substances that activate or strengthen the effect of probiotic bacteria with the po-

Corresponding Author:
e-mail: metatlipinar@medipol.edu.tr
ORCID
Neda Taner: 0000-0002-6141-8676
Eşref Tatlıpınar: 0000-0001-8422-3188
Nihal Büyükuslu: 0000-0003-1420-0989
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tential synergistic effect caused by the combination of probiotics and prebiotics. [3]

The history of the relationship between gut health and human disease has followed a development from Hippocrates (460-370 BC) to the present day. Within the understanding of modern medicine, the first studies related to intestinal bacteria and digestive physiology, which started with T. Escherich in 1886, has become a common scientific knowledge field including prebiotic, probiotic and symbiotic definitions that form today's common terminology. [4,5]

Functional foods are food itself and, additive ingredients, that provide the human body's need for essential nutrients, provide additional benefits in human physiology and metabolic functions, thereby helping to prevent disease and achieve a healthier life. [6] On the other hand, these foods are also defined as nutrients that can be consumed in the form of nutriment with a daily diet, without containing synthetic compounds and have health and well-being properties. [7]

With the development of healthy eating awareness, consumers expect to benefit from health benefits as well as food. Functional foods have become one of the fastest growing sectors of the food industry as a result of these expectations that consumers show to new products and quality. One of these functional foods is probiotics that have received great interest in recent years. As of the definition given above probiotic bacteria are found in the normal human intestinal flora. Probiotics are naturally present in fermented milk and other fermented foods, but many products originating from yoghurt, sour cream, milk powder, sweets, fruit juice, ice cream, baby milk or butter, butter, mayonnaise, meat and oats can also be added externally.[6]

One of the questions most asked by consumers who see a balanced intestinal flora in relation to an effective immune system is the benefit of probiotic use on healthy people. A response to a healthy microflora composition and the benefits to the host is usually that the answer is that it can prevent complaints that may arise from occasional imbalances, even if they are speculative, even in a balanced system. On the other hand, probiotics have been proven to have effects such as gastrointestinal complaints, normalization of reduced bowel mobility or reduction of long-term risks (cancer, ischemic heart disease) as well as health benefits such as prevention or alleviation of common infectious diseases (eg colds) or atopic diseases caused a general wonder to awaken. However, in no way should probiotic consumption consume a healthy lifestyle and a balanced diet.[8]

Probiotic bacteria have been shown in several publications in recent years as being very useful for human health in research. It has been shown to be beneficial in cancer and inflammatory bowel disease, preventing inflammation, diarrhea and

constipation, preventing inflammation, colonization of the intestinal flora, and prevention of colonization of pathogenic bacteria in the intestine.[9]

This research is to investigate the structure and properties of probiotics, their effects on human health, their usage areas, their effect mechanisms and their reliability in order to reach the desired point by determining information about university students' consumption of probiotic foods and probiotics which are increasingly used nowadays.

METHODOLOGY

This section comprises the methods and tools used in the research and defined in sub-headings as of 'Research Model', 'Research Universe and Sampling', 'Data Collection Techniques' and 'Analysis of Data'.

Research Model

The screening model was used in this study. The research aimed to determine the probiotic nutrient consumption status, probiotic consumption frequency and information about probiotic foods of the students who continue university education.

Research Universe and Sampling

The universe of this research was Istanbul Medipol University. The questionnaire was applied to 100 students selected randomly among the students who continued their education at Istanbul Medipol University.

Data Collection Techniques

The research data were collected by the researcher with the questionnaire technique. The survey form developed as a data collection tool is preferred because it is the most appropriate tool in the data collection. The evaluation and preparation of the questionnaire were consisting of literature expert opinions and the examinations and review of thesis and researches related with the subject.

The questionnaire consists of three parts. In the first part, it is aimed to measure the information about the students and their families, in the second part the probiotic food consumption situation and in the third part the information about the probiotic products.

The questionnaire was applied to the students participating in the research under the supervision of the researcher. The necessary explanations about the questionnaire were made to the students by the researcers and, it was accepted that the students who participated in the research gave accurate and impartial answers to the questions.

Analaysis of Data

The data collected about the sub-problems that are searched for within the framework of the research problem are first processed in the data coding tables on the computer. Then statistical analyzes on the data were performed using SPSS (Statistical PacketforSocialSciences) 20 package program.

Findings are shown in the tables as female and male gender, number and percentage. Frequency (f), percent (%), chi square, arithmetic mean (x) and standard deviation (ss) were used to determine the personal and family characteristics of the students. A significance level of 0.05 was taken to test the differences.

The following formula was used in the calculation of the BMI of the students in the study, and the evaluations were made according to the following classifications.

BMI = (Body Weight (kg)) / (Thickness (m2))

• BMI below 18.5	Underweight
• 18.5- 24.9	Normal Weight
• 25.0- 29.9	Excess Weight
• 30.0- 39.9	Obese
• Morbid (serious) obesity	Over 40,0 °

RESULTS AND DISCUSSION

Participants selected through coincidental sampling are students from the Medipol University who responded to the survey. Demographic data and values for these students and their families were first collected for measurement results of knowledge of probiotic food consumption and probiotic products of research and thesis subjects. The results are summarized in Table 1 below.

Findings / Part 1. Participatory demographic data

Demographic data and values of the participants: General

Demographic data and values related with the enrolled students and their families were considere as of the measurement results of knowledge of probiotic food consumption and probiotic products. The results are tabulated in Table 1-5 and summarized in Figures 1-6 in the foolowin sections.

Table 1. Participants' demographic data and values

Characteristics	(n=100)	Percent Exhibitor (%)	% Mean ± SD	Median (Min-max)	p-value
Gender					
Female	54	54	71.04 ± 12.08	73.5 (44-92)	0.55
Male	46	46	72.5 ± 12.7	73.0 (48-100)	
Department of Education					
Pharmacy	22	22	70.9 ± 14.08	73.0 (48-100)	0.67
One-language speech	18	18	79.39 ± 11.14	68.0 (53-92)	
Medicine	25	25	77.6 ± 8.44	75.0 (57-92)	
Law	34	34	75.6 ± 14.9	69.5 (44-100)	
Physiotherapy	1	1	-	-	
Classs					
1	25	25	72.0 ± 14.9	76.0 (46-100)	0.88
2	30	30	69.6 ± 13.57	68.0 (44-100)	
3	30	30	73.12 ± 10.39	75.0 (48-92)	
4	10	10	75.66 ± 25.06	73.0 (63-92)	
Living Area					
Bay	3	3	60.33 ± 17.21	53.0 (48-80)	0.54
District	23	23	79.15 ± 13.16	74.0 (48-100)	
Province	23	23	80.09 ± 12.87	72.0 (53-92)	
Big city	51	51	74.63 ± 11.79	73.0 (44-99)	
Mother Education					
Illiterate	2	2	-	-	0.79
Educated	3	3	-	-	
Primary school graduate	19	19	70.86 ± 16.55	70.5 (44-100)	
Secondary school graduate	17	17	71.45 ± 11.24	73.0 (48-92)	
High school graduate	32	32	-	-	
University	27	27	73.08 ± 27.3	74.5 (48-92)	
Father Education					
Educated	1	1	-	-	0.88
Primary school graduate	16	16	72.25 ± 12.7	70.5 (50-100)	
Secondary school graduate	15	15	70.16 ± 13.16	73.0 (44-100)	
High school graduate	17	17	-	-	
University	51	51	72.66 ± 11.33	74.0 (48-92)	
Economic Income Level					
Very low	1	1	-	-	0.35
Middle	77	77	72.27 ± 12.8	73.0 (48-100)	
High	19	19	69.9 ± 12.2	73.5 (48-100)	
Very high	2	2	-	-	

Participants' mean height (170.4 ± 14.08) in terms of arithmetic mean ± SD (Std.) body weight (66.45 ± 14.8) and ages (21.35 ± 3.25)

Demographic data and values of the participants: Participant gender differentiation

The values for the data table are also summarized in the figures below. Figure 1 shows the status of the participants in terms of gender differentiation. Total mean values of both sexes (71.04 ± 12.08) (72.50 ± 12.7) p> 0.05 in both males and females, respectively, show no statistically significant difference.

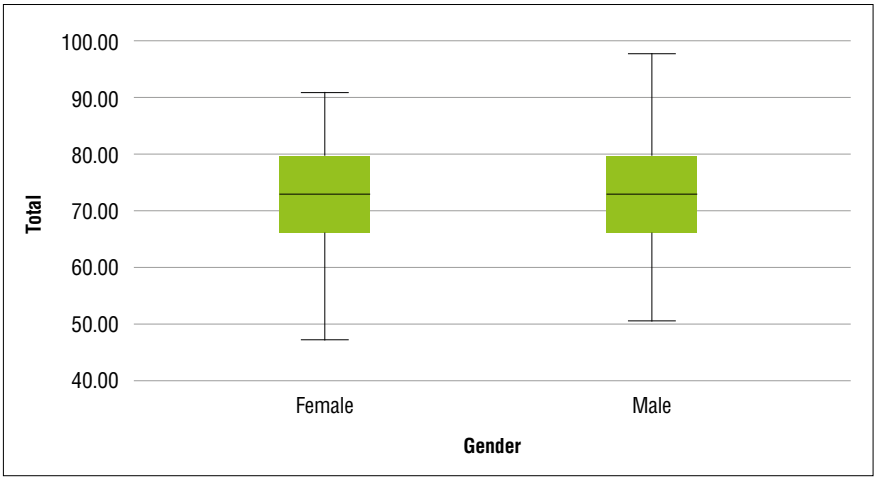

Figure 1. Participant gender differentiation

Demographic data and values of participants: Differences in occupational section preferences of participants

Figure 2 exhibit the training sections preferred by the participants. Participants have a more widespread preference in the field of pharmacy and law, while a language preference in speech and medicine-physics is relatively less favorable than the other two disciplines. However, there is no statistically significant difference between all departmental preferences.

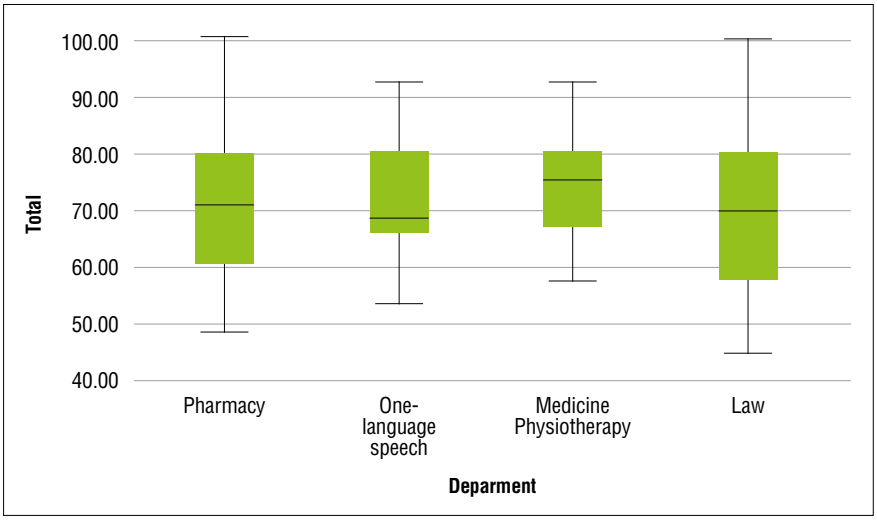

Figure 2. Participants' preference for occupational sections

Demographic data and values of participants: Difference in educational level of participant parents

The results of the differences in educational level of participant parents were exhibited in Table 2, Figure 3 and, Table 3, Figure 4, as of the data of fathers and mothers, respectively. There is a wide range in terms of different graduation categories, but no statistical significance is calculated.

Educational level of fathers:

Table 2. Father: Case Summary

	Father	Cases					
		Current Missing Total		Current Missing Total		Current Missing Total	
		N	Percent	N	Percent	N	Percent
Total	Primary School Graduate	16	94.1%	1	5.9%	17	100.0%
	Secondary School Graduate	29	90.6%	3	9.4%	32	100.0%
	Graduated from a University	43	84.3%	8	15.7%	51	100.0%

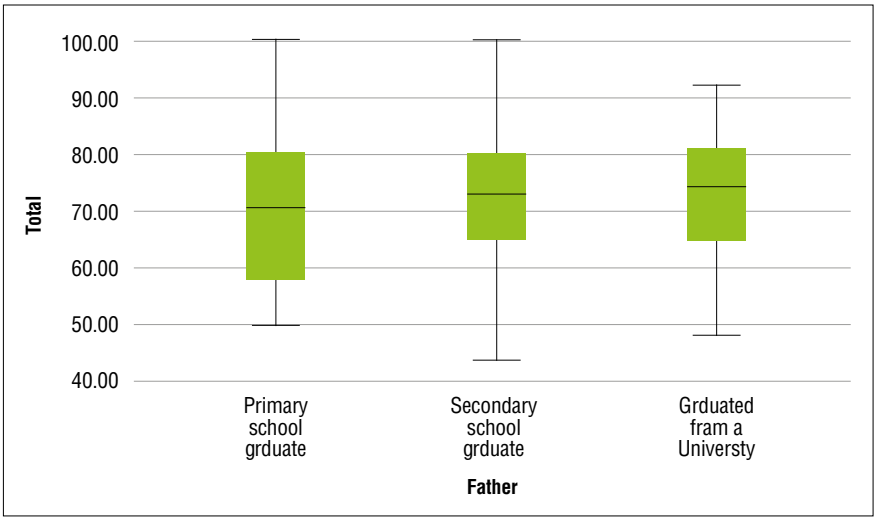

Figure 3. Difference in educational level of participant parents (father)

Educational level of mothers:

Table 3. Mother: Case Summary

	Mother	Cases					
		Current		Missing		Total	
		N	Percent	N	Percent	N	Percent
Total	Primary School Graduate	22	91.7%	2	8.3%	24	100.0%
	Secondary School Graduate	42	85.7%	7	14.3%	49	100.0%
	Graduated from a University	24	88.9%	3	11.1%	27	100.0%

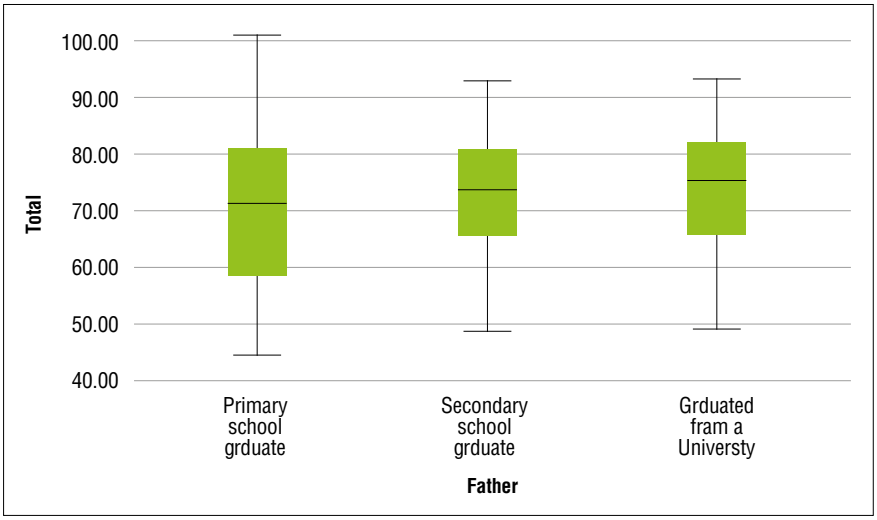

Figure 4. Difference in educational level of participant parents (mother)

Demographic data and values of participants: Differences in economic income level of participant families

The level of income of the participating families is shown in table 4 and figure 5. In terms of income levels, participants are from low, middle income families. However, there is no statistical difference between these groups.

Table 4. What do you think? Economic Level: Case Study Summary

	What do you think? -Economic Level	Cases					
		Current		Missing		Total	
		N	Percent	N	Percent	N	Percent
Total	Low and Medium	68	87.2%	10	12.8%	78	100.0%
	High	20	95.2%	1	4.8%	21	100.0%

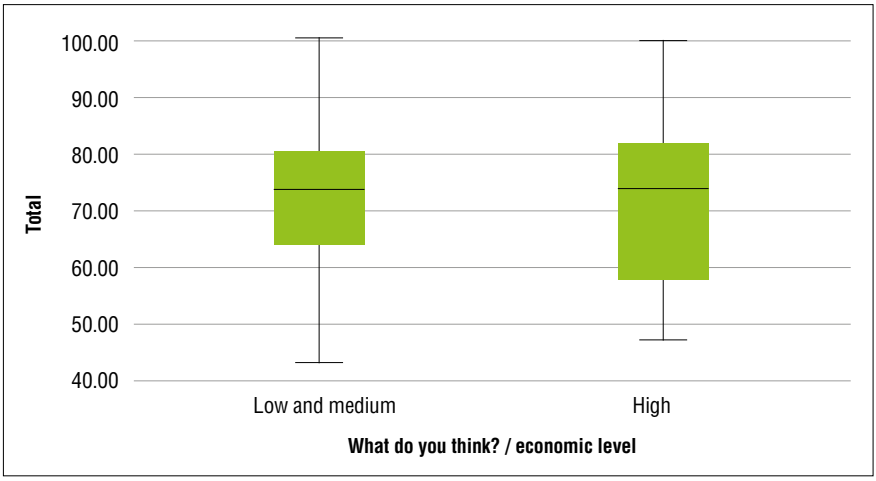

Figure 5. Differences in economic income level of participant families

Participants' demographic data and values: Life zones

Participants' families prefer to live in the cities. However, there is no statistical difference among the participants from the smaller settlement areas.

Table 5. Life: Case Summary

	Life	Cases					
		Current		Missing		Total	
		N	Percent	N	Percent	N	Percent
Total	Town and village	3	100.0%	0	0.0%	3	100.0%
	District	22	95.7%	1	4.3%	23	100.0%
	Province	17	73.9%	6	26.1%	23	100.0%
	Big city	46	90.2%	5	9.8%	51	100.0%

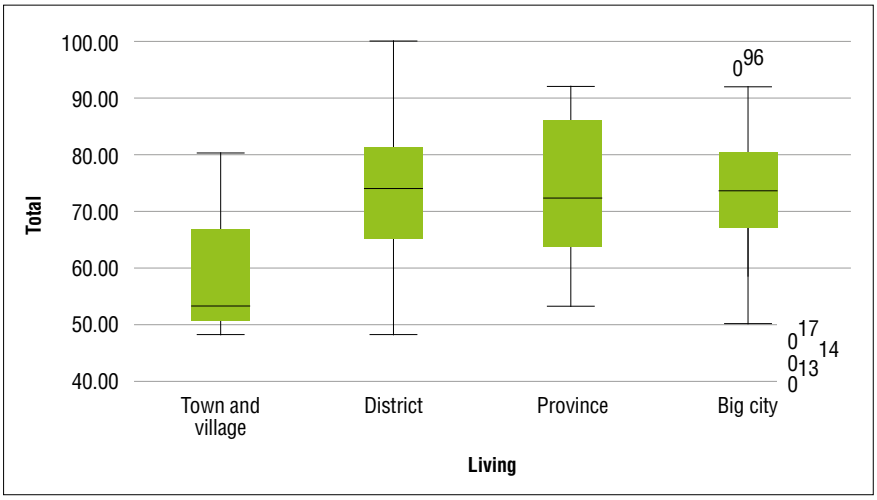

Figure 6. Living area preferences of participating families

Findings / Part 2. Measurement of students' knowledge of probiotic food consumption and probiotic products

This section is based on the answers obtained from questionnaire questions 11-31. Table 6 summurizes the general information related with all questionarry questions.

Table 6. General informations and summaries related with all questionary questions

Questionnaire questions numbers	Number of tables refering the summury results	Results
11	**-**	**Questionnaire question 11** was questioned whether probiotic description is known or not. From the responses "I know the answer" is 77%, "I do not know the answer" is 21%.
12	**7**	-
13	**-**	**Questionnaire question 13** was questioned whether students have problems related with feeding. 85% of the students have no problem.
14	**8**	
15	**-**	**Questionnaire question 15** was questioned students' probiotic fattening consumption. 52% of 100 students answered yes, 8% answered no. There is no idea about the consumption of 40% students.
16	**9**	-
17-20	**10**	-
21	**11**	-
22	**12**	-
23	**13**	-
24		**Questionnaire question 24** was questioned the use of probiotic products as nutritional supplements in students. The percentage that does not use is 68, and the percentage of users who use it is 20. The percentage of people who do not know about the use of probiotic products is 12.
25	**14**	-
26	**15**	-
27	**16**	-
28	**17**	-
29		**Questionnaire question 29** was questioned the students, about the ideal use time probiotics. Duration does not include five different time periods, from one week to over three years. 47% of the respondents answered as not knowing about the usage time period. The 20 %group, gave response for the time period as 1 to 3 months.
30	**18**	-
31	**19**	-

Questionnaire question 12:

This questionnaire question mainly questioned whether the disease (s) benefit or not from probiotic food consumption. Mainly the section measures information in 11 categories including constipation, diarrhea, allergy, lactose intolerance, inflammatory bowel diseases, high cholesterol, urogenital infections, irritable bowel syndrome, helicobacter pylori infection, acute pancreatitis and others. These are summarized in the table 7 below.

Table 7. Questionnaire Question 12: Which disease (s) do you think benefit from probiotic food consumption?

Disease	% Yes	%No
12 1 Constipation	65.0	35.0
12 2 Diarrhea	43.0	57.0
12 3 Allergy	20.0	80.0
12 4 Lactose intolerance	27.0	73.0
12 5 Inflammatory bowel diseases	35.0	65.0
12 6 High cholesterol	15.0	85.0
12 7 Urogenital infections	10.0	90.0
12 8 Irritable bowel syndrome	42.0	58.0
12 9 *Helicobacter pylori infection*	29.0	71.0
12 10 Acute pancreatitis	10.0	90.0
12 11 Other		100.0

Yes answers indicate that students have knowledge only about constipation, without consisting of sufficient information of probiotics usage in other diseases.

Questionnaire question 14:

In questionnaire question 14, it is questioned whether and if there were any diseases diagnosed in the students. The answers to this question are summarized in table 8.

Table 8. Questionnaire Question 14: If you have a diagnosed any health problem.

Disease	% Yes	%No
14 1 Diabetes	4.0	96.0
14 2 Kidney disease	2.0	98.0
14 3 Dental problems	1.0	99.0
14 4 Anemia	8.0	92.0
14 5 Eye illness	6.0	94.0
14 6 Cardiovascular disease	1.0	99.0
14 7 Other	9.0	91.0

Questionnaire question 16:

This questionnaire question, questioned the students' reasons for consuming probiotic food. Belowmentioned table 9 summarizes the answers.

Table 9. Questionnaire Question 16: What are your reasons / reasons for consuming probiotic food?

Preference factor	% Yes	%No
16 1 I find it delicious	19.0	81.0
16 2 I see the benefits of digestive system	38.0	62.0
16 3 I think that strengthens my immune system	30.0	70.0
16 4 I am consuming because of my health problems	2.0	98.0
16 5 I consume on advice	10.0	90.0
16 6 Other		100

Questionnaire question 17-20:

The answers given by the students to ***the questions 17-20 of the questionnaire*** is summarized in the following table 10.

Table 10. Questionnaire Question 17-20 Answers

Question	% No Idea	% Yes	% No
Questionnaire Question 17. Do you see the benefit of the probiotic foods you use?	41	53	6
Survey Question 18. Do you read the packaging labels of the probiotic foods you bought?	40	45	15
Questionnaire Question 19. Do you know the microorganisms in the probiotic foods you consume?	40	30	30
Questionnaire Question 20. Are you proposing for the consumption of probiotic foods?	39	38	23

Questionnaire question 21:

It is questioned that which health problems contributed to the consumption of probiotic nutrients. The returns from the students are summarized in the below-mentioned.table 11.

Table 11. Questionnaire Question 21: Do you think that the consumption of probiotic food contributes to the elimination of the following health problem/problems?

Problems	%Yes	%No
21 1 Circulatory system problems	6.0	94.0
21 2 Digestive system problems	51.0	49.0
21 3 Immune system problems	36.0	64.0
21 4 Obesity	16.0	84.0
21 5 Other	2.0	98.0

Questionnaire question 22:

In this question, students were questioned about the reasons of why they do not consume probiotic foods. In table 12, relevent “yes” or “no” responses as tabulated below.

Table 12. Questionnaire Question 22: What are your reasons / reasons for not consuming probiotic foods?

Causes	% Yes	% No
22 1 Do not know	17.0	83.0
22 2 Not needing	24.0	76.0
22 3 Expensive find	12.0	88.0
22 4 No tasteless find	10.0	90.0
22 5 Other	6.0	94.0

Questionnaire 23 questions:

Students' were questioned of their probiotic food consumption frequency. Probiotic yoghurt takes the first order of 2-3 times a week and consumes 26% from eleven different products. Table 13 summarizes the situation.

Table 13. Questionnaire Question 23: Consumption frequency of probiotic foods in students

	Food consumption frequency					
Food	**0**	**Everyday**	**2-3 times a week**	**1 time per week**	**Once in a month**	**I do not consume**
23 1 Probiotic yogurt	12.0	15.0	26.0	17.0	10.0	20.0
23 2 Probiotic milk	13.0	3.0	15.0	15.0	14.0	40.0
23 3 Kefir	12.0	1.0	5.0	9.0	16.0	57.0
23 4 Kefir cheese	15.0	1.0	2.0	1.0	7.0	74.0
23 5 Bread with sourdough yeast	16.0	7.0	2.0	10.0	17.0	48.0
23 6 Tarhana	10.0	3.0	22.0	24.0	32.0	9.0
23 7 Boza	14.0	-	3.0	4.0	24.0	55.0
23 8 Redfish	15.0	-	1.0	-	1.0	83.0
23 9 Kambucha tea	16.0	1.0	1.0	2.0	-	80.0
23 10 Natural turnip juice	11.0	3.0	5.0	10.0	30.0	41.0
23 11 Pickled olives	13.0	15.0	16.0	9.0	15.0	32.0

Questionnaire 25 questions:

Survey question 25, investigated the reasons of students' supplement.consumptions. The tendency to consume the most important product among the four elements is the recommendation (15%) taken around. Table 14 exhibits the collective results.

Table 14. Questionnaire Question 25: What is the reason for consuming a supplementary probiotic product?

Causes	**% Yes**	**% No**
25 1 Health problems	8.0	92.0
25 2 Advertisements	5.0	95.0
25 3 Recommendation	15.0	85.0
25 4 School education	4.0	96.0

Questionnaire 26 questions:

This questionnaire question 26, examined how the students hear of about the supplementary probiotic products. Among the six different learning sources, "friends, acquaintances, family and similar factors" is more prominent than others. Table 15 summarizes the results of this case

Table 15. Question 26: Where did you hear about the supplementary probiotic products?

Resources	% Yes	% No
26 1 Specialist (doctor, pharmacist or dietitian)	9.0	91.0
26 2 Friends, acquaintances, family etc.	11.0	89.0
26 3 Advertisements (newspapers, magazines, television)	6.0	94.0
26 4 Education, conferences, informal meetings	5.0	95.0
26 5 Pharmacies and sales points	6.0	94.0
26 6 Internet	6.0	94.0

Questionnaire 27 questions:

Questionnaire question 27, questioned the determination of the criteria why students took into account when purchasing supplemental probiotic products. According to five different criteria, the preference factor of the students in purchasing tendency is the content of the product (20%) and table 16 summarizes the results for this parameter.

Table 16. Questionnaire Question 27: What are the criteria / criteria to consider when buying a supplementary probiotic product?

Criteria	%Yes	%No
27 1 Price	4.0	96.0
27 2 Brand	9.0	91.0
27 3 Contents	20.0	79.0
27 4 Recommendation	6.0	94.0
27 5 Appearance	2.0	98.0

Questionnaire 28 questions:

The questionnaire question 28, questioned which 28 students used the supplementary probiotic products as brands. Table 17 summarizes the results and points out that Enterogermina (13%) is the most preferred product.

Table 17. Questionnaire Question 28: Which one do you use as a supplementary probiotic product?

Probiotic products	% Yes	% No
28 1 NBL Probiotic Goldschafts	3.0	97.0
28 2 Enterogermina®	13.0	87.0
28 3 BIFIFORM® Drops	3.0	97.0
28 4 NTBIOTIC Capsule	-	100.0
28 5 Natrol Acidophilus Capsule	-	100.0
28 6 Other	5.0	95.0

Questionnaire 30 questions:

The questionnaire question 30, questioned about the participating students how they recognize the sources of information about probiotics. It is found that, the first order tendency is to acquire information through 61% of health personnel. The answers were documented below in table 18.

Table 18. Survey Question 30: Which do you see as a source of information about probiotics?

A source of information on probiotics	% Yes	% No
30 1 Internet	36.0	64.0
30 2 Written-visual media	24.0	76.0
30 3 Through my friend	7.0	93.0
30 4 Through the health personnel	61.0	39.0
30 5 Medical courses	21.0	79.0

Questionnaire 31 questions:

The most comprehensive questionnaire surveyed among participant students in the questionnaire was ***Question 31,*** and it analyzes 20 sub parameters of "How are reinforcing probiotic products affecting our health". Table 19 is summarizing this data..

Table 19. Survey Question 31: How do you think reinforcing probiotic products are affecting our health?

	I strongly disagree	I do not agree	Partially Agree	I agree	Absolutely I agree
31 1 It contains useful items in health.	2.0	2.0	20.0	36.0	26.0
31 2 Helps strengthen the immune system.	7.0	13.0	41.0	25.0	-
31 3 Does not affect the regulation of the digestive system.	37.0	38.0	2.0	5.0	3.0
31 4 Contains a high number of microorganisms.	2.0	3.0	26.0	37.0	16.0
31 5 Prevents milk-induced discomfort (lactose intolerance).	4.0	8.0	31.0	20.0	13.0
31 6 Supports bone development.	2.0	8.0	15.0	29.0	30.0
31 7 There is no therapeutic effect.	23.0	31.0	21.0	4.0	7.0
31 8 It facilitates digestion by accelerating the transit of consumed foods.	4.0	10.0	13.0	35.0	21.0
31 9 Causes cancer.	42.0	34.0	6.0	5.0	1.0
31 10 Prevents disease-causing microorganisms from setting in the gut	5.0	6.0	23.0	29.0	24.0
31 11 Antibiotic-induced diarrhea is good.	1.0	7.0	28.0	28.0	20.0
31 12 Causes allergic diseases.	21.0	29.0	21.0	10.0	5.0
31 13 Allows living microorganisms to remain in balance in the mouth cavity.	6.0	8.0	33.0	23.0	15.0
31 14 Helps to lose weight.	8.0	14.0	36.0	22.0	8.0
31 15 Provides the synthesis of vitamins (B12, Folic acid).	4.0	8.0	32.0	24.0	16.0
31 16 Supports bone growth by increasing calcium absorption in the intestines.	3.0	4.0	27.0	31.0	18.0
31 17 Affects oral and dental health negatively.	34.0	29.0	10.0	5.0	5.0
31 18 Organizes intestinal functions in old age.	3.0	5.0	16.0	39.0	24.0
31 19 It is not safe to use probiotic-added foods in children.	15.0	38.0	16.0	9.0	7.0
31 20 The living organisms living in the intestines ensure that the microorganisms are in balance.	11.0	17.0	38.0	20.0	100.0

Probiotics are defined as live microorganisms that improve health by promoting health when they are taken in defined quantities. Many of these are obtained by fermentation of dairy products. *Lactobacillus* and *Bifidobacterium* are the most frequently found bacteria.[10]

The use of probiotic dairy products is increasing rapidly in developed countries. The widespread use of such products has great importance in terms of community health. Increasing consumption especially during childhood will contribute to healthier growth of new generations.

The main reason behind the wide use of prebiotics and probiotics is that many diseases are directly or indirectly related to the impairment of the balance of microbial flora and, that microbial flora controls this balance of prebiotics and probiotics. Particularly, if the side effects are negligible, the greatest advantages can be considered.[8]

The use of antibiotics, immunosuppressive agents and radiation for the treatment of infectious diseases can cause changes in the present balance by affecting the host flora. For this reason, the use of probiotics as an ecological method for the prevention and treatment of diseases has been an interesting research area for scientists.[11]

While the therapeutic effects and areas of the usage mentioned for probiotics, do not apply to all probiotics, the right microorganism and correct selection of strains is crucial so that the expected effect can be observed. [12] Therefore, it is very valuable to determine which indications the probioty is acting on, and more importantly, to select the optimum strain to obtain the maximum benefit, in other words, to select the ideal probiotic for each disease and disorder.[13]

Although they support human health with these positive effects, probiotics are not drugs taken to improve health. When the consumption of probiotic foods is stopped, the intestinal flora returns to its original state and the positive effect is lifted. For this reason, probiotics are only microorganisms that have positive effects when they are taken in the body regularly with probiotic foods. The positive health effects of probiotic foods can only be observed by cultivating acid-resistant probiotic bacteria in culture, using the cultures selected from pure cultures, and consuming the products for a long time without interruption.[3]

These foods need to meet certain conditions in order to be able to show desired effects. It should contribute to the nutrition of the individual, help to protect the health and bring it to a better state. At the same time, these characteristics should be based on good nutrition science and medicine. Again, appropriate daily intake quantities should be determined in terms of medical and nutritional knowledge. It should be proven that the consumption of food is reliable. Qualitative, quantitative, physicochemical properties of the components should be determined. If the nutrient is functionalized through processing; should not be lost in nutritional properties. Nutrition is rarely preferred, but it should be a food that is

often preferred to daily nutrition. Nutrient or any component should not have the ability to be used as a medicine. The functional component in foods should be resistant to digestion and should not show any health-affecting properties when taken over daily recommended amounts.

Much of the positive effects of prebiotics and probiotics studies have not yet been proven and the mechanism of action of proven efficacy has not been determined precisely. [14] Therefore, it may be useful to clarify the issues such as precise indications, mechanisms underlying the effect, determination of correct strains, selection of suitable host-microorganism-related prebiotic and, establishment of appropriate symbiotic combinations by making more randomized controlled studies related to this important topic.

The following table is important as a result of this thesis work.

Table 20. Measurement of knowledge of probiotic food consumption and probiotic products by participating students

Survey QUESTION	Parameter	% Yes
Questionnaire Question 12: Which disease (s) do you think probiotic food consumption might benefit?	12 1 Constipation	65.0
Questionnaire Question 14: If you have a diagnosed health problem:	14 4 Anemia	8.0
Questionnaire Question 16: What are your reasons / reasons for consuming probiotic food?	16 2 I see the benefits of digestive system	38.0
Questionnaire Question 17: Do you see the benefit of the probiotic foods you use?		53
Questionnaire Question 18: Do you read the packaging labels of the probiotic foods you bought?		45
Questionnaire Question 19: Do you know the microorganisms in the probiotic foods you consume?		30
Questionnaire Question 20: Are you proposing for the consumption of probiotic foods?		38
Questionnaire Question 21: Do you think that consumption of probiotic food contributes to the elimination of the following health problem / problems?	21 2 Digestive system problems	51.0
Questionnaire Question 22: What are your reasons / reasons for not consuming probiotic foods?	22 2 Not needing	24.0
Questionnaire Question 23: Consumption frequency of probiotic foods in students	23.1 Probiotic yogurt	26.0 (2-3 times a week)
Questionnaire Question 25: What is the reason for consuming a supplementary probiotic product?	25 1 Health problems	8.0
Questionnaire Question 26: Where did you hear about the supplementary probiotic products?	26 2 Friends, acquaintances, family etc.	11.0

Questionnaire Question 27: What are the criteria / criteria to consider when purchasing a supplementary probiotic product?	27 3 Contents	20.0
Questionnaire Question 28: Which one do you use as a supplementary probiotic product?	28 2 Enterogermina®	13.0
Questionnaire Question 29: Optimal use of probiotics	1-3 months	20
Questionnaire Question 30: Which do you see as a source of information on probiotics?	30 4 Through the health personnel	61.0
Questionnaire Question 31: How do you think reinforcing probiotic products have an impact on our health?	I agree + I absolutely agree	
	31 20 The living organisms living in the intestines ensure that the microorganisms are in balance.	20+100

In the context of the above table results, this study presents a summary of the cross-sectional results of the questionnaire on the measurement of knowledge of probiotic food consumption and probiotic products of participating students.

These results are of great importance in support of the literature on probiotic consciousness, relevance and use discussed above.

As a result, probiotic foods, which have numerous benefits in terms of health protection and positive development, do not attract as much interest in the consumer diet. The underlying reason for this is that probiotic foods are more expensive than those produced by conventional methods. Productivity to probiotic appetite and its addition to the diet are only due to the full knowledge of the positive effects of these foods on health. It should be considered that probiotic foods are not medicines and should not be discontinued when consumed, otherwise the intestinal flora will soon return to its former state.

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Formulation and In vivo Pharmacodynamics studies of Nanostructured Lipid Carriers for Topical Delivery of Bifonazole

Running head: *Nanostructured Lipid Carriers as vesicles for Topical Delivery of Bifonazole*

Aman Deep[1], Manish Kumar[1*], Rakesh Pahwa[2], Sumeet Gupta[1], Shailendra Bhatt[1], Beena Kumari[3], Prabhat Kumar Upadhyay[5], A. Pandurangan[1]

1 M.M College of Pharmacy, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala, Haryana, India

2 Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra-136119, India

3 Department of Pharmaceutical Sciences, Indira Gandhi University, Meerpur, Rewari, Haryana, India, Pharmaceutics, Rewari, India

4 Institute of Pharmaceutical Research, GLA University, Mathura, Uttar Pradesh,, Pharmaceutical Chemistry, Mathura, India

ABSTRACT

The main objective of the present study was to developed and evaluated the Bifonazole (BFZ) loaded Nanostructured lipid carriers (NLCs) for topical delivery of BFZ. BFZ-NLCs were deve560ted for Particle size, EE, DL and drug release profiles. The *in-vitro* release studies show better drug release over 24h as compared to the marketed formulation. Ex-vivo skin permeation and Pharmacodynamic studies indicated that NLCs get effectively reduced the fungal infection. *In-vitro* antifungal activity study shows that the BFZ-NLCs were more effective in inhibiting the growth of Candida Albicans. Therefore, the study concludes that NLCs showed a continuous release profile and has the prospective for treatment of topical fungal infections.

Corresponding author:
M.M College of Pharmacy, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala, Haryana, India
Telephone number: 07017548594
E-mail address: manish_singh17@rediffmail.com
ORCID
Deep Aman 0000-0002-1103-4228,
Manish Kumar 0000-0001-9405-3311,
Rakesh Pahwa 0000-0002-4399-328X,
Beena Kumari 0000-0002-6333-5081,
Prabhat Upadhyay 0000-0001-6825-7126,
Shailendra Bhatt 0000-0001-9405-3311,
Pandurangan A 0000-0002-0379-9853
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INTRODUCTION

In the recent scenario, fungal infections are widely spread all over the world. Billions of people are treated every year with either topical or severe systemic fungal infections[1]. Although, antifungal drugs are effective in treating fungal infections but they are associated with severe toxicities like liver damage or they may affect estrogen levels or may cause allergic reactions. For example, the antifungals with azole groups are known to have caused anaphylaxis.

BFZ is an azole derivative and active against the fungal infections such as moulds, yeasts, dermatophytes and Gram-positive bacteria. BFZ is mainly preferred for the treatment of topical infections as like tinea pedis, cutaneous candidiasis. And it is virtually insoluble in water with 1-2 h of half-life[2,3].

Mycelium fungi can deeply penetrate the skin layers and causes the fungal infection. To overcome this problem, improvement in the activity of the active agent for the antifungal treatment is required[4]. Thus, the NLCs based gel was developed for the deeper penetration and retention of the active agents throughout the skin layers. Hence, the purpose of the recent study was to develop the NLCs based gel of BFZ for the fungal infection ultimately increases the effectiveness of the drug.

Nowadays lipids used in the novel drug delivery system. Because lipids plays a important role in improving the bioavailability and enhances the solubility of the lipophilic drugs[5]. NLCs are the advanced form of the nanoparticles. Hence, they overcome the limitations over the conventional formulations and SLNs[6,7]. Previously, SLNs concerned lot of consideration as a drug delivery system[8]. Because they help to improves the bioavailability and increases the solubility of poorly water soluble drugs[9]. However, depending on the drug, a variety of possible problems can occur, such as insufficient drug loading and drug leaking during storage. Later on, NLCs are developed and designed by mixing the solid lipid with the liquid lipid, which leads to exceptional nanostructures with enhanced properties for remedial loading, modification of the drug release profile and stability[10,11].

NLCs are the advanced form of the nanoparticles and have the reason that they formed a less ordered lipid matrix with many imperfections has the capacity to integrate big quantities of drugs[12]. The primary site of action of Terbinafine is the stratum corneum in fungal infections residing superficially on the skin layers[13,14].

In this context, topical NLC may prove to be a potential option for increasing the concentration of drug by controlled targeting up to deeper skin layers. SLNs

involve the incorporation of the solid lipid whereas NLCs involve the drug entrapment into the solid and liquid lipid mix which may contribute to make it a sustained release formulation and thereby overcoming the limitation of SLNs. Topical NLC formulation aims to reach the target site with required concentration to achieve its therapeutic action with minimal adverse effects[15,16].

Therefore NLCs, the newer generation of SLNs were chosen to overcome these limitations and provide better therapeutic prospects. As a result, the objective of the present study was to prepare and optimize. BFZ loaded NLC for topical administration and prepared formulations for *in vitro* release, *ex vivo* permeation and *in vivo* (Pharmacodynamics) studies which may be found to be more effective than the SLNs developed before.

METHODOLOGY

Materials

BFZ was received as a gift sample from Vital Laboratories Private Limited (Gujarat, India). Stearic acid and Castor oil was obtained from Central Drug House Ltd. Vardaan House, Daryaganj, New Delhi (India). Tween80 was obtained from Central Drug House Ltd. Vardaan House, Daryagan (New Delhi, India). Sodium hydroxide pellets, HCL (Concentrated), Methanol AR were obtained from Qualikems Fine Chemicals (Mumbai, India). All reagents and solvents used were of systematic rank.

Experimental design of BFZ loaded NLCs

A 3^2 full factorial was applied to design the experiments. Polymer ratio and Castor oil were used as independent variables, whereas particle size, percentage entrapment efficiency and percentage Drug loading were kept as dependent variables. Formulations F1 to F9 were prepared using three different levels of lipid ratio and surfactant and the response parameters were evaluated (Table 1).

Selection of lipids:

The semi quantitative method was used to check the solubility of BFZ in the various solid lipids[17].the predetermined amount of the drug was precisely weighed in the series of test tubes. Different lipids were included increasing sum to particular test tubes and warmed till medication is absolutely solubilized. The temperature of test cylinders was kept up 10°C over the softening purpose of lipid utilized and shaken irregularly to break down the medication. Test tubes were watched outwardly for any medication buildup. The measure of lipid required for solubilizing fixed measure of medication was resolved[18,19].

Selection of solid lipids

The semi quantitative method was used to check the solubility of BFZ in the various solid lipids[20]. The predetermined amount of the drug was precisely weighed in the series of test tubes. Different lipids were included increasing sum to particular test tubes and warmed till medication is absolutely solubilized. The temperature of test cylinders was kept up 10°C over the softening purpose of lipid utilized and shaken irregularly to break down the medication. Test tubes were watched outwardly for any medication buildup. The measure of lipid required for solubilizing fixed measure of medication was resolved[21].

Selection of liquid lipid (oils) and surfactants

The solubility of BFZ in various liquid lipids (Castor oil, Oleic acid, Isopropyl myristate, Cremophore EL) and surfactants (Tween 20, Tween 80, Span 80 and Pluronic F 127) was determined by adding excess amounts of drug in 3 ml of oils in small vials. The vials were tightly stoppered and were continuously stirred to reach equilibrium for 72 h at 25^{0}C in a mechanical shaker. After that, High Speed Centrifuge (3K30, SIGMA, Germany) were used to centrifuge the mixtures at 15000 rpm for 45 min at 37^{0}C[22]. The upper layer was separated out and solubility was determined by UV Spectrophotometer at 254nm. The solubility studies were done in triplicate and results reported as ±SD.

Selection of binary lipid phase

The solid and liquid lipid with the best-solubilizing potential for BFZ were mixed in different ratios viz., 95:5, 90:10, 85:15, 80:20, 70:30, and 60:40 in array to found the miscibility of the two lipids. Lipid mixtures were agitated at 200 rpm for 1 h at 85^{0}C using a magnetic stirrer (Remi instruments Ltd., Mumbai, India). Smearing a cooled sample of the solid mixture onto a filter paper was used to find out the miscibility between the two components, the visual observation is used to determine the presence of any liquid oil droplets on the filter paper. The binary mixture who shows the melting point above 40^{0}C which did not expose the presence of oil droplets on the filter paper was selected for the development of BFZ – loaded NLCs[23].

Preparation of NLCs

The weighed amount of drug was added to the lipid phase which was heated at 10–15^{0}C above the melting point of solid lipid and simultaneously, aqueous surfactant solution was heated at the same temperature (85^{0}C). Then the lipid mixture was poured in the hot aqueous surfactant solution using a magnetic stirrer (Remi instruments Ltd., Mumbai, India) at 12,000 rpm for 30 min, to prepare the primary emulsion[24].

This primary emulsion was converted to the NLC system using high pressure homogenizer (Stansted Fluid Power Ltd., Harlow, UK) at 15000 PSI. The obtained NLC dispersion was cooled down to room temperature. The NLC dispersion was lyophilized for long term stability. Mannitol (5% w/v) was added as cryoprotectant. The samples were frozen at -78°C for 10 h followed by lyophilization for 36 h. The lyophilized formulation was reconstituted with phosphate buffer pH 6.8 as per the requirements for later experiments.

Evaluation and Characterization of BFZ loaded NLCs

Particle size analysis

The particle size analysis of NLC formulations was done by photon correlation spectroscopy (PCS) with a Zetasizer (Malvern Instruments, Worcestershire, UK). The PCS provides the mean particle size (z-average).

Entrapment Efficiency (EE) and Drug-loading capacity (DL)

For EE and DL, the drug-loaded NLC dispersion was uniformly mixed by gentle shaking. 1.0 ml of this dispersion was diluted with 9.0 ml methanol, centrifuged using High-Speed Refrigerated Centrifuge (3K30, SIGMA, Germany) for 45 min at 15,000 rpm and then filtered using Millipore membrane (0.2 µL). The analytical method employed was as per the method reported[25]. Hence, UV absorption spectra of stock solution (10 µg/mL) were scanned for absorbance in the region of 400–200 nm at 254 nm. Serial dilutions of standard solutions were prepared and absorbance was recorded at 254 nm. The calibration curve was prepared and the method was validated. The filtrate was collected and appropriately diluted with methanol and measured spectrophotometrically (Shimadzu, model UV-1601, Kyoto, Japan) at λ_{max} of 254 nm. The percent entrapment efficiency (EE %) was calculated using the following equation[26].

$$\% \text{ EE} = \frac{\text{W (Total)} \times \text{W (Free)}}{\text{W (Total)}} \text{ x 100}$$

$$\% \text{ DL} = \frac{\text{W (Total)} \times \text{W (Free)}}{\text{W (Lipid)}} \text{ x 100}$$

W_{total} = the weight of drug

W_{free} = weight of drug in supernatant

W_{lipids} = weight of lipid

Evaluation of BFZ loaded NLC

***In vitro* drug release study**

In vitro drug release studies of NLCs were performed using dialysis bag technique. The activation of dialysis membrane was carried out. The experiments were carried out under sink conditions. 10 mg of each formulation i.e., (F1-F11) was loaded into a cellulose membrane dialysis bag immersed in 200 mL of pH 6.8 phosphate buffer containing 0.8% tween80 solution magnetically stirred at 32^{0}C at pH 6.8. Samples were taken at predetermined intervals from the receiver solution, replaced with equal volumes of fresh solvent, and spectrometrically assayed for drug concentration at λ_{max} 254 nm. The correction for the cumulative dilution was calculated. The release studies were performed in triplicate[27,28].

Differential scanning Calorimetry (DSC) study

Drug lipid interaction in NLCs formulations and crystallinity of drug was analyzed by performing DSC analysis. Samples were analyzed using SII Nanotechnology EXSTAR DSC 6220 in scanning range of 30-300°C at a heating rate of 10°C/min. Plain drug, lipid, Drug-lipid physical mixture and NLCs formulation DSC scans were recorded and compared.

Transmission electron microscopy (TEM)

TEM studies were determined for the NLCs using TEM (TECNAI-G2, 200 kV, HR-TEM, FEI, The Netherlands). A drop of NLC was placed on a paraffin sheet and carbon coated grid was put on sample and left for 1 min to allow NLCs to adhere on the carbon substrate. The remaining NLC was removed by adsorbing the drop with the corner of a piece of filter paper. Then the grid was placed on the drop of phosphotungstate (1%) for 10 s. The remaining solution was removed by absorbing the liquid with a piece of filter paper and samples were air dried and examined by TEM.

Preparation of BFZ loaded NLCs Based Gels

The gels were prepared by dispersing 1% w/w Carbopol 940 in the selected NLCs formulations and subsequently neutralizing the Carbopol_ dispersion using triethanolamine (TEA). The final concentrations of BFZ in the NLCs gels were maintained at 0.5, 1 and 1.5% w/w and were coded as G1, G2 and G3 respectively.

Evaluation of NLCs based gel:

Viscosity

Brookfield viscometer (Brookfield engineering laboratories, Inc., MA, USA) was used to determined the viscosity of the optimized NLCs gel formulation (Brook-

field engineering laboratories, Inc., MA, USA) with spindle No. 62 at 10 rpm at temperature of 37 ± 0.5^{0}C.

Determination of pH

Weighed quantity (1gm) of the NLCs preparation was taken and put in the 100 mL volumetric flask and made up the volume up to 50ml with distilled water (0.2% strength). The pH was determined using pH meter (pH Tutor Bench Meter, EUTECH Instruments, Singapore).

Spreadability

Glass plate was taken and marked a circle of 1cm diameter. Then 0.5g of NLCs gel was placed within a circle, over which another glass plate was placed. Half kg of weight was placed over the glass plate for 5-10 min. gel spreading diameter was noted and compare with the earlier one.

***Ex vivo* permeation studies**

Ex vivo study was carried out using full thickness rat abdominal skin. In this work due to easy availability, the skin of albino rat was used. The species used was Wistar Albino Rats of 18–25 weeks and weight of 150–200 g of either sex. The abdominal skin was removed and dipped into phosphate buffer saline (PBS) pH 7.4. Hairs were removed from the skin by hair removal cream. The subcutaneous fat was removed with a scalpel. The skin was mounted on the Franz diffusion cell and the receptor chamber was filled with 20 ml diffusion medium. The dispersion medium comprised of PBS pH 6.8 containing 0.8% v/v of Tween-80. The skin was situated on the receptor chamber with the stratum corneum facing upward in the receptor chamber and after that the donor chamber was clipped set up. The abundance skin was cut off and the entire get together was put on a magnetic stirrer to consistently mix the medium present in the receptor compartment. The distribution cell was set in the dispersion mechanical assembly to settle at 32^{0}C. The test formulations (Drug dispersion, Marketed formulation, optimized formulation, optimized gel formulation i.e., 1% cream) equivalent to 20 mg drug were applied to the skin. Samples were withdrawn from the receptor compartment at predetermined time intervals, and immediately replaced with fresh diffusion medium. The studies were performed for 24 h according to the clinical application time and samples were analyzed spectrophotometrically at λ_{max} 254 nm. Drug permeation, Flux (μg/cm^2/h) and permeability coefficient (Pb) [cm^{-2}h^{-1}] studies were calculated using the formulas mentioned[29,30].

In vitro antifungal activity

In vitro antifungal activity of the optimized formulation was determined by using cup plate method. In this method, firstly we prepared the dextrose agar media. Then sterilization was done by autoclaving and the sterilized media was transferred into the Petri plate for solidify[31]. After that inoculation of the fungal suspension was done in solidified media. The borer was used for the bore well must be Presterilized (equivalent to McFarland standard no. 0.5). Presterilized stainless steel borer was used to bore wells in the media[32]. NLCs based gel; marketed formulation and BFZ STD solution were placed in respective bored well. Petri plates were allowed to incubate for growth of fungi and zone of inhibition was measured after incubation period[33].

In vivo Pharmacodynamics

The *in vivo* Pharmacodynamics was performed according to the guidelines provided by institutional animal ethics committee (IAEC) (MMCP-IAEC-17).

According to the procedure required 12 male albino rats (weight 170-200gm) for Pharmacodynamic study. Proper diet and water was given for 24-48 h. Then the animals were divided into four different groups and each group contains three rats. Group 1 is control (untreated rats), groups 2, 3 and 4 consisted of rats induced with fungal infection and treated with Bifonazole loaded NLCs based Carbopol 940 gel, commercial Bifonazole cream (commercial formulation i.e., 1% cream) and BFZ dispersion in water, respectively[34]. All sterilized materials were used.

Preparation of fungal inoculums

Candida albicans culture was obtained from Microbiology Department, MMIMS, Mullana, Ambala, India. The Candida albicans was subculture and allow for 24 hr growing at 25^{0}C and Adjusted the conidial suspension density to 1 $\times$ 10^6 CFU/ml by hemocytometer and it was used for the inoculum[35].

Orientation of cutaneous candidiasis infection

Firstly, 2 cm area on the back of the rat were make hairless by using hair removal cream and make a hairless square. On the same day, sand paper was used to abrade the skin. Ethyl alcohol was used to disinfect the skin and 0.2ml conidial suspension was adhered to the skin by using sterile cotton and kept for 2-3 days[35,36]. Then the infected tissues of the skin was excite to the Sabouraud dextrose agar media with the help of sterile scalpel (at 25^{0}C for 2 days) [36]. Appropriate dilution was done to accomplish countable colony forming unit (CFU) development. CFU was checked utilizing a computerized colony forming dependent on countable CFU values. This was preceded till acceptance of contagious

disease was affirmed in view of the quantity of CFUs.

Treatment

BFZ loaded NLC based Carbopol 940 gel, BFZ dispersion and commercial formulation i.e., 2% cream (equivalent to 4 mg drug on daily basis) were applied topically and the results in terms of reduction in fungal burden were compared. Treatment was started after confirmation of induction of fungal infection by counting the number of CFUs. After initiation of treatment, quantitative analysis of fungal burden was performed by the above mentioned procedure.

RESULTS AND DISCUSSION

Selection of components

The criteria for selection of excipients for developing BFZ-NLC include pharmaceutical acceptability, non-irritant and non-sensitizing to the skin and that they fall under GRAS (generally regarded as safe) category. As per the results of solubility studies, BFZ exhibited maximum solubility in Stearic acid, Castor oil and Tween 80. Therefore, BFZ-NLC was prepared using Stearic acid as solid lipid, Castor oil as liquid lipid and Tween 80 as surfactant. Based on the visual observation of smear test, dual lipid phase was selected in the ratio 1:1 w/w (solid: liquid lipid ratio) for designing NLC.

Particle size

The particle size of the optimized NLCs was found to be in the nanometric size range (160.4 nm) with low polydispersity index (0.338 ± 0.16) (figure 1.). it was observed the particle size decreased as the concentration of the liquid oily phase was increased. Furthermore. The increased concentration of the surfactant also influences the particle size of the Preparation.

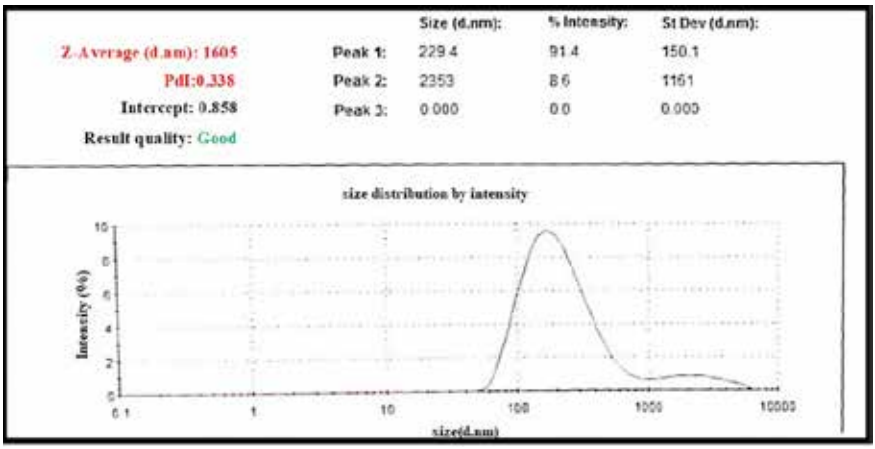

Figure 1: Particle size of optimized BFZ loaded NLCs formulation.

Entrapment efficiency and drug loading

The EE and DL was found to be 98.17 ± .69 and 19.6 ± 1.23. The EE and DL of the optimized BFZ-NLCs was Having the capability of lipid to integrate the drug and surfactant into the matrix. The solid lipid matrix encloses tiny oil section in which drug solubility is higher which increases their total drug loading capacity. And liquid lipid also affects the entrapment efficiency and helps in the loading of larger amount of drug (table 2).

Optimization of BFZ loaded NLCs

The 3^2 factorial design was used to study the responses for all formulations on the basis of variables. The responses observed for all formulations were Particle size (Y_1), Entrapment Efficiency (Y_2) and Drug Loading (Y_3). Table 2 shows the experimental design of Stearic acid and castor oil nanoparticles and the results of measured responses. The effect of the combination of polymer and surfactant (Tween 80) on entrapment efficiency and drug loading was studied using the response surface plot and the results of the response surface plot are given in Figure 2 (a, b, c). Based on the results obtained in preliminary experiments, the amount of Stearic acid and castor oil (X_1) and concentration of Surfactant (X_2) were found to be major variables affecting the Particle size (Y_1), Entrapment efficiency (Y_2) and % Drug loading (Y_3) of the nanoparticles. In case of particle size, the results showed that an increase in PS due to an increase in the polymer concentration and a decrease in the volume of organic phase. In case of drug entrapment efficiency, the results indicate that an increase in drug entrapment due to an increase in polymer concentration and a decrease in the solvent.

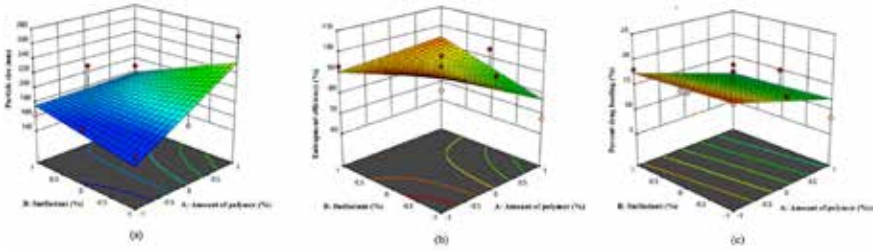

Figure 2: Response surface plots of factors (particle size, entrapment efficiency, drug loading

Response surface plots

Three-dimensional response surface plots generated by the Design Expert are presented in Fig 2(a) for bifonazole nanoparticles. Fig 2(**a**) depicts the response surface plots for the particle size of bifonazole nanoparticles which show an increase in PS due to an increase in the lipid concentration and a decrease in the volume of the aqueous phase[26,28]. Fig 2(b) depicts the response surface plot for Drug entrapment efficiency which indicates an increase in drug entrapment due to an increase in lipid concentration. Fig 2(c) shows due to the concentration of lipids and surfactant the drug loading capacity was increased.

Quadratic model was found to be significant for particle size, entrapment efficiency and drug loading.

Data Analysis

The data generated by evaluation of the formulations were subjected to statistical analysis using 3^2 full factorial designs with the help of design expert software version 9.0.5 (state-Ease, Inc., Minneapolis, USA). A statiscal model incorporating interactive and polynomial terms was used the evaluated the responses.

$$Y=b_0+b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_1^2+b_{22}X_2^2$$

Where Y is the independent variable. Where *b*0, the intercept is the arithmetic mean of the main effects (regression coefficients) b1, b2, b3, b12, b13, b23 and b123 were calculated by use of signs in the columns, by adding or subtracting the value of the obtained responses, *Y*. Finally, the values are summed up and divided with the number of formulations. Where X_1 and X_2 are the coded levels of the autonomous factors and X_1X_2 are the interaction and polynomial terms, respectively.

Based on the information acquired from the streamlined details, a general factual model can be depicted as for the above information. The model created can be

described by utilizing the polynomial condition speaking to the separate reaction information. This can be given as takes after:

Particle Size = $184.70+22.17X_1 - 7.33X_2 - 20.50\ X_1\ X_2$

Entrapment efficiency = $91.40 - 5164X_1 + 1.07\ X_2 + 7.53X_1X_2$

Drug Loading = $14.83 - 3.01X_1 - 0.4383X_2$

From the above polynomial equations, 3D response surface graphs were generated, which were used to predict the responses of dependent variables at the intermediate levels of independent variables. Three dimensional response surface plots generated.

Evaluation of NLCs

***In vitro* drug release study**

The in vitro drug release profiles (fig. 3) were used to determine % cumulative drug release (CDR) varied widely b/w 56.38±2.26 – 92.5±1.18%. This variability was observed due to variation in particle size among all the ten formulations.

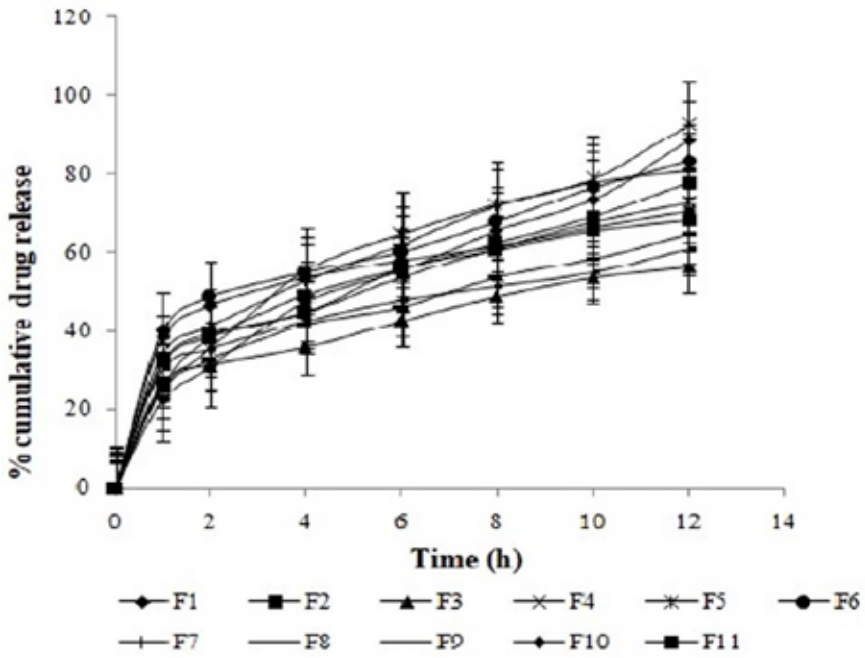

Figure 3: *In vitro* release of BFZ loaded NLCs formulations (F1-F11).

Formulation F5, F6 & F7 exhibited more than 80% CDR and among these F5 displayed maximum CDR of 92.5 ± 1.18% due to small sized particles and optimum entrapment efficiency NLCs developed from Span 80 (F1-F4) showed show & incomplete CDR OF (56.38 ± 2.26 – 79.35 ± 1.32) and when compared to F5 – F7 made with Tween 80 (81.2 ± 2.1 – 92.5 ± 1.18). The determinants of this variability may be attributed to particle size and PDI that was in turn affected by type of copolymer used (Harada et al., 2011). Thus F8 – F11 prepared with PF127 produced large sized Nanostructured lipid carriers. This led to incomplete drug release which was varied from (61.1 ± 2.12 – 70.56 ± 1.82 %).

Differential Scanning Calorimetry (DSC)

The DSC thermogram shows the disappearance of drug peak in the optimized formulation (F5) as shown in Fig 4. It means drug is fully enclosed inside the lyophilized drug-loaded NLCs.

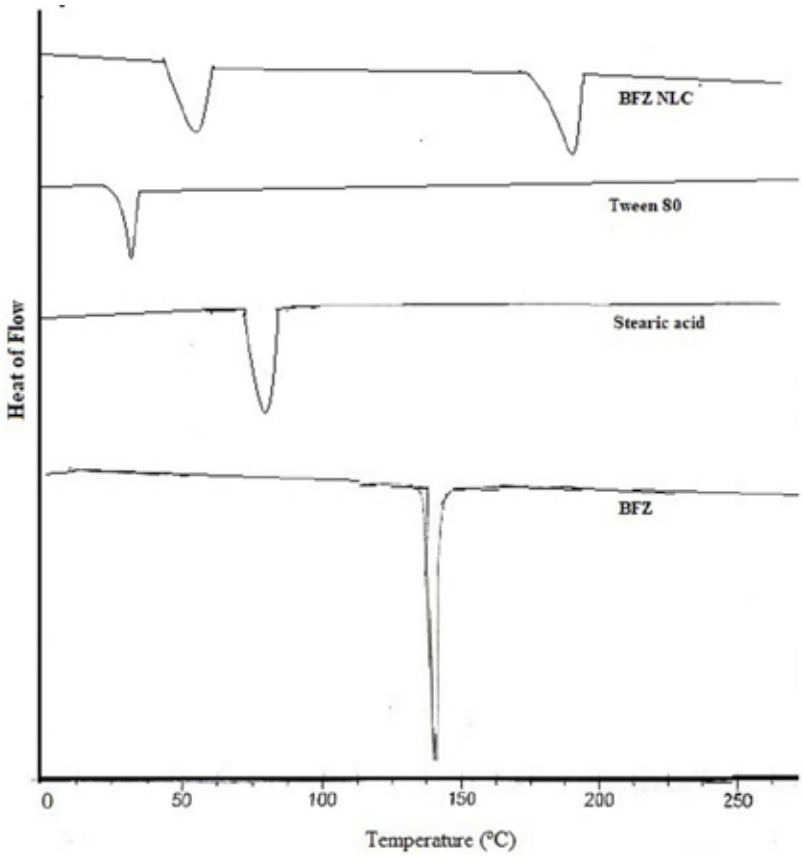

Figure 4: DSC curve of drug, lipids and surfactant.

Transmission electron microscopy (TEM)

The TEM studies were carried out to get more insights into the morphology of the NLCs systems. From the study it was observed, that after loading the drug into placebo, the particle size of the formulation increased. This might be due to the accommodation of the drug in sufficient space in the lipid matrix. The TEM images (Fig 5) show the drug enclosed in the lipid matrix. The TEM images of BFZ-NLCs show uniform size distribution of lipid nanoparticles having coarsely spherical shape. The uniformity in particle size distribution correlates with the small PDI (0.38) obtained via photon correlation spectroscopy. The particle size after the TEM study was found to be in the range of 160–500 nm.

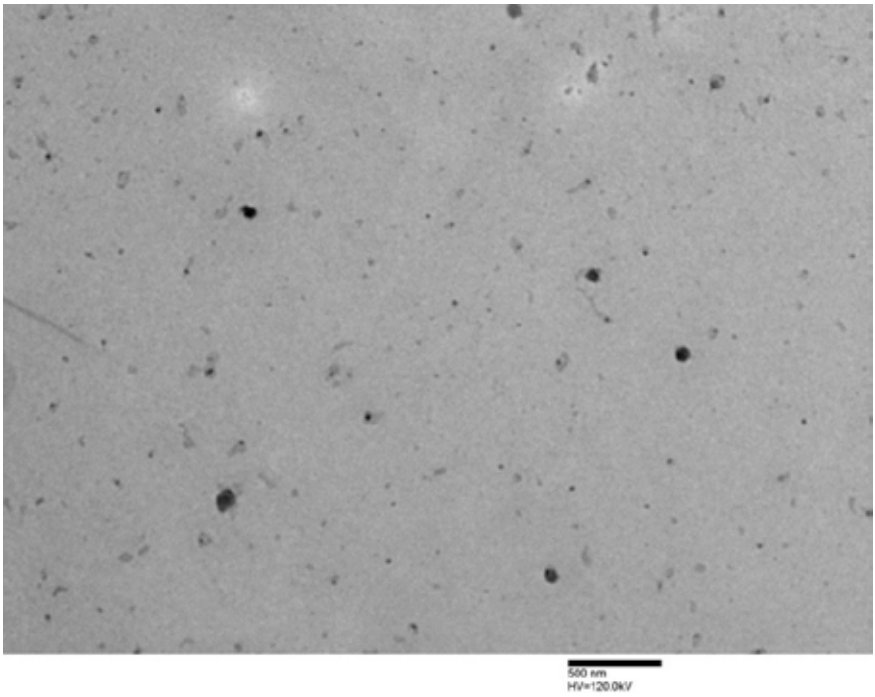

Figure 5: TEM image of optimized formulation.

Preparation of BFZ – NLCs based gel

Carbopol 940 in various concentrations 1%, 1.5%, and 2% was used to formulate the BFZ-NLCs into the gel. Gel (1.5%) was found to be suitable for gelling the NLC because of desirable consistency.

Viscosity

Brookfield viscometer was used to determine the viscosity of the optimized NLCs gel formulation. The viscosity was found to be 593 ± 0.98 cps.

Determination of pH

pH Meter is used to determine the pH of the optimized NLCs Gel formulation in triplicate at 26^{0}C. and the pH was found to be 5.6 ± 0.07. The pH of the NLC-loaded gel was within the acceptable range for topical formulations and compatible with the pH of the skin.

Spreadability

The ideal gelling formulation is readily spread on the site of application. The increased diameter due to the spreading of test gel formulation is found to be 6.5 ± 0.05 cm. the obtained value shows the good Spreadability of the test formulation and which is essential for the topical delivery

Ex vivo permeation studies

The aim if the recent study was determined the permeation of drug through the skin with controlled release effect. Drug loaded Nanostructured lipid carriers can easily penetrate the skin layers. *Ex vivo* permeation studies was performed for drug dispersion, marketed formulation, optimized formulation (F5) and gel formulation (G5) as shown in Fig 6.

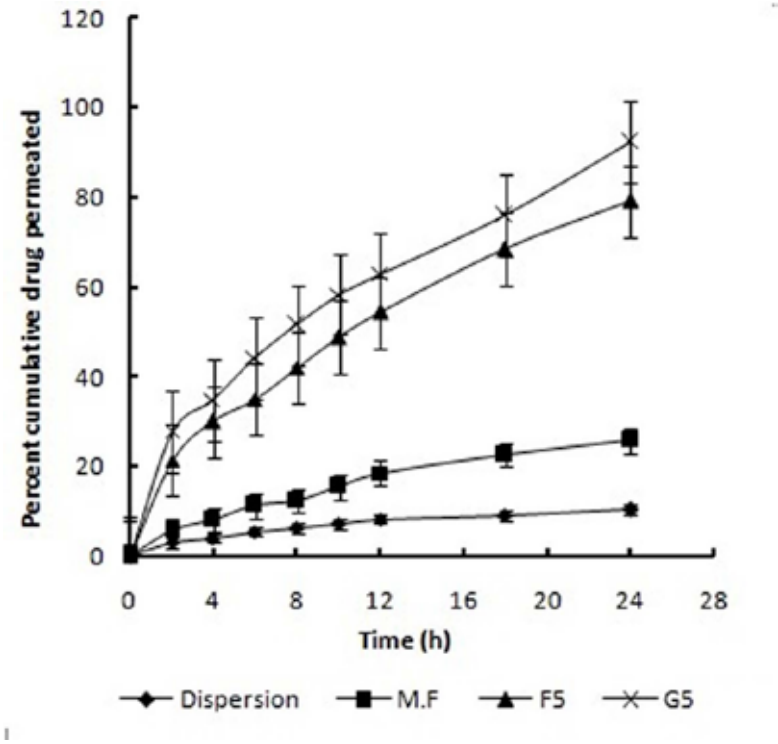

Figure 6: Comparative ex-vivo permeation of BFZ loaded optimized NLC (F5), Dispersion, M.F and G5.

The drug was dispersed within the lipid matrix which was further incorporated into Carbopol gel which adhered to the skin and increases the contact time. NLCs based gel and optimized formulation shows their skin targeting ability. This is desirable for the topical application. NLCs incorporated into gel may induce structural change of particle structure due to evaporation of water resulting in the transition of lipid matrix into a highly ordered structure causing drug expulsion[34]. From this, it could be concluded that NLCs may play an important role in controlling the release of TH from NLCs as well as targeting of drug to the skin.

In *vitro* antifungal activity

The mean zone inhibition value of the NLCs gel was bigger than the marketed formulation but less than the BFZ standard solution.

NLCs based gel having the higher antifungal activity as compared to the market-

ed formulation. This is due to the higher solubility of BFZ which helps to dipper penetration of BFZ loaded NLCs through the skin layer and inhibit the ergosterol synthesis[35] (Table 4).

In vivo Pharmacodynamic

Fungal burden was quantitatively analyzed in terms of colony forming units (CFUs) after initiation of the treatment. CFUs were counted using a colony counter (Microbiology lab, MMIMRS, Mullana, Ambala, India) (Table 5) gives the quantitative analysis of fungal burden.

Control group did not show any growth, as infection was not induced to this group. Group treated with NLCs showed a significant decrease in fungal burden after 5 days (671 ± 40.675 CFUs) (p value < 0.001) as compared to CFUs before initiation of treatment (2,55,000 ± 3.505.551CFUs) (p value < 0.001). Group treated with marketed formulation also showed a significant decrease in fungal burden after 5 days (1674 ± 154.65CFUs) (p value < 0.001), but it was higher as compared to the group treated with developed formulation. Also, the TH dispersion in water showed an initial reduction in fungal burden after which it almost came to a

Steady state[36,37]. These results showed that the NLC reduced the fungal burden in a shorter duration of time as compared to marketed formulation and dispersion. Thus, TH was found to be more effective when formulated as NLC based gel because of improved contact, adhesion, occlusion and sustained release.

BFZ loaded NLCs was successfully produced by high pressure homogenization technique using Stearic acid as solid lipid, Castor oil as liquid lipid and Tween 80 as surfactant. Therefore, BFZ loaded NLCs were capable of treating the fungal infection. And it can be concluded that the use of NLCs was far better than the conventional creams/gels.

TABLES

Table 1: 3^2 full factorial design layout for preparation of NLCs of BFZ

Formulation Code	Bifonazole (mg)	Lipid ratio (Castor oil: Stearic acid) (% W/W) X_1	Surfactant (% W/V) X_2	Dependent variables
F1	200	1:1 (-1)	-1 (1%)	
F2	200	1:1 (-1)	+1 (3%)	$Y_{1=}$ Particle size
F3	200	4:1 (1)	-1 (1%)	$Y_{2=}$ % entrapment efficiency
F4	200	1:1 (-1)	0 (2%)	Y_3=Drug loading
F5	200	4:1 (1)	+1 (3%)	
F6	200	2:1 (0)	0 (2%)	
F7	200	2:1 (-1)	-1 (1%)	
F8	200	4:1 (1)	0 (2%)	
F9	200	2:1 (0)	+1 (3%)	
*F10	200	1.5:0.5	(1.5%)	
*F11	200	2.5:1.5	(2.5%)	

Table 2: Results of 3^2 factorial design of BFZ loaded nanoparticles

Formulation Code.	Lipid ratio (%) X_1	Surfactant (%) X_2	Particle size (nm) Y_1	Entrapment efficiency (%) Y_2	Drug loading (%) Y_3
F1	1:1 (-1)	1 (-1)	162.50	98.02 ±1.00	18.70 ±1.25
F2	1:1 (-1)	3 (+1)	174.20	96.30 ±1.50	16.50 ±0.58
F3	4:1 (+1)	1 (-1)	270.62	67.60 ±1.23	08.38 ±2.36
F4	1:1 (-1)	2 (0)	169.20	97.78 ±1.12	17.70 ±3.20
F5	4:1 (+1)	3 (+1)	160.40	98.17 ±0.69	19.6 ±1.23
F6	2:1 (0)	2 (0)	168.30	94.65 ±0.98	15.04 ±2.14
F7	2:1 (0)	1 (-1)	163.40	93.06 ±1.02	18.90 ±1.23
F8	4:1 (+1)	2 (0)	210.30	82.54 ±1.62	10.07 ±2.75
F9	2:1 (0)	3 (-1)	189.60	92.76 ±1.71	11.75 ±3.12
***F10**	1.5:0.5	1.5	182.70	93.16 ±0.98	14.67 ±1.29
***F11**	2.5:1.5	2.5	184.60	94.31 ±0.56	15.45 ±1.87

Table 3: *In vitro* release profile of BFZ loaded NLCs

Time (h)	Formulation code										
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
0	0	0	0	0	0	0	0	0	0	0	0
1	22.31	26.45	26.90	31.70	25.53	40.40	34.60	30.40	25.30	38.34	32.34
2	30.74	38.22	31.40	38.30	35.45	48.50	41.30	35.40	32.70	46.43	39.90
4	47.56	49.30	35.61	44.60	55.43	54.94	52.78	42.56	41.60	53.40	44.60
6	55.34	55.60	42.32	53.76	64.65	60.23	61.90	47.60	45.90	57.90	55.90
8	65.40	62.34	48.80	61.20	72.30	67.80	71.68	51.30	53.70	61.60	60.80
10	73.42	69.21	53.60	67.56	78.6	76.54	77.90	54.80	58.34	66.40	65.30
12	79.35	78.12	56.38	72.62	92.50	83.40	81.20	61.10	64.80	70.56	68.34

Table 4: Comparison of NLCs based gel, marketed formulation and Standard drug for antifungal activity

S. no	Formulation	Mean zone of inhibition in cm (n=3) (Mean ± SD)
1	BFZ Standard	1.45 ± 0.12
2	NLCs based gel	1.29 ± 0.09
3	Marketed formulation	1.15 ± 0.87

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COMPLIANCE WITH ETHICAL STANDARDS

For this study, prior clearance from an institutional animal ethics committee (approval number MMCP-IAEC-17) was obtained.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors alone are responsible for content and writing of the paper.

DISCLAIMER

The authors alone are responsible for the content and writing of the paper.

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Thiol Functionalization of *Sesbania* Gum and Its Evaluation for Mucoadhesive Sustained Drug Delivery

Shakuntla Verma[1], Munish Ahuja[1*]

Drug Delivery Research Laboratory, Department of Pharmceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar-125001(Haryana)

ABSTRACT

The objective of present study was to improve muco-adhesiveness of *sesbania* gum by thiol functionalization. Thiolated *sesbania* gum was synthesized by reacting *sesbania* gum with mercaptoacetic acid in the presence of catalytic amount of acids. The modified gum was characterized physic-chemically and for biocompatibility. Thiolated *sesbania* gum was tested as mucoadhesive polymer for pharmaceutical applications by formulating its composite beads in the sodium alginate using metformin as a model drug. Thiolation onto *sesbania* gum was confirmed by Fourier transform infrared spectroscopy and energy dispersive X-ray –scanning electron micrographs. The degree of thiol substitution was found to be 1.72mmol/gm. The results of thrombogenic and haemolytic potential studies confirmed the biocompatibility of Thiolated *sesbania* gum. The comparative evaluation of composite beads of thiolated *sesbania* gum with *sesbania* gum and alginate alone beads revealed that thiolation of *sesbania* beads improves the bioadhesion property of *sesbania* gum.

Keywords: *Sesbania* gum, thiolation, bead, biocompatible, mucoadhesive

INTRODUCTION

A group of naturally occurring neutral polysaccharides i.e. galactomannans are most abundant raw material for industrial and pharmaceutical application due to easy availability, biodegradability, sustainability and non-toxic characteristics[1].

Corresponding author:
Dr MunishAhuja,
Drug Delivery Research Laboratory Department of Pharmaceutical Sciences, G.J. University of Science and Technology, Hisar,
Telephone number: +91-1662-263515
Fax: +91-1662-276240
E-mail address: munishahuja17@yahoo.co.in
ORCID
Munish Ahuja 0000-0001-6723-140X,
Shakuntla Verma 0000-0001-7683-7067.
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Galactomannans are composed of linear β– (1 ⟶ 4) glycosidic linked mannan backbone with α-galactose side chain residues at C-6 of mannose[2]. The various properties of galactomannans including molecular weight, mannose: galactose ratio (M:G) and attachment of galactose side chain residue on mannan backbone are believed to be responsible for their different rheological and physicochemical properties. Despite of various potential advantages of natural galactomannans, they owned certain limitations including less microbial stability and biodegradability. In order to overcome these problems and to enhance their utility, galactomannans are chemically modified via thiolation, carboxymethylation, microwave assisted grafting and many more. Thiolated polymers designated as thiomers havethiol group bearing side chain along the polymeric backbone[3-5]. These thiol groups are able to form disulfide linkage with mucosal glycoproteins resulting in higher mucoadhesiveness which further improve the therapeutic efficacy of the drug delivery system[6]. Numerous studies conducted earlier reported that thiolation modification of natural polysaccharide such as xanthan gum[7], gellan gum[8], pectin[9], tamarind seed polysaccharide[10], Psyllium husk[11], chitosan[12], alginate[13] and hyaluronic acid[14] improved their mucoadhesive properties.

Sesbania gum,a seed galactomannan, belongs to genus *Sesbania* and family *Faboideae*. The M:G ratio and average molecular weight of *Sesbania* gum is 2:1 and 2.3-3.4×105Da, respectively[15]. The gum form highly viscous suspension in aqueous system. *Sesbania* gum have been explored as diclofenac sodium loaded topical gelling agent[16] and for colon targeting drug delivery of metronidazole[17-18]. Carboxymethyl functionalized *sesbania* gum has been evaluated as thickening agent in printing cotton fabrics with reactive dyes[19]. Also, the cross linking on *sesbania* gum using dialdehyde group was performed with changed thermal and swelling properties[20]; *Sesbania* gum was evaluated as filter-aid agent with enhancing leachability of rare-earth ore with ammonium sulphate solution as lixiviant[21]; Oxidized *sesbania* gum was formed using sodium hypochlorite and evaluated as wrap sizing agent for fine cotton yarns[22]; High adsorption capacity towards metal ions having epoxy functional groups into *sesbania* gum was evaluated[23]. However, there are no reports on thiol modification of *sesbania* gum.

In the present investigation thiol functionalization of *sesbania* gum was carried out. The modified gum was characterized usingfourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), energy dispersive X-ray micro-analysis (EDX) and thermo gravimetric analysis (TGA) studies. Thiolated *sesbania* gum was tested as mucoadhesive polymer by formulating composite beads with sodium alginate by ionic gelation method. The beads were evaluated for entrapment efficiency, *in vitro* release and mucoadhesive study. It was aimed

to prepare metformin hydrochloride (model drug) loaded *sesbania* gum-sodium alginate (SG-Alg) and thiolated *sesbania* gum-sodium alginate (TSG-Alg) composite beads to achieve controlled drug delivery system.

METHODOLOGY

Materials

Sesbania gum and metformin hydrochloride were obtained as gift samples from Badar Enterprises (Jodhpur, Rajasthan, India). Mercaptoacetic acid, sodium alginate and methanol were purchased from Thomas Baker Chemicals Pvt. Limited (Mumbai, India). Sodium dihydrogen phosphate, hydrochloric acid and calcium chloridewere purchased from SD Fine-Chem Limited (Mumbai, India). Ellman's reagent [5,5'–dithiobis-(2-nitrobenzoic acid); M_v = 396.34 g/mol; 0.03% w/v], di-sodium hydrogen phosphate, and *L-Cysteine* were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).Freshly excised chick intestine was procured from local butcher house (Hisar, India).

Synthesis of thiolated *sesbania* gum

The synthesis of thiolated *sesbania* gum was done by esterification of native *sesbania* gum employing mercaptoacetic acid (80%, w/v) in the presence of hydrochloric acid as catalyst. A dispersion of *sesbania* gum was prepared by adding 2 g of *sesbania* gum powder in 200 ml cold distilled water with the aid of magnetic stirrer. The dispersion was reacted with mercaptoacetic acid (7.56 g) in the presence of 5 ml of 7N HCl by refluxing at temperature of 70 ± 2°C for 2h. The above reaction mixture was poured in 500ml methanol. White precipitate of thiolated *sesbania* gum so obtained was filtered using Whatman filter paper, washed with methanol and dried in oven at a temperature of 50±2°C[24].

Calculation of thiol group content

The content of thiol group substitution in TSG was determined by well-establishedEllman'smethod[25]. An aqueous dispersion (0.2% w/v) of native *Sesbania* gum (control) and thiolated *Sesbania* gum was prepared. A volume of 2.5 ml of each prepared suspension was diluted with 2.5 ml of phosphate buffer (0.5 M, pH 8.0) followed by addition of 5 ml of Ellman's reagent in it and allowed to react for 2 h at an ambient temperature in dark. Absorbance of the above reaction mixture was measured at 450 nm using UV spectrophotometer (UV-1800, Shimadzu, Japan). The number of thiol group substitution was calculated using calibration curve of *L-cysteine* (standard) with Ellman's reagent as detailed above.

Physiochemical characterization of *sesbania* gum and thiolated *sesbania* gum

Both the gums i.e. native *sesbania* gum and thiolated *sesbania* gum were characterized for organoleptic and physical properties. Color, odour and taste like organoleptic characterization were done manually, while the physical properties such as density (bulk and tapped), angle of repose, Hausner's ratio, Carr's index and swelling index were calculated using standard procedures as follows-

pH determination

For pH determination of native and thiolated *sesbania* gum, a 2% w/v dispersion of each of the gum in distilled water was mixed with vigorous shaking for 10 min[26]. The pH was measured using calibrated pH meter (Waterproof *pHTestr 10,* EUTECH instruments, OAKTON®, Singapore).

Bulk density and tapped density

Accurately weighed amount of 5 gm of *sesbania*/thiolated *sesbania* gum powder sample was introduced into 100 ml measuring cylinder and the volume occupied by the powder was recorded as bulk volume. The measuring cylinder was tapped on a wooden frame till obtaining constant volume, which was taken as tapped volume[27]. The bulk density and tapped density was calculated as follows:

$$Bulk\ p = \frac{Amonut\ of\ sample\ taken}{Bulk\ volume} \quad (1)$$

$$Tapped\ p = \frac{Amonut\ of\ sample\ taken}{Tapped\ volume} \quad (2)$$

Angle of repose

The angle between horizontal surface and apexof cone shaped pile of powder is characterized as the angle of repose. A glass funnel having the orifice diameter of 5 cm was fixed using a stand, 4 cm above the horizontal surface. The weighed amount of powder was then allowed to pass through the glass funnel followed by the measurement of diameter and height of the pile of powder[27]. The angle of repose was found using the equation:

$$\tan \Theta = \frac{Height\ of\ pile}{Diameter\ of\ pile} \quad (3)$$

Where, Θ is the angle of repose

Hausner ratio and Carr's Index

Hausnerratio and Carr's index provide flow properties and compressibility of powders. The values of bulk density and tapped density were used to determine these parameters using the formula:

$$Hausner\ Ratio = \frac{\rho_{Tapped}}{\rho_{Bulk}} \quad (4)$$

$$Carr's\ Index = \frac{\rho_{Tapped} - \rho_{Bulk}}{\rho_{Tapped}} \quad (5)$$

Swelling Index

Swelling behaviour of *sesbania*/thiolated *sesbania* gum was determined by using modified method reported in previous literature[28]. A dispersion (1% w/v) of *sesbania* gum/thiolated *sesbania* gum powder was prepared and the initial (at 0 h) and final volume (at 24 h) occupied by the powder sediment was noted. The swelling index was determined using the given below equation:

$$Swelling\ Index = \frac{V_{Final} - V_{Initial}}{V_{Initial}} \times 100 \quad (6)$$

Where, $V_{initial}$ and V_{final} are initial and final volume of powder, respectively

Moisture content

Moisture content affects the quality and stability of the product. However, it can be determined using thermogravimetric approach so, the moisture content of s*esbania* gum and chemically modified *sesbania* gum was calculated by evaporating them in a petridish (4g each) at 80°C in an oven until constant weight obtained[29]. The percentage moisture content was calculated via given formula:

$$Moisture\ content\ (\%) = \frac{W_{Initial} - W_{Final}}{W_{Initial}} \times 100 \quad (7)$$

Where, $W_{initial}$= Initial weight of sample and W_{final}= Final weight of sample after evaporation

Fourier transformInfrared spectroscopy (FT-IR)

Native *sesbania* gum and thiolated s*esbania* gum were subjected to spectrophotometer (IR-Affinity-1, Shimadzu, India)for functional properties confirmation at an ambient temperature using KBr pellets (Pellets were prepared by compress-

ing the material for 30 sec at the pressure of 75 kg/cm^{2}in IR hydraulic press, CAP-15T, PCI Analytics, Mumbai, India) and scanned in the wave number range of 4000-400 cm^{-1}.

Thermal Analysis

Thermogravimetric analysis study was conducted to investigate thermal stability and decomposition of *sesbania* gum and thiolated *sesbania* gum with the elevation of temperature. TGA analysis was performed employing Mettler Toledo TGA analyser (DSC3 PLUS, California, USA) in temperature ranging from 30-410°C under nitrogen atmosphere at a heating rate of 2°C per minute.

Scanning electron microscopy- Energy dispersive X-ray micro-analysis (SEM-EDX)

The size and surface morphological analysis of *sesbania* gum and thiolated *sesbania* gum were determined using scanning electron microscope (Apreo SEM, Thermo Scientific™). The specimens were coated with gold and mounted on specimen stubs using double adhesive carbon tape. Electron micrographs were captured at an accelerating voltage of 20kV at different magnifications. Presence of different element in native *sesbania* gum and thiolated s*esbania* gum were recorded using EDX image (AZtech,Oxford X –Max^N).

Biocompatibility studies

A comparative biocompatibility study of thiolated s*esbania* gum was performed against native *sesbania* gum for evaluation of its clot formation capability using thrombogenic and haemolytic potential. Gravimetric method was used to determine thrombogenic potential as discussed in previous literature[30]. The equal amounts of *sesbania* gum and thiolated *sesbania* gum (500 mg) was dispersed in phosphate buffer (20 ml, pH 7.2) for 24 h at room temperature. After complete hydration of both gums, samples were kept in whole citrated human blood (0.2 ml) followed by mixing of 0.1M $CaCl_2$ (0.2 ml) and then distilled water (5 ml) after 45 min. A volume of 5 ml of formaldehyde (38 %) was added for fixing the clot formed which was dried further and weighed. The following equation was used to determine percentage thrombose:

$$Thrombose = \frac{Wt.\ of\ sample - Wt.\ of\ negative\ control}{Wt.\ of\ postive\ control - Wt.\ of\ negative\ control} \times 100 \quad (8)$$

Weight of positive control indicates weight of the clots without sample while weight of negative control represents weight of residue without blood and samples

ASTM (American Society for Testing and Materials) standard was used to determine haemolytic potential as described in literature[30]. The same procedure was followed as mentioned above for hydration and clot formation of pure gum with its thiolated form. After incubating the samples in B.O.D. incubator (NSW- 152, Super Deluxe Automatic, India), centrifugation (Research centrifuge, TC 4100 D, Khera Instruments Pvt. Ltd., Delhi) was done at 10,000 rpm for 15 min for complete leaching of the unclotted blood. The absorbance of obtained supernatant fluid was analysed at λ_{max} of 540 nm in UV Visible spectrophotometer (UV-1800, Shimadzu, Japan). The following equation was used to calculate the haemolytic index:

$$\textit{Haemolytic index (\%)} = \frac{\textit{Absorbance of sample - Absorbance of negative control}}{\textit{Absorbance of postive control - Absorbance of negative control}} \text{ X100} \quad (9)$$

Fabrication of drug loaded composite beads of thiolated *sesbania* gum with sodium alginate

Composite beads of thiolated s*esbania* gum (TSG-Alg) and s*esbania* gum with sodium alginate (SG-Alg) were prepared by extrusion through a hypodermic needle in a crosslinking solution of calcium chloride. Previously, *sesbania* gum/ thiolated *sesbania* gum (500mg) and sodium alginate (500mg) was dispersed in 0.1 N NaOH (20 ml) and deionized water, respectively. Both the suspensions i.e. *sesbania* gum/thiolated *sesbania* gum and sodium alginate were mixed followed by addition of model drug i.e. metformin hydrochloride (150mg) with continuous stirring. To obtain the adequate composite beads, the prepared suspensions were dropped into $CaCl_2$ solution (30 ml) using hypodermic needle (24 #) from a height of 26 cm over the period of 2 min, and the beads formed were kept for 10 min so that cross-linking could take place[31]. On completion of cross-linking reaction, obtained composite beads were filtered, taken out and dried in petridish at ambient temperature. For comparison purpose, metformin hydrochloride loaded sodium alginate beads (Alg) were also prepared using above mentioned procedure.

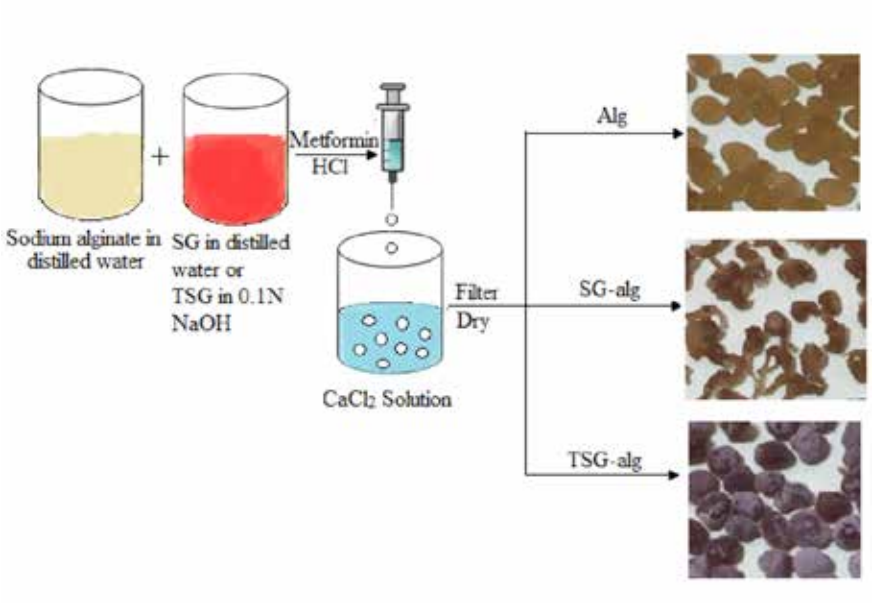


Figure 1: Schematic representation of bead formulation.

Characterization of drug loaded composite beads

The beads of various batches so obtained were evaluated for percentage yield, percentage entrapment efficiency, swelling behaviour, Fourier-transform infrared spectroscopy, scanning electron microscopy and *in-vitro* release behaviour.

Percentage yield

The yield (%) of SG-Alg,TSG-AlgandAlgwere calculated by using the given formula:

$$Yield\ (\%) = \frac{Wt._{Beads}}{Wt._{Polymer+Metfor.HCl}} X100 \quad (10)$$

Where,

$Wt._{Beads}$ = Total weight of beads produced

$Wt._{Polymer+Metfor.HCl}$ = Total weight of *sesbania* gum/thiolated*sesbania* gum/sodium alginate and drug used in the formulation of beads

Entrapment efficiency

The amounts of drug in gum included or not included in the beads were evaluated spectrophotometrically.Briefly, about 100 mg of crushed beads from each batch i.e. composite beads and sodium alginate beads were digested in 50 ml phosphate

buffer (pH 6.8) using probe sonication (Q55, QSonica, USA) for 5 min (amplitude 40%). Aliquots from the filtrate, remaining after filtration of polymer debris were assayed using a spectrophotometer at 234 nm (UV 1800, Shimadzu, Japan).The amount of entrapped metformin hydrochloride was calculated as follows:

$$\textit{Entrapment efficiency (\%)} = \frac{\text{Metfor.}HCl_{Practical}}{\text{Metfor.}HCl_{Theoritical}} \text{ X100} \quad (11)$$

Where,

$\text{Metfor.HCl}_{\text{Practical}}$= amount of metformin hydrochloride found in beads

$\text{Metfor.HCl}_{\text{Theoritical}}$= amount of metformin hydrochloride calculated to be present in thebeads

In-vitro release study

The release of metformin hydrochloride from SG-Alg, TSG-Alg and Alg beads were performed using USP dissolution test apparatus (Paddle Type, TDL-08L, Electrolab, Mumbai, India) in 900 ml dissolution medium (0.2 M phosphate buffer, pH 6.8). The beads having metformin hydrochloride equivalent to 100 mg were tied in muslin cloth and suspended under the paddle[32]. Then the paddle was immersed in phosphate buffer solution for 24 h at 50 rpm and the temperature was maintained at (37±0.5°C. At predetermined interval of time, aliquots of 5ml sample were removed and replaced by the same volume of fresh dissolution medium to maintain the sink condition during the whole test. The withdrawn samples were filtered using syringe filter (0.45µm) and analysed using UV-Visible spectrophotometer (UV 1800, Shimadzu, Japan) at 234 nm.

Swelling study

The swelling behaviour of SG-Alg, TSG-Alg and Alg beads were determined in solutions of different pH (1.2, 6.8 and 7.2) at (37±0.5)°C up to 24 h. Briefly, about 100 mg of beads from each batch was kept in 200 ml buffer solution[31]. Weight of swollen beads after blotting the excess liquid adhered on the surface was recorded at different interval of time until constant weight. The % swelling was calculated using the given formula:

$$\textit{Swelling (\%)} = \frac{\text{Wt.}_S\text{-Wt.}_D}{\text{Wt.}_D} \text{ X100} \quad (12)$$

Where, Wt._S and $\text{Wt.}_D$are the weights of swollen and dry beads, respectively.

Mucoadhesive study

Mucoadhesivestudy of SG-Alg, TSG-Alg and Alg beads were carried out by wash off method using freshly excised chick intestine purchased from local butcher house (Hisar, India)[9]. Adipose and connective tissue of the isolated intestine was removed properly. Then, tissue was adhered with mucosal surface (facing outside) on glass slide using cyanoacrylate glue. About 150 beads of each batch were adhered by pressing lightly on mucosal surface. The prepared glass slide was hung into the beaker having phosphate buffer solution (pH 6.8) on the USP tablet disintegration test machine[33] for 24 h. Total number of beads detached was noted at specific time interval. The whole study was carried out in triplicate manner.

RESULTS AND DISCUSSION

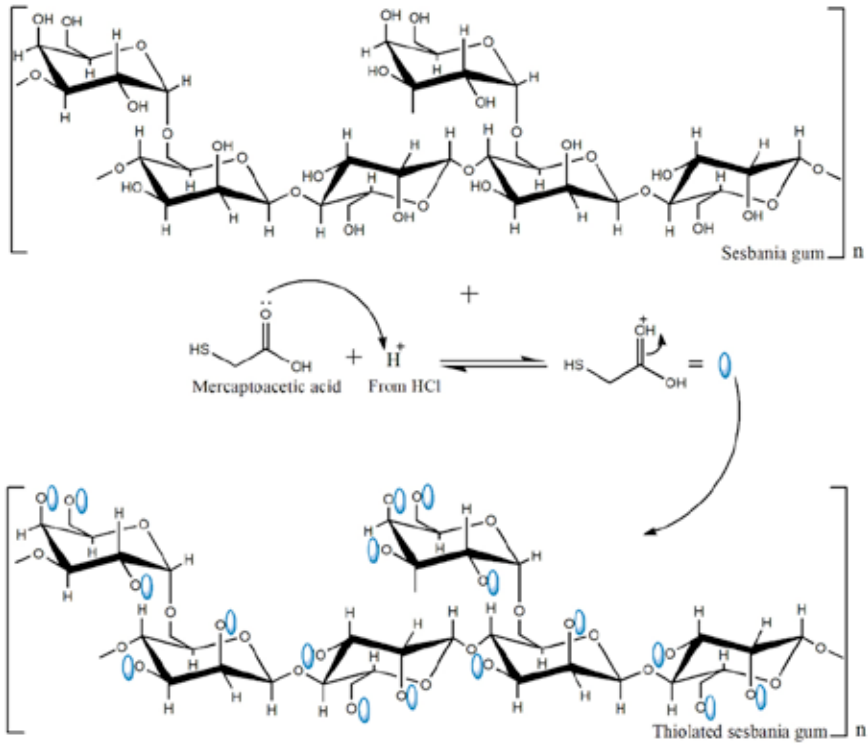


Figure 2: Schematic diagram for thiolation of *sesbania* gum.

Sesbania gum, a galactomannan has been chemically modified by employing mercaptoacetic acid under acidic conditions for thiol derivatization as shown in figure 2. In the first step of thiolation process, the hydroxyl groups (-OH) exhibited on *sesbania* gum are substituted by chlorine which further react with the carboxyl

groups (-COOH) of mercaptoacetic acid having sulfhydryl (-SH) present at terminal to form thiolated s*esbania* gum[3]. The air dried reaction product so obtained was copper red in colour with the characteristic odour. It was soluble in alkaline medium. The % yield of modified s*esbania* gum was found to be 89.65%. The number of thiol groups substitution was found to be 1.72mmol/gm, which was determined by Ellman's method.

The physicochemical properties of s*esbania* gum were considerably altered after thiolation. An aqueous dispersion (2.5% w/v) of thiolated*sesbania* gum was found to be more acidic than the *sesbania* gum. The pH of *sesbania* gum gets decreased after thiolation as shown in table 1. Similar results were earlier reported for thiolated starch, which were attributed to the higher affinity of thiol group for accepting electron pair and donation of H^+ ions[29]. The bulk and tapped densities after chemical modification of *sesbania* gum were increased. The Hausner ratio and Carr's index were calculated from the bulk and tapped density. The Hausner ratio is indicator of interparticulate friction. The lower values of Hausner ratio on thiolation of *sesbania* gum points towards lesser friction among the thiolated*sesbania* gum particles as compared to the *sesbania* gum[27]. Further the Carr's Index which refers to the bridge strength and stability of powder was also found to diminish on thiol functionalization. The angle of repose which is traditionally used to characterize the flow properties of powders also shows a decrease in value on thiolation. On the basis of the result of Hausner ratio, Carr's Index and angle of repose measurements, it can be concluded that the *sesbania* gum can be characterized as passable to very poor flow properties powder, whereas thiolated *sesbania* gum powder exhibit fair to good flow behaviour.

Table 1. Different parameters of *sesbania* and thiolated *sesbania* gum

Parameters	Sesbania gum	Thiolatedsesbania gum
pH	6.2±0.1	2.9±0.1
Bulk density (g/cm^3)	0.51±0.02	0.81±0.02
Tapped Density (g/ cm^3)	0.75±0.01	1±0.01
Angle of repose (°)	42.26±0.5	21.8±0.7
Hausner's ratio	1.49±0.01	1.22±0.01
Carr's index	32.65±0.03	19±0.04
Swelling (%)	30±1.24	10±1.11
Moisture content (%)	7.7±0.6	2.8±0.4

Data are presented as mean± SD (n=3)

In terms of swelling, thiolated *sesbania* gum has found less swelling power than pure *sesbania* gum as shown in table 1. It can beexplained by the fact that the native *sesbania* gumhas large numbers of hydroxyl groups which form intermolecular hydrogen bond with water molecules while in case of thiolated *sesbania* gum,-SH groups present in thiolated *sesbania* gumform weak H-bonds as compared to –OH groups because the thiol groups have less polarity and dipole moment as compared to the corresponding alcohols[34].

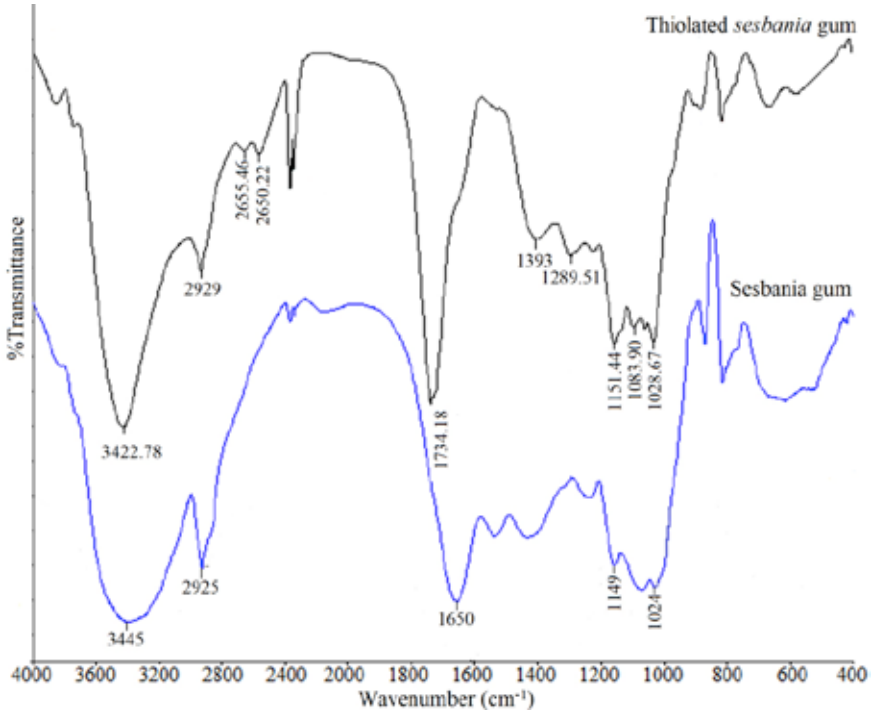


Figure 3: FT-IR spectrum of thiolated *sesbania* gum and *sesbania* gum.

Figure 3 represent FT-IR spectra of *sesbania* and thiolated *sesbania* gum. The broad and strong absorption band in native s*esbania* gum which appears at 3445 $cm^{-1}$is due to O-H group stretching. Thepresence of C-H linkage of alkane is shown at 2925 cm^{-1}, while the peak at 1650 cm^{-1} is attributed to C=O stretching of primary alcohols and two small peaks at 1149 cm^{-1} and 1024 cm^{-1} are due to C=O stretching of tertiary alcohol[22]. FTIR spectrum of thiolated *sesbania* gum showed a narrow band at 3442.78 cm^{-1}which is due to free O-H group stretching of mercaptoacetic acid, while the presence of C-H linkage of alkane is shown at 2929 cm^{-1}; C-H bending at 1734.18 cm^{-1}; O-H bending at 1393cm^{-1}; C=O stretching at 1289.51cm^{-1} and 1028.67cm^{-1};C=O stretching of primary and tertiary alcohol at

1083.90cm^{-1} and 1151.44cm^{-1}, respectively; small peak at 2655.46cm^{-1} is related to O-H stretching;one extra stretch at 2650.22cm^{-1} is due to –SH stretching of thiol group which confirms thiolation of *sesbania* gum. Thiol bands are not easy to detect using FT-IR spectroscopy; therefore, various other studies were performed.

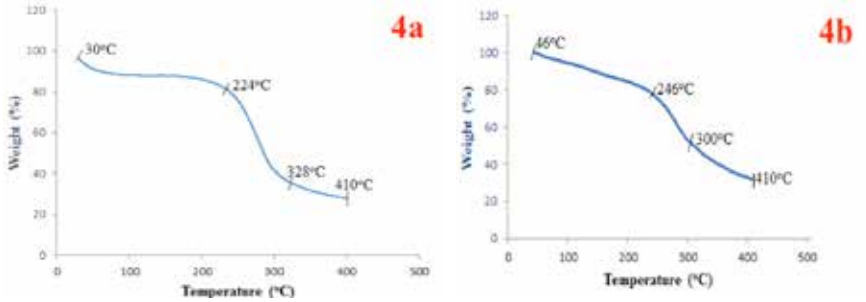
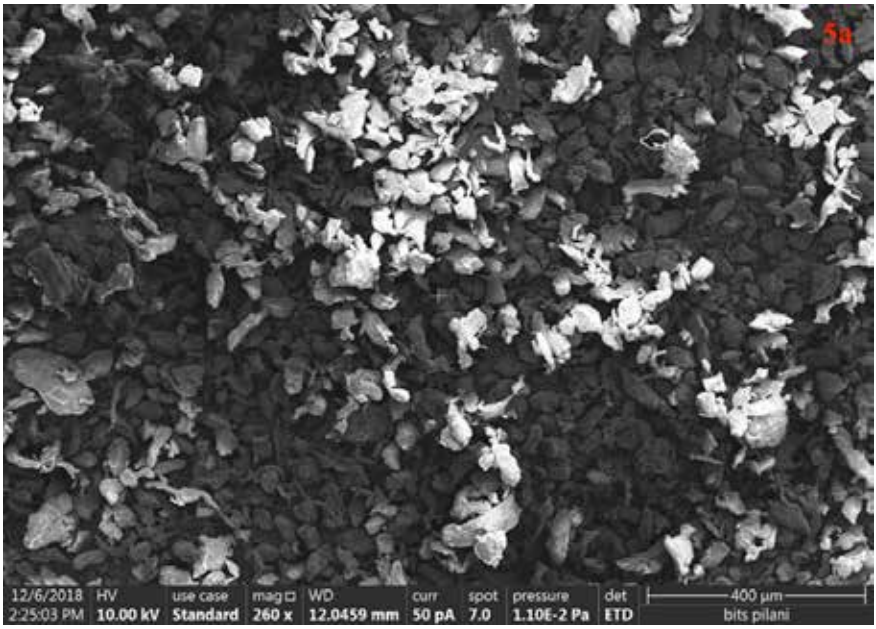
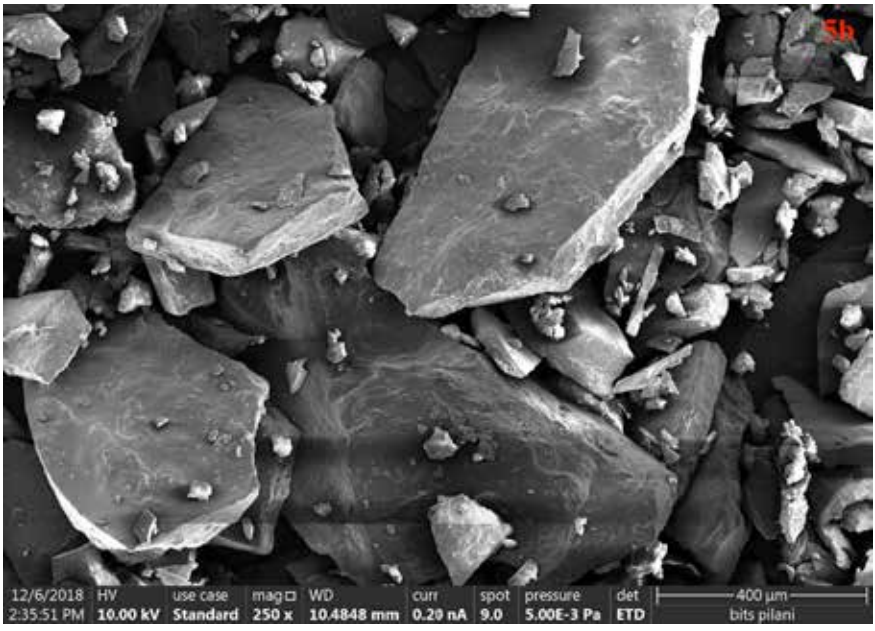
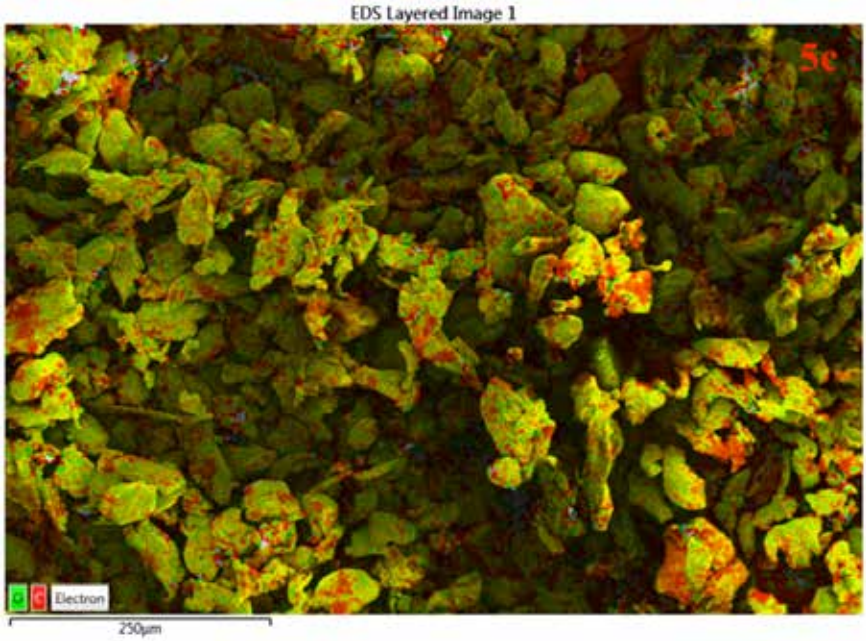


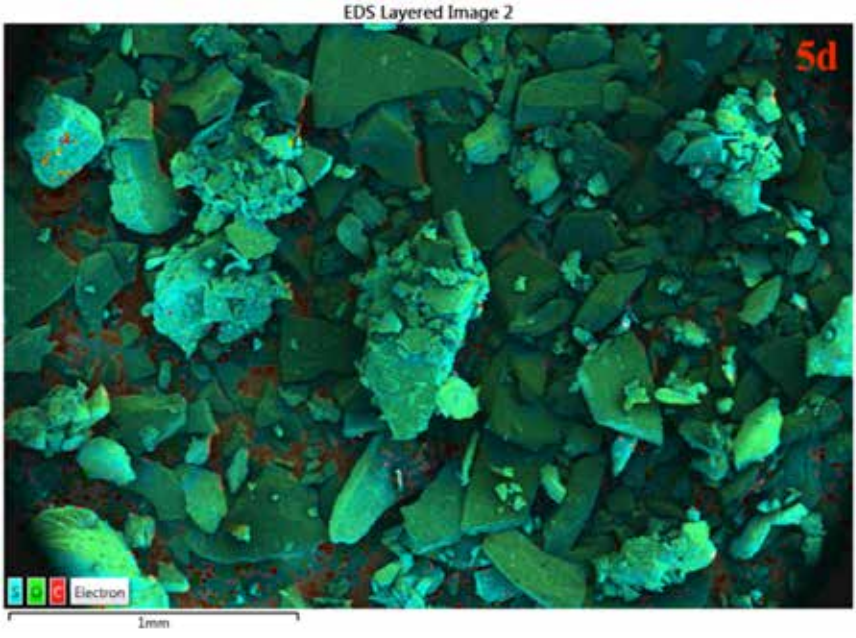
Figure 4: TGA plot of *sesbania* and modified thiolated *sesbania* gum.

Figure 4 exhibits thermal plots of *sesbania* and thiolated *sesbania* gum showing weight loss (%) with temperature while reviewing the thermogram, it was observed that decomposition occurs in three successive phases showing sigmoid curve. In native *sesbania* gum, first stage of decomposition occurs from 30°C to 224°C with 14% weight loss while 48% weight loss occurred during second stage in temperature range of 225°C-328°C. In third stage of thermal degradation from 329°C-410°C, 8% loss of weight was observed. In thiolated *sesbania* gum, first stage of decomposition was from 46°C to 246°C in which 24% weight loss occurred. In second stage of degradation which occurred between 247°C and 300°C, 24% weight loss occurred while in third stage (301°C-410°C), 21% weight loss occured. At the end of the thermal study at 410°C, a residue of 30% of *Sesbania* gum and 31% of thiolated *sesbania* gum was left which indicate that there is no difference between thermal stability of *sesbania* gum and thiolated *sesbania* gum[24]. The weight loss during first stage of degradation is due to desorption of bound water and dehydration because of loss of hydroxyl groups from the polysaccharide backbone[35], while the weight loss during the second and third stages can be ascribed to depolymerisation and the pyrolysis resulting in the evolution of gaseous products such as carbon monoxide, carbon dioxide, methane etc[36].


5a
12/6/2018 HV use case mag WD curr spot pressure det 400 µm
2:25:03 PM 10.00 kV Standard 260 x 12.0459 mm 50 pA 7.0 1.10E-2 Pa ETD bits pilani


5b
12/6/2018 HV use case mag WD curr spot pressure det 400 µm
2:35:51 PM 10.00 kV Standard 250 x 10.4848 mm 0.29 nA 9.0 5.00E-3 Pa ETD bits pilani


EDS Layered Image 1
5c
O
C
Electron
250µm


EDS Layered Image 2
5d
Si
O
C
Electron
1mm

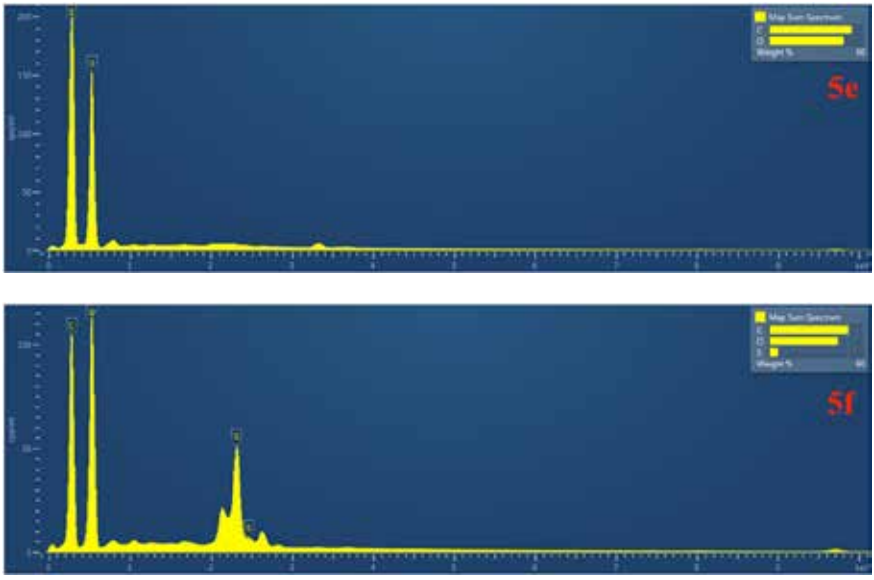


Figure 5: Field emission-scanning electron micrographs – Energy dispersive X-ray analysis (FE-SEM-EDX) of *sesbania* gum (a, c, e) and modified *sesbania* gum (b, d, f).

Scanning electron micrographs of *sesbania* and thiolated s*esbania* gum have been shown in figure 5. As clearly shown in photographs of s*esbania* gum (fig. 5a), polyhedral flakes are present while SEM micrographs of thiolated *sesbania* gum (fig 5b), show presence of plate shape particles. Fig 5shows the EDX layered images of *sesbania* (c) and thiolated s*esbania* gum (d). The presence of carbon is indicated by red color while green color represents oxygen in figure 5(c). In the EDX layered image of thiolated s*esbania* gum (fig. 5d), additional blue color shows the presence of sulphur. Further, the EDX spectrum of thiolated s*esbania* gum (fig. 5f) also shows the additional peak of sulphur at 2.3keV, which confirms the presence of sulphur in thiolated s*esbania* gum. This peak is not present in the EDX spectrum of *sesbania* gum (fig 5e).

Biocompatibility study was performed on native *sesbania* and thiolated *sesbania* gum for analysis of its clot formation capability. The test revealed that clot formation in native *sesbania* gum (0.132 g in 2 ml citrated whole blood) and thiolated-*sesbania* gum (0.157 g in 2 ml citrated whole blood) was less than as compared to positive control clot weight (0.181 g in 2 ml of citrated whole blood). The % thrombose of *sesbania* and thiolated *sesbania* gum was calculated i.e. 71% for *sesbania* gum and 85% for thiolated *sesbania* gum which concludes that both *sesbania* and thiolated *sesbania* gum can be non-thrombogenic. Further, hemolytic index of *sesbania* and thiolated *sesbania* gum was found to be 2.85% and 1.30% respectively which is also considered as safe and suitable for drug delivery applications[30].

Thiolated *sesbania* gum was further tested for drug delivery applications by using it as a mucoadhesive polymer. Since thiolated *sesbania* gum as such does not form ionically gelled beads, it was used in combination with sodium alginate to prepare composite beads using metformin hydrochloride as a model drug and $CaCl_2$ as a cross-linking agent. For comparative study, composite beads of *sesbania* gum with sodium alginate and the beads of sodium alginate alone were also prepared. The composite beads of thiolated *sesbania* and alginate beads (TSG-Alg), *sesbania* gum and sodium alginate (SG-Alg) and alginate alone beads (Alg) were obtained in a percentage yield of 122.3%, 141.2% and 97.4%, respectively. The entrapment efficiency of TSG-Alg, SG-Alg and Alg beads was found to be 99.96%, 99.26% and 89.03%, respectively.

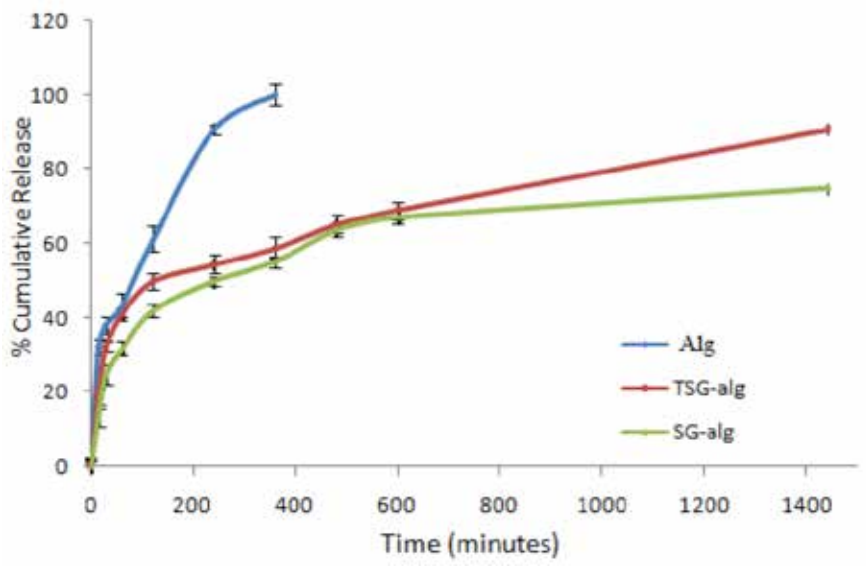


Figure 6: *In-vitro* drug release of Alg, SG-Alg and TSG-Alg composite beads.

Figure 6 represents the comparative *in-vitro* release profile of metformin from the Alg, TSG-Alg and SG-Alg composite beads. The drug release behaviour was studied in phosphate buffer (pH 6.8) to evaluate its release kinetics. It can be seen in the figure thatTSG-Alg composite beads released 90.68% of drug in 24 h whereas SG-Alg composite beads released 74.85% of metformin in the same time. As compared to composite beads, Alg beads released almost 100% of the drug in 6 h. This indicates that composite beads of *sesbania* gum/thiolated*sesbania* gumwith alginate are more effective in sustaining the release of metformin as compared to the beads of alginate alone. It can be observed from the profile

that almost similar pattern of release is observed for the release of metformin from composite beads of SG-Alg and TSG-Alg. Only at 24th h there was somewhat greater difference in the release of metformin from the TSG-Alg beads. Further to check whether there is any significant difference in the release of metformin from the two composite beads, the release data was evaluated for determining f_1 and f_2 value. On comparing the release data of metformin from TSG-Alg and SG-Alg beads, the f_1 and f_2 value were found to be 13.07 and 55.58, respectively which indicates that the release profile of metformin from the two beads can be considered to be similar. Further the release rate data was fitted into various kinetic models for determining the release kinetic and mechanism of release (Table 2). The results revealed that the release of metformin from the alginate (Alg) and composite beads of *sesbania* gum and alginate beads (SG-Alg) follows Higuchi square-root kinetics while in case of composite beads of thiolated sesbania gum ad alginate (TSG-Alg), the release data fits best into 1st order kinetics. The value of '*n*' the release exponent of Korsemeyer and Peppas equation (n<0.45) which indicates that the release of metformin from all batches of the beads occurs primarily by diffusion through matrix.

Table 2 Modelling and release kinetics of TSG-Alg,SG-Algand Alg.

Formulation	R^2				'n'
	Zero order	Istorder	Higuchi square-root	Korsemeyer-Peppas	
TSG-Alg	0.693	0.950	0.908	0.900	0.330
SG-Alg	0.626	0.782	0.887	0.937	0.421
Alg	0.871	0.966	0.981	0.969	0.300

Table 3 lists the results of swelling behaviour and *ex-vivo* bioadhesion study of TSG-Alg, SG-Alg composite beads and alginate beads in phosphate buffer (pH 6.8).The results of swelling study support the release rate profile. The Alg beads dissolved within 6h releasing almost all the drug. On the other hand, SG-Alg beads continued to swell till 24h sustaining the release of metformin, while TSG-Alg beads continued to swell till 12h and then started to erode thereby releasing drug at slightly faster rate than the SG-Alg beads.

Table 3. Swelling behaviour and *Ex-vivo*bioadhesion study of Alg, SG-Alg and TSG-Alg

Time (h)	Swelling (%)			Ex-vivobioadhesion time (%)		
	Alg	SG-Alg	TSG-Alg	Alg	SG-Alg	TSG-Alg
0.5	45.21±1.23	77.41±2.55	20±1.32	100	100	100
1	223.48±1.44	183.81±2.12	153.33±1.22	100	100	100
2	486.67±2.13	294.23±2.34	320.34±1.12	95.4	100	100
4	256.45±1.47	483.87±2.17	520±1.25	50.9	100	100
6	21.11±1.69	516.13±1.99	706.67±1.09	30	86.6	100
12	-	541.93±1.33	893.33±1.11	28	80	100
24	-	554.83±1.76	320±2.05	5	73	93.3

The mucoadhesive ability of the metformin loaded beads of the different batches of the beads was evaluated comparatively by determining bioadhesion (table 3). It can be observed from the results that Alg beads could adhere to the intestinal mucosal tissue only upto 6h. On the other hand, composite beads of SG-Alg shows 100% adhesion till 4 h and at the end of 24 h of the study 73% of the beads still adhered to the intestinal mucosal tissue. In case of composite beads of TSG-Alg, 100% of the beads were found adhering till 12 h and at the end of 24 h study period 93.3% of beads were found adhering to the intestinal mucosal tissue. The results thus conform the higher mucoadhesivity of TSG-Alg as compared to the SG-Alg beads. The literature already reported that sulfhydryl group (-SH) present in thiolated polymers form strong covalent disulphide bond with glycoproteins present in mucus which was clearly indicated in its results also[33]. However, hydroxyl group (-OH) present in s*esbania* gum form weak hydrogen bond or show weak Van der Waal's interaction with mucus glycol-proteins and show less mucoadhesionproperty[37]. Similar results were earlier observed in thiolated pectin[9] and thiolated alginate beads[38].

This study introduces the thiolation modification on *sesbania* gum with their characterization. The *sesbania* gum was esterified using mercaptoacetic acid to formulate thiolated *sesbania* gum which was characterized physiochemically, structurally, morphologically, and thermally. Thiolated *sesbania* gum was also found biocompatible as compared to native *sesbania* gum in the biocompatibility study. Fabrication of metformin drug loaded composite beads with sodium alginate i.e. Alg, SG-Alg and TSG-Alg beads were done with characterization. The thiolated *sesbania* gum shows 90.68 % *in-vitro* drug release following 1st order kinetics. Thiol group on *sesbania* gum enhance the mucoadhesive strength which can also be explored for pharmaceutical applications.

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Antioxidant Properties of Capsule Dosage Form From Mixed Extracts of *Garcinia Mangostana* Rind and *Solanum Lycopersicum* Fruit

Mahalul Azam[1], Willy Tirza Eden[2,*], Arulita Ika Fibriana[1], Sri Ratna Rahayu[1]

1 Public Health Department, Faculty of Sport Science, Universitas Negeri Semarang, Indonesia
2 Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Indonesia

ABSTRACT

Several studies showed both *Garcinia mangostana* L. and *Solanum lycopersicum* L. have shown important role as natural sources of antioxidant compounds. Hence, capsule supplement from mixed extracts of *Garcinia mangostana* L. and *Solanum lycopersicum* L. was prepared. This study aims to investigate the antioxidant properties from the capsules supplement contained with mixed extracts of *Garcinia mangostana* rind (GMR) and *Solanum lycopersicum* fruit (SLF). Antioxidant activity of capsule dosage form was measured using DPPH, ABTS, and FRAP assays. In addition, the total phenolic content and total flavonoid content of the capsules preparation were also evaluated. Total phenolic content was 0.7082 ± 0.1372 mg GAE/capsule and total flavonoid content was 11.7769 ± 3.9504 µg QE/capsule. The strong correlation observed between antioxidant capacity by ABTS method and the total phenolic contents (R^2= 0.995, P<0.05) indicated that phenolic compounds in capsule preparation related with its antioxidant activity.

Keywords: Antioxidant, DPPH, ABTS, FRAP, *Garcinia mangostana, Solanum lycopersicum*

Corresponding author:
Willy Tirza Eden
Telephone number: (+62)85226059090
Email address: willytirzaeden@mail.unnes.ac.id
(Received 13 February 2020, accepted 17 April 2020)
ORCID
Mahalul Azam, 0000-0002-2441-5433
Willy Tirza Eden, 0000-0002-4157-6060
Arulita Ika Fibriana, 0000-0003-4057-1644
Sri Ratna Rahayu, 0000-0003-3514-2351

INTRODUCTION

Antioxidant is a molecule that has the ability to protect organisms from damage caused by free radical-induced oxidative stress. Oxidative stress is considered to be linked with numerous degenerative diseases such as cancer, cardiovascular disease, Alzheimer's disease and Parkinson [1-3]. Human body can balance the oxidative state by synthesizing glutathione and enzymes (e.g., catalase and superoxide dismutase) which produced internally, or taking exogenous antioxidants like the vitamin C, vitamin E, carotenoids, and polyphenols [4-5]. Taking dietary antioxidant supplementation has been found to be a promising method of countering the effects of oxidative stress [6-9]. Natural products began to receive much attention as sources of safe antioxidants nowadays [10-12]. Some species of medicinal plants have antioxidant and pharmacological activities which related to the existence of phenolic compounds [13-14].

Mangosteen (*Garcinia mangostana* L., family Guttiferae) is known as "the queen of tropical fruits" because of its tasty flavor. The pericarp of mangosteen has been used traditionally by Southeast Asian for treating diarrhea, skin infection and wounds, amoebic dysentery, etc [15-16]. *G. mangostana* rind (GMR) contains a lot of water soluble antioxidant compounds. Various kinds of xanthones, such as prenylated and oxygenated xanthones in GMR had been proven to have strong antioxidant activity [17-19]. Tomato (*Solanum lycopersicum* L., family Solanaceae) is one of the most consumed vegetables worldwide. *S. lycopersicum* fruit (SLF) are considered as important sources of dietary antioxidants, such as carotenoids, in particular α-carotene, β-carotene, lycopene, lutein, and cryptoxanthin [20-21]. Anthocyanins, the flavonoid constituents in highly pigmented fruits including tomato, have been reported to possess potential antioxidant, anti-inflammatory, anticancer, and antidiabetic activity [22-24].

GMR and SLF each has been widely investigated for their antioxidant activities, yet the antioxidant properties of mixed extracts of GMR and SLF has not been reviewed. Due to the widely marketed nutritional supplements containing GMR, we are challenged to create capsule dosage form as supplements prepared from both plant extracts. This study aims to investigate the antioxidant properties from the capsules supplement contained with mixed extracts of *G. mangostana* rind (GMR) and *S. lycopersicum* fruit (SLF), also its total phenolic and flavonoid contents. Several methods were applied to measure antioxidant capacity, including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP).

METHODOLOGY

Chemical and Reagents

Aluminium chloride hexahydrate, Folin-Ciocalteu's phenol reagent, ascorbic acid, sodium carbonate, quercetin, and gallic acid were purchased from Merck Millipore. Dimethylsulfoxide **(**DMSO**)** and AR grade methanol from Sigma-Aldrich. Aerocyl and amylum manihot for preparation of GMR and SLF capsules were purchased from local industries. The commercial grade solvents were used for extraction.

Plant Material

The *G. mangostana* and *S. lycopersicum* fruit were collected from Gatutkaca, a local Jamu (Indonesian traditional medicine) industry and identified by Eling Purwantoyo, M.Si. (Department of Biology, Universitas Negeri Semarang). The voucher specimen (204579) was deposited at the Biology Laboratory, Department of Biology, Universitas Negeri Semarang, Indonesia.

Preparation of Plant Extracts

Extraction of *G. mangostana* and *S. lycopersicum* using hydroalcoholic solvent was based on previous studies which performed hydroalcoholic extractions to obtain antioxidant compounds [25-26]. The dried pericarp of *G. mangostana* (1000 g) were macerated using 70% ethanol/water (1:3) at room temperature for 72 h. After filtration, the filtrate was concentrated followed with the addition of aerocyl (30 g). From this process, 50 g of condensed extract was obtained. For the *S. lycopersicum* extract, fresh fruits of tomatoes (1000 g) were crushed and blended using 70% ethanol/water (1:1). After filtration, the filtrate was concentrated followed with the addition of aerocyl (20 g). 65 g of condensed tomato extracts were obtained.

Preparation of Capsule Contains Mixed Extracts of GMR and SLF

The condensed extract of GMR were added with 1500 g filling agents (amylum manihot) to produce dry powder of GMR extracts (1448 g). The same procedure was also conducted to the condensed extract of SLF. 850 g filling agents (amylum manihot) were added to produce dry powder of SLF extracts (774 g). Both dried extracts were mixed at a ratio of GMR:SLF (2:1) and capsulated with number 0 capsule shell (average weight 450 mg/capsule).

DPPH-radical Scavenging Activity Assay

The diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was performed based on the method of Zongo (2010) with slight modification [27]. In the microplate well, 10, 20, 30, 40, 50 μL of the capsule powder (50 mg/mL in DMSO)

or standard solution was mixed with 100 μL of the DPPH-radical (100 μg/mL in methanol) and left to stand at room temperature for 15 min in the dark. The absorbance was measured at 517 nm. Ascorbic acid (Vitamin C) was used as references. This experiment was conducted in triplicates.

ABTS Assay

In the ABTS free radical assay, the method of Jemli (2015) was adopted with minor changes [28]. Briefly, ABTS reagent solution was freshly prepared by mixing 2 mM of ABTS solution with 70 mM of potassium persulfate, stored in the dark at room temperature for 16 h before use. ABTS·+ solution was then diluted with 80% methanol to obtain an absorbance reading of 0.700 ± 0.005 at 743 nm. The 100µL of sample solution with various concentration was added to 100µL of ABTS solution. The absorbance was measured at 734 nm after 1 minutes of mixture reaction. All the measurements were carried out three times repetition. A standard curve was obtained by using ascorbic acid standard solution at various concentrations (ranging from 25 to 125 g/mL). The scavenging activity of different concentrations of sample against ABTS radical were also measured to calculate IC_{50}, and the procedure was similar to the DPPH scavenging method described above.

FRAP Assay

FRAP was measured by spectrophotometric assay as previously described [28]. 100µL of sample at different concentration, 100µL of phosphate buffer (0.2 M, pH 6.6), and 100µL of potassium ferricyanide $K_3Fe(CN)_6$ (1%) were mixed and incubated at 50°C for 20 min, to reduce ferricyanide into ferrocyanide. The reaction was stopped by adding 100µL of 10% (w/v) trichloroacetic acid followed by centrifugation at 3000 rpm for 10 min. Lastly, 100µL of the top layer was mixed with 100µL of distilled water and 25µL of ferric chloride solution (0.1%) and the absorbance at 710 nm was calculated by plotting absorbance against the corresponding sample concentration. All the determinations were performed triplicates. Ascorbic acid (vitamin C) was used as a reference compound.

Total Phenolic Contents

Folin–Ciocalteu's method with slight modification was applied to determine the total phenolic content [29]. In a 96-well plate, 12 µL of capsule powder solutions (250 µg/mL in DMSO) or standard gallic acid solutions were added, followed by 50 µL of DI water and 12 µL of Folin-Ciocalteu (50%, v/v in DI water). After 10 min, 125 µL of 7 % Na_2CO_3 and 100 µL of DI water were added. The mixture was allowed to stand for 15 min at 45°C and the absorbance was determined at 765 nm. Total phenolic content was calculated from gallic acid standard curve with linear relation of r^2=0.9727. Data were expressed as mg of gallic equivalent (GAE) per capsule.

Total Flavonoid Contents

In order to investigate the total flavonoid content, a colorimetric method was applied [27]. In a 96-well plate, 100 µL of the capsule powder (100 µg/mL in DMSO) or standard quercetin solutions and 100 µL of 2 % $AlCl_3$ in methanol were added and mixed thoroughly. The reaction mixture was kept at room temperature for 15 min and the absorbance was recorded at 435 nm. The total flavonoid content was calculated using quercetin standard curve with linear relation of R^2=0.9936. Data were expressed as mg quercetin equivalent (QE) per capsule.

RESULT AND DISCUSSION

According to previous studies, *G. mangostana* rind contains phytochemicals such as xanthones, terpenes, anthocyanins, tannins, and phenols, which exert numerous biological effects, including antioxidant activity [30-31]. It is believed that antioxidants can help to overcome oxidative damage in human body, which is associated with many degenerative diseases such as atherosclerosis, coronary heart diseases, aging, and cancer [32-33]. *S. lycopersicum* fruit, which is being widely consumed either fresh or processed in products, possess carotenoids, such as lycopene and β-carotene that are apparently the main tomato micro constituents that responsible for the effect of tomato product on antioxidant activity [34]. Different solvent was used in the extraction of GMR and SLF. The extraction of GMR and SLF by using 70% ethanol/water 1:3 and 1:1, respectively, was conducted according to the polarity of major compounds contained in GMR, which is xanthone, and SLF, which is carotenoid. In this study, the antioxidant activity of capsule dosage form prepared from mixed extracts of GMR and SLF was being determined.

Total Phenolic Contents

It is important to measure the total phenolic compounds correctly in such medicinal plants, the better to assess their antioxidant capacity. Under the basic reaction conditions, a phenol loses an H^+ ion to produce a phenolate ion, which reduces Folin-Ciocalteu reagent [35]. The change is monitored spectrophotometrically. Results of Total Phenolic Contents (TPC) determination by Folin-Ciocalteau method are summarized in Table 1. The greater amount signifies the presence of different constituents having phenolic moiety in their structures. The phenolic content with respect to gallic acid was found to be 0.7082 ± 0.1372 (mg Gallic Acid Equivalent/capsule).

Table 1. The Total Phenolic Content of Capsule Preparation from Mixed Extract of *G. mangostana* rind (GMR) and *S. lycopersicum* fruit (SLF)

Sample	Equation	R^2	TPC (mg GAE/capsule)	Mean TPC (mg GAE/capsule)
Capsule (1st repetition)	y = 4.1289x + 0.029	0.9863	0.8588	
Capsule (2nd repetition)	y = 4.07x + 0.0346	0.9987	0.6643	
Capsule (3rd repetition)	y = 3.8011x + 0.0854	0.9596	0.6241	0.7082 ± 0.1372
Capsule (4th repetition)	y = 4.2799x + 0.0458	0.9438	0.7974	
Capsule (5th repetition)	y = 4.2376x - 0.0213	0.9343	0.9564	

Total Flavonoid Contents

The Total Flavonoids Content (TFC) of the capsule dosage form was determined by a colorimetric assay using quercetin as standard (Table 2). The greater amount signifies the presence of more flavonoids moieties in the constituents. The flavonoid content with respect to quercetin was found to be 11.7769 ± 3.9504 (µg Quercetin Equivalent/capsule).

Table 2. The Total Flavonoid Content of Capsule Preparation from Mixed Extract of *G. mangostana* rind (GMR) and *S. lycopersicum* fruit (SLF)

Sample	Equation	R^2	TFC (µg QE/capsule)	Mean TPC (µg QE/capsule)
Capsule (1st repetition)	y = 0.0155x + 0.1738	0.9453	11.5552	
Capsule (2nd repetition)	y = 0.0162x + 0.1863	0.9885	6.4782	
Capsule (3rd repetition)	y = 0.0156x + 0.175	0.9726	12.8523	11.7769 ± 3.9504
Capsule (4th repetition)	y = 0.0173x + 0.1334	0.9780	17.4097	
Capsule (5th repetition)	y = 0.0154x + 0.1524	0.9752	10.5889	

Most antioxidant activities from plant sources correlate with phenolic and flavonoid contents. The next section discuss about the antioxidant activity and the correlation between phenolic and flavonoid contents; and antioxidant activity.

Antioxidant Activity

In this study, the antioxidant activities were determined by *in vitro* assays, including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP). All three assays are based on the reaction of electron transfer, where the color change would serve as an indication of the antioxidant's ability to reduce radicals [36].

Table 3. IC_{50} Values of Capsule Preparation from Mixed Extract of *G. mangostana* rind (GMR) and *S. lycopersicum* fruit (SLF)

Assays	Sample (mg/mL)	Positive control (Vitamin C) (µg/mL)
DPPH	5.8837 ± 1.4586	184.7211 ± 9.1777
ABTS	6.8098 ± 2.8832	83.6069 ± 8.2220
FRAP	13.7393 ± 2.3856	80.6294 ± 9.5560

Values represent means ± SD (standard deviations) for triplicates experiment

As summarized in Table 3, the measurement using DPPH method resulted the lowest IC_{50} for capsule dosage form (sample), whereas FRAP method resulted the lowest IC_{50} for standard reference (ascorbic acid). Therefore, it is possible that capsule contain mixed extracts of SLF and GMR exhibit different antioxidant mechanism than ascorbic acid. Vitamin C acts as a scavenger of ROS and by one-electron reduction of lipid hydroperoxyl radicals via the vitamin E redox cycle, hence it has the ability to protect against lipid peroxidation (radical chain reaction) [37-39].

Since the capsules were prepared from mixing 50 g of GMR extracts and 65 g of SLF extracts and filling agents, we calculated the estimation of the IC_{50} value of each extracts that may contribute to the antioxidant activity (Table 4).

Table 4. Estimation of IC_{50} Value of Each Extracts

Assays	GMR extract (µg/mL)	SLF extract (µg/mL)
DPPH	200.10	100.50
ABTS	231.60	115.80
FRAP	467.27	233.63

It has been proposed that samples with IC_{50} lower than 50 μg/mL are very strong antioxidants, with 50-100 μg/mL are strong, with 100-250 μg/mL are moderate, with IC_{50} greater than 250 μg/mL are weak antioxidants, and with IC_{50} greater than 500 μg/mL are inactive [40]. Meanwhile, Molyneux (2004) stated that IC_{50} of 200-1000 μg/mL is less active but still has an antioxidant potential [41]. Thus, generally, GMR and SLF extracts are considered to have moderate antioxidant activity.

In addition, we conducted the correlation analysis of the values of total antioxidant capacity obtained by three assay methods, also the correlation analysis of the TFC and TPC to the antioxidant capacity. As shown in Table 5, TPC and ABTS assay indicated a strong correlation (R^2= 0.995).

Table 5. Correlation Coefficient (R^2) among Antioxidant Assays and Total Phenolic and Flavonoid Contents

	DPPH	ABTS	FRAP
ABTS	0.182	–	–
FRAP	0.395	0.190	–
TPC	0.133	0.995*	0.255
TFC	0.358	0.221	0.217

*Correlation is significant at $P < 0.05$

The strong correlation between TPC and antioxidant capacity of ABTS assay shown that phenolic content of GMR and SLF capsule was responsible for its antioxidant activity. The present study revealed that strong correlation between total phenolic and ABTS assay was in agreement with previous studies [42-44]. The ABTS assay is based on the generation of a blue/green $ABTS^{+\cdot}$, which is applicable to both hydrophilic and lipophilic antioxidant systems. The previous investigation by Floegel (2011) stated that the high-pigmented and hydrophilic antioxidants were better reflected by ABTS assay [42]. There was no correlation between antioxidant activity as determined by DPPH, ABTS, and FRAP assays (R^2= 0.182 to 0.395, P>0.05). This might be due to the potential of an antioxidant against free radicals of DPPH and ABTS and inevitably does not equal with its ability to reduce ferric to ferrous [45].

Therefore, based on the finding of this study, capsule dosage form containing mixed extracts of GMR and SLF possesses *in vitro* antioxidant potential. Capsule dosage form of mixed GMR and SLF extracts would be an interesting subject to be further investigated for its *in vivo* antioxidant activity study in animal models. Further experiments needed to obtain a standardized natural-based supplement for combating harmful effects of oxidative stress in human body.

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Cytolysin potential of some of the di and triterpenoids from the seeds of *Guilandina bonducella L.*

Ayesha ASIF[a], M. Asif SAEED[b]

a Faculty of Pharmacy, University of Central Punjab, Lahore, 54000, Pakistan
b College of Pharmacy, University of the Punjab, Lahore, Pakistan. (Retired)

ABSTRACT

Cytolysin potential of the constituents of *Guilandina bonducella* seeds was evaluated. For this purpose, di and triterpenes had been investigated. Two di and four triterpenes from the EtOAc extract of *G. bonducella* seeds were isolated, purified by chromatographic and re-crystallization methods and identified by comparative spectroscopic data. Cytolysin potential of these compounds was carried out on brine shrimp's (*Artemia salina*) larvae (nauplii), compared with positive controlled colchicine's reaction and evaluated by LD_{50}.

Diterpenes (neocaesalpin C, neocaesalpin D) and triterpenes (α-amyrin, β-amyrin, lupeol and lupeol acetate) exhibited a marked cytolytic reaction, even though their intensities differ from each other and with the colchicine. EtOAc extract of *G. bonducella* seeds contained diterpenes (neocaesalpin-C and neocaesalpin-D) and triterpenes (α-amyrin, β-amyrin, lupeol and lupeol acetate). These compounds had cytotoxic ability to the brine shrimp's larvae.

Keywords: Cytolysin potential; ditepenoid and triterpenoid componds; *Guilandina obonducella*; LD_{50}.

INTRODUCTION

Guilandina bonducella L. or *Caesalpinia bonduc* (L.) Roxb. or *Caesalpinia bonducella* (L.) Fleming or *Caesalpinia crista auct*. Amer., commonly called as 'fever nut' 'bonduc nut' or 'nicker nut' (Katkaranja) by the indigenous people. It

Corresponding author:
College of Pharmacy, University of the Punjab, Lahore, Pakistan. (Retired)
b At Present Address = 155 Mamdot Block, Mustafa Town, Lahore, 54580, Pakistan.
E-mail address: moasif1605@yahoo.com
ORCID
Ayesha ASIF, 0000-0002-1387-735x
M. Asif SAEED, 0000-0002-6003-7871
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is an important medicinal plant, belongs to the family Caesalpiniaceae. It had been reported in phratry medicine and in ancient Ayurveda, Siddha, Unani and Homoeopathic Holy Scripturies[1, 2]. This plant is a prickly shrub or a small tree, distributed in subtropical and temperate regions of Pakistan and abundantly established itself in the Punjab Province during the bedewed season[3].

Leaves and roots of this plant had been extensively employed in canonical medicines for the treatment of enumerating diseases[4-7]. It had been investigated that various solvent extracts of leaves of this plant exhibited substantial antidiarrhoeal, cytotoxic and antibacterial activities[4]. Anti-inflammatory, antioxidant, antimicrobial, antifungal, antipyretic, analgesic, anti-asthmatic, anti-diabetic, anti-filarial, anti-tumor, adaptogenic, anti-convulsant, anti-spasmodic, nootropic, anti-feedant, anti-amoebic, anti-oestrogenic, anxiolytic, diuretic, hepato-protective and immuno-modulatory activities of the seeds of this herb had also been reported[4-9]. The consequences of methanolic extract of the whole herb on the lipid peroxidation (LPO), glutathione content (GSH), superoxide dismutase (SOD) and catalase (CAT) was carry out by Gupta et al. and concluded that a significant diminish in the intensity of the tumor and packed feasible cell counts were detected[8,9] while its impression on hematology and hepato-renal functions in mice were ascertained by Kumar et al.[11,12].

As regards the presence of phtochemical ingredients were concerned, phtochemical compounds belonging to different classes such as alkaloids, glycosides, di- and triterpenoids, saponins, phytosterols, phenolic compounds, flavonoids and carbohydrates from various solvent extracts had been isolated from various species of the genus *Guilandina*[2,3]. Phytochemical screening of *G. bonducella*'s leaves and seeds, revealed the presence of non-toxic[13-17] and cytotoxic flavonoids[18]. Large number of diterpenenoid[13-15, 16, 17, 19-25, 27-31] and triterpenoid[32, 33] compounds chiefly from the ethanolic extracts of the seeds and other parts of this plant had previously been isolated and characterized by many research workers. Many fatty acids triglycerides, including palmatic, stearic, octadec-4-enoic and octadeca-2,4-dienoic acids from the seed kernels of this species had also been isolated and identified[35].

No attempt had been made to isolate and evaluate the harmful effects of its constituents. Our phytochemical and biological investigation of local natural products have led to the isolation of terpenoid compounds from *G. bonducella* seeds. In the present communication, we delineate the cytolysin potential of some of the di and triterpenoids, isolated from the seeds of this species, on brine shrimp's (*Artemia salina* Leach) larvae (nauplii) followed by fractionation, to isolate and characterize its active compounds whose cytolysin potential was evaluated by computing their LD_{50}.

METHODOLOGY

General Experimental Procedures

Unless otherwise stated, the chemicals used were of analytical grades. Concentrations were carried out under reduced pressure at bath temperatures not exceeding 50° C. Melting points were determinate on Perfit apparatus with the help of open capillary tubing and were unadmonished. UV spectra of the compounds were measured on Hitachi 270-30 spectrophotometer in MeOH while IR spectra were procured as KBr disc or as thin films on NaCl discs on Pye-Unicam SP-8-400 spectrophotometer. ^{1}HNMR spectra were obtained in deuterated DMSO-d_6 solvent on Bruker NMR at 270 MHz using tetramethylsilane (TMS) as an internal standard. ^{13}CNMR spectra were carried out on Bruker AM-300 NMR, spectrometers with 75 MHz, at 27±1.5°C and with 0.2-0.5 mM/ml sample concentrations, using 10 mm tubes and deuterated DMSO-d_6 as a solvent. Tetramethylsilane (TMS) was used as an internal reference. Chemical shifts were calculated for both ^{1}HNMR and ^{13}CNMR spectra in δ (ppm). EI and FD mass spectra were recorded on a Varian MAT-312 double focusing mass spectrometer using direct inlet method. FAB (positive) in glycerin, were conducted on JEOL JMS-110 spectrometer. Column chromatography was carried out on silica gel 60 (70-230 mesh ASTM No. 7734 of E. Merck, Damstadt, Germany), monitoring its fractions by analytical TLC. Both the analytical and preparatory TLC were performed with silica gel $PF_{254+366}$ (from E. Merck, Damstadt Germany) on 10×20 or 20×20cm glass plates. Analytical TLC with a depth of 0.25 mm thicknesses and preparatory TLC with 0.75 mm thick was utilized, where the samples were applied as thin sports on analytical TLC and as narrow bands on preparatory TLC. Spots on chromatograms were visualized by a combination of UV fluorescence, exposing on 254/365 nm UV light, or with I_2 vapors, or with anisaldehyde / H_2SO_4 spraying reagent or with Liebermann-Burchard spraying reagent[36]. The separated bands on preparatory TLC were scraped off and eluted with methanol.

Plant Materials

Ripened seeds of *Guilandina bonducella* L. were accumulated from the uncultivated and wasted areas of Lahore region of Punjab (Central plain areas of Pakistan) in July / August 2018. These were authenticated by Prof. Dr. Zaheer-Ud-Khan, in-charge herbarium, Department of Botany, Government College University, Lahore, Pakistan. A voucher specimen of the sample (No. **P-cog. 0156**) was kept in Herbarium of Pharmacognosy Section, Faculty of Pharmacy, University of Central Punjab, Lahore for further reference. The seeds were air dried at laboratory temperature and stored in an amber glass bottle after pulverizing.

Extraction and Isolation

8.0 kg of seed powder was soaked in MeOH for three weeks. It was percolated and the filtrate was concentrated under reduced pressure then to dryness to generate dark-brown 130g of a residue. The dried residue was partitioned between light petroleum ether (40-60°C) and H_2O. The aqueous layer was further concentrated and segmented between EtOAc and H_2O. The EtOAc extract was condensed by removing the solvent under reduced pressure and 461g (about 5.75% yield) of the material was obtained. 300g of the EtOAc extract was incorporated with a minimum amount of silica gel using methanol and after drying, it was pulverized into a fine powder. It was then adsorbed over silica gel column and chromatographed in light petroleum ether (40–60°C). The column was eluded with 100% light petroleum ether, petroleum ether-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH, while increasing the amount of latter solvent gradually. The fractions holding similar compounds were pooled after monitored by analytical TLC. The pooled fractions were evaporated to dryness under reduced pressure.

Compound-1

Compound-1 was eluded from the silica gel column with light petroleum ether/$CHCl_3$ (95:5) with the initial 50 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$ (90:10). It was obtained as colorless prisms like crystals (85mg, with 0.03% yield) and had mp. 262-264°C after re-crystallization with hot MeOH. This compound gave a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (70:30, 80:20 and 90:10) were used as solvent systems. $[\alpha]D^{28}$–52°(*c* = 0.036). FABMS, m/z: 489.2116 (Calcd for $C_{24}H_{36}O_9$. Na+: 489.2100). EIMS, m/z (rel. intens. %): 448 M^+–H_2O (1), 430 M^+–2×H_2O (11), 406 M^+–CH_3COOH (25), 388 M^+–CH_3COOH – H_2O (17), 370 M^+–CH_3COOH –2×H_2O (53), 346 M^+–2×CH_3COOH (44), 328 M^+–2×CH_3COOH –H_2O (89), 310 M^+–2×CH_3COOH –2H_2O (100). IR (KBr) cm^{-1}: 3586 (broad OH), 2948, 1734 (a strong ester group), 1364, 1257, 1229, 1036. UV λ_{max} (MeOH) nm (logε): 216 (4.16). ^{1}HNMR, δ: 5.68 (H-1, d, *J* = 2.9), 5.56 (H-2, ddd, *J* = 2.8, 4.2, 13.4), 2.32 (H-3α, dd, *J* = 13.1, 13.1), 1.40 (H-3β, dd, *J* = 4.2, 13.4), 2.43 (H-6α, dd, *J* = 5.8, 13.5), 1.88-1.94 (H-6β, m, *J* = 10.9, 13.1), 4.70 (H-7, ddd, *J* = 5.6, 10.6, 10.6), 1.93-1.97 (H-8, m, *J* = 10.6, 12.9), 3.31 (H-9, ddd, *J* = 2.6, 12.5, 12.5), α2.52(H-11, dd, *J* = 2.7, 12.6), β1.47 (dd, *J* = 12.7, 12.7), 3.88 (H-14, dq, *J* = 4.6, 7.3), 5.82 (H-15, s), 1.58 (Me-17, d, *J*= 7.3), 1.22 (Me-18, s), 1.14 (Me-19, s), 1.18 (Me-20, s), 1.98(CH_3COO, s), 2.13 (CH_3COO, s). ^{13}CNMR, δ: 74.4 (C-1), 67.6 (C-2), 35.4 (C-3), 40.5 (C-4), 78.6 (C-5), 36.5 (C-6), 66.2 (C-7), 47.9 (C-8), 32.6 (C-9), 45.5 (C-10), 38.6 (C-11), 106.4 (C-12), 171.3 (C-13), 33.6 (C-14), 113.6 (C-15), 175.2 (C-16), 13.2 (C-17), 28.2 (C-18), 25.6 (C-19), 17.8 (C-20), 170.5, 170.8 (CH_3CO), 20.6,

21.2 (CH_3CO) (Fig. 1). The compound-1 was identified by comparing its spectral data with the reported data and with CAS ID = C00033244 as neocaesalpin C[19].

Compound-2

Compound-2 was eluded from the column with light petroleum ether/$CHCl_3$ (85:15) with further 51 to 90 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$ (85:15). 153mg (with 0.052% yield) of this compound was obtained as colorless needle like crystals with mp 213-215°C after re-crystallization from hot EtOH. It appeared on TLC at hRf = 40 (with petroleum ether/$CHCl_3$ 90:15) and gave a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (70:30, 80:20 and 90:15) were used as solvent systems. $[\alpha]D^{25}$ +71.6°(*c* = 0.091). FABMS m/z: 433.2235 (Calcd for $C_{24}H_{32}O_7$. H+: 433.2227). EIMS, m/z (rel. intens. %): 414 (M^+-H_2O (7), 372 M^+-CH_3COOH (10), 354 $M^+-CH_3COOH-H_2O$ (42), 312 $M^+-2\times CH_3COOH$ (59), 294 $M^+-2\times CH_3COOH-H_2O$ (100). IR (KBr) cm^{-1}: 2946 (broad OH), 1790, 1769, 1733, 1375, 1259, 1234. UV λ_{max} (MeOH) nm (logε): 281 (4.26). ^{1}HNMR, δ: 5.70 (H-1, d, *J* = 3.2), 5.62 (H-2, ddd, *J* = 3.1, 4.8, 13.1), 2.34 (H-3α, dd, *J* = 13.1, 13.1), 1.38 (H-3β, dd, *J* = 4.8, 13.1), 1.70 (H-6α, ddd, *J* = 2.1, 2.5, 12.8), 1.58 (H-6β, ddd, *J* = 4.2, 12.8, 12.8), α 2.01-2.07m; β1.18 m(H-7), 1.75 (H-8, ddd, *J* = 4.2, 10.3, 10.3), 3.42 (H-9, br d, *J* = 10.3), 5.92 (H-11, br s), 2.68 (H-14, dq, *J* = 4.3, 7.3), 5.87 (H-15, d, *J* = 0.8), 0.92 (Me-17, d, *J* = 7.4), 1.13 (Me-18, s), 1.04 (Me-19, s), 1.06 (Me-20, s), 2.03 (CH_3COO, s), 2.09 (CH_3COO, s). ^{13}CNMR, δ: 73.4 (C-1), 67.8 (C-2), 36.3 (C-3), 40.8 (C-4), 76.8 (C-5), 26.6 (C-6), 23.7 (C-7), 37.8 (C-8), 36.6 (C-9), 45.5 (C-10), 111.3 (C-11), 151.5 (C-12), 161.9 (C-13), 33.6 (C-14), 110.9 (C-15), 170.8 (C-16), 14.6 (C-17), 27.4 (C-18), 24.8 (C-19), 19.8 (C-20), 170.3[b], 170.5[b] (CH_3CO), 20.6, 20.8 (CH_3CO) (Fig. 1). The compound-2 was identified by comparing its spectra data with the reported data and with CAS ID = C00033245 as neocaesalpin D[19].

Compound-3

Compound-3 was eluded from the column with light petroleum ether/$CHCl_3$ (80:25) with further 91 to 135 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$ (80:25). 73mg of this compound (0.025 % yield) was obtained as light yellow needles and with mp 183-184°C after re-crystallization from hot acetone. This compound indicated a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (60:40, 70:30 and 80:20) were used as solvent systems. EIMS, m/z (rel. intens. %): 426 [$C_{30}H_{50}O$, M^+] (24), 411 [M-Me]$^+$ (18), 408 [M-H_2O]$^+$ (24), 218 [M-$C_{14}H_{24}O$]$^+$ (100), 207 [M-$C_{16}H_{27}O$]$^+$ (15), 203 [M-$C_{15}H_{27}O$]$^+$ (56) and 189 [M-$C_{16}H_{29}O$]$^+$(68). IR (Thin film)cm^{-1}: 3512 (broad OH), 3058, 1638 and 822 (trisubstituted double bond). ^{1}HNMR, δ: 1.98 (ddd, *J* = 8.1, 9.6, 4.0H_z, H-1), 1.92 (m, H-2), 3.13 (dd, *J* = 5.5, 8.0H_z, H-3), 1.27

(m, H-5), 1.52 (m, H-6), 1.31 (m, H-7), 1.57 (dd, *J* = 2.1, 9.1H_z, H-9), 1.67 (dd, *J* = 9.1, 3.5H_z, H-11), 5.24 (m, H-12), 1.51 (dd, *J* = 9.1, 3.5H_z, H-15), 1.30 (dd, *J* = 16.1, 8.3H_z, H-16), 1.40 (m, H-19), 1.57 (dd, *J* = 2.1, 9.1H_z, H-21), 1.65 (*dd, J* = 9.1, 3.5H_z, H-22), 0.84 (brs, H-23), 0.95 (brs, H-24), 0.90 (brs, H-25), 0.74 (brs, H-26), 1.06 (brs, H-27), 0.78 (brs, H-28), 0.92 (3H, d, *J* = 6.7 H_z, H-29), 0.81 (d, *J* = 6.9 Hz, H-30). ^{13}CNMR, δ: 40.1 (C-1), 27.5 (C-2), 78.1 (C-3), 37.5 (C-4), 55.6 (C-5), 20.7 (C-6), 32.7 (C-7), 40.6 (C-8), 48.1 (C-9), 37.5 (C-10), 23.6 (C-11), 124.3 (C-12), 138.7 (C-13), 41.5 (C-14), 28.8 (C-15), 27.7 (C-16), 34.1 (C-17), 59.8 (C-18), 40.1 (C-19), 39.2 (C-20), 31.2 (C-21), 42.8 (C-22), 28.6 (C-23), 16.1 (C-24), 16.2 (C-25), 17.5 (C-26), 24.1 (C-27), 28.8 (C-28), 17.5 (C-29),20.7 (C-30) (Fig. 1). The compound-3 was recognized by comparing its spectra data with the reported data and with CAS ID = C0003737 as α-Amyrin[33].

Compound-4

Compound-4 was obtained from the column with light petroleum ether/$CHCl_3$ (80:30) with further 136 to 175 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$/MeOH (75:25:3). 67 mg of this compound (0.023% yield) was obtained as light yellow needles after re-crystallization from hot EtOH and with mp. 197–198°C. This compound showed a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (60:45, 70:35 and 80:25) were used as solvent systems. EIMS, m/z (rel. intens. %): 426 [$C_{30}H_{50}O$, M^+] (16), 411 [M-Me]$^+$(17), 408 [M-H_2O]$^+$(18), 393 [M-Me-H_2O]$^+$(34), 257 [M-C_{11} H_{21} O]$^+$(20), 218 [M-C_{14} $H_{24}O$]$^+$(100), 207 [M-C_{16} H_{27} O]$^+$(11), 203 [M-C_{15} H_{27} O]$^+$(46) and 189 [M-C_{16} H_{29} O]$^+$(58). IR, (Thin film)cm^{-1}: 3510 (broad OH), 3055, 1636 and 820 (trisubstituted double bond). ^{1}HNMR, δ: 1.31 (ddd, *J* = 8.2, 6.1, 11.1H_z, H-1), 1.60 (m, H-2), 1.37 (m, H-6), 1.40 (m, H-7), 1.87 (dd, *J* = 4.7, 3.5H_z, H-11), 1.60 (dd, *J* = 3.1, 4.0H_z, H-15), 1.53 (m, H-16), 2.77 (dd, *J* = 11.3, 6.2H_z H-18), 1.37 (m, H-19), 1.87 (dd, *J* = 4.7, 3.5H_z, H-21), 1.45 (dd, 3.5, 4.0H_z, H-22), 1.03 (3H , s, Me-23), 0.82 (6H, s, Me-24), 0.94 (3H, s, Me-25), 1.05 (3H, s, Me-26), 1.12 (3H, s, Me-27), 0.80 (brs, Me-29) and 0.91 (3H, brs, Me-30). ^{13}CNMR, δ: 40.1 (C-1), 27.7 (C-2), 78.3 (C-3), 37.5 (C-4), 55.4 (C-5), 20.7 (C-6), 32.7 (C-7), 39.2 (C-8), 48.1 (C-9), 37.5 (C-10), 23.6 (C-11), 122.6 (C-12), 145.5 (C-13), 41.5 (C-14), 27.6 (C-15), 27.7 (C-16), 34.1 (C-17), 48.1 (C-18), 48.0 (C-19), 31.2 (C-20), 34.0 (C-21), 37.5 (C-22), 28.6 (C-23), 16.1 (C-24), 16.0 (C-25), 17.5 (C-26), 27.5 (C-27), 28.6 (C-28), 32.7 (C-29), 23.6 (C-30) (Fig. 1). The compound-4 was identified by comparing its spectra with reported data and with CAS ID = C0003738 as β-Amyrin[33].

Compound-5

Compound-5 was received from the column with light petroleum ether/$CHCl_3$ (60:40) from further 176 to 216 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$/MeOH (70:30:5). 149 mg of this compound (0.051% yield) was obtained as white needles after re-crystallization from hot Me_2CO-MeOH (1:1 mixture) and with mp. 216–217°C. This compound displayed a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (60:45, 70:35, 80:25) were used as solvent systems. EIMS, m/z (rel. intens. %): 426 [$C_{30}H_{50}O$, M $^+$](21), 411 [M-Me]$^+$(26), 408 [M-H_2O]$^+$(32), 393 [M-Me-H_2O]$^+$(36), 385 [M-41]$^+$(14), 220 [M-C_{15} H_{26}]$^+$(82), 218 [M-C_{14} $H_{24}O$]$^+$(56), 207 [M-C_{16} H_{27}]$^+$(24), 189 [M-C_{16} H_{29} O]$^+$(100) and 139 [M-C_{21} H_{35}]$^+$(71). IR, (Thin film)cm^{-1}: 3452 (broad OH), 3076, 1645 and 883 (exomethylene group). ^{1}HNMR, δ: 4.78 and 4.65 (2H, brs, 1H each, H-29), 3.22 (1H, dd, J = 9.6 H_z, J = 4.7 H_z, H-3), 1.64 (3H, brs, Me-30), 1.07 (3H, s, Me-26), 0.96 (3H, s, Me-23), 0.98 (3H, s, Me-27), 0.84 (3H, s, Me-25), 0.81 (3H, s, Me-28) and 0.80 (3H, s, Me-24). ^{13}CNMR, δ: 38.63 (C-1) 27.53(C-2), 78.82(C-3), 38.75 (C-4), 55.30(C-5), 18.32(C-6), 34.28 C-7), 40.84(C-8), 50.46C-9), 37.12 (C-10), 20.98(C-11), 25.27(C-12), 38.18 (C-13), 42.86 (C-14), 27.41(C-15), 35.52 (C-16), 92.94(C-17), 48.24(C-18), 47.79 (C-19), 150.66 (C-20), 92. 91(C-21), 39.88(C-22), 28.06(C-23), 15.49(C-24), 16.16(C-25), 15.92(C-26), 14.54 (C-27), 18.15 (C-28), 109.28 (C-29) and 19.26(C-30) (Fig.1). The compound-5 was recognized by comparing its spectral data with the reported data and with CAS ID = C00029492 as being Lupeol[31, 32 34].

Compound-6

Compound-6 was prevailed from the column with light petroleum ether/$CHCl_3$ (50:50) with further 217 to 257 fractions (50ml each) and by preparatory TLC after using petroleum ether/$CHCl_3$/MeOH (60:40:7). 67 mg of this compound (0.022% yield) was obtained as white molded acicular crystals after re-crystallization from hot MeOH and with mp. 213-214°C.This compound demonstrated a single spot on three-dimensional TLC when petroleum ether/$CHCl_3$ (50:50, 70:30 and 80:20) were used as solvent systems. EIMS, m/z (rel. intens. %): 468 [$C_{23}H_{52}O_2$, M $^+$](56), 453 [M-Me]$^+$(12), 427 [M-C_3H_5](8), 408 [M-AcOH]$^+$(21), 393 [(M-Me)-AcOH]$^+$(4), 249 [M-C_{16} H_{27}]$^+$(26), 218 [M-$C_{16}H_{26}$ O_2]$^+$(39), 189 [(M-C_{16} H_{27})-AcOH]$^+$(65), 181[M-C_{21} H_{35} O]$^+$(16) and 121[(M-$C_{21}H_{35}$O)-AcOH]$^+$(49). IR (Thin film) cm^{-1}: 3077 (broad OH), 1712 (ester carbonyl), 1648 and 884 (exomethylene group). ^{1}HNMR, δ: 4.74 and 4.64 (2H, brs, 1H each, H-29), 4.24 (1H, dd, J = 9.8H_z, J = 4.5H_z, H-3), 2.13 (3H, s, CH_3COO), 1.68 (3H, dd, J = 1.28 H_z, Me-30), 1.08 (3H , s, Me-26), 0.96 (3H, s, Me-23), 0.96 (3H, s, Me-27), 0.89 (3H, s, Me-25), 0.77 (3H, s, Me-28) and 0.78 (3H, s, Me-24). ^{13}CNMR, δ: 38.46

(C-1), 23.78 (C-2), 81.08 (C-3), 37.84 (C-4), 55.44 (C-5), 18.25 (C-6), 34.36 (C-7), 40.98 (C-8), 50.49 (C-9), 37.13 (C-10), 21.08 (C-11), 25.17 (C-12), 38.16 (C-13), 42.98 (C-14), 27.54 (C-15), 35.68 (C-16), 43.12 (C-17), 48.09 (C-18), 48.35 (C-19), 152.14 (C-20), 30.15 (C-21), 40.06 (C-22), 28.08 (C-23), 16.59 (C-24), 16.27 (C-25), 16.07 (C-26), 14.54 (C-27), 18.08 (C-28), 19.37 (C-29), 109.53 (C-30), 21.35 (CH_3COO) and 170.88 (CH_3COO), (Fig. 1). The compound-6 was identified by comparing its spectral data with the reported data and with CAS ID = C0003750 as being Lupeol acetate[33, 34].

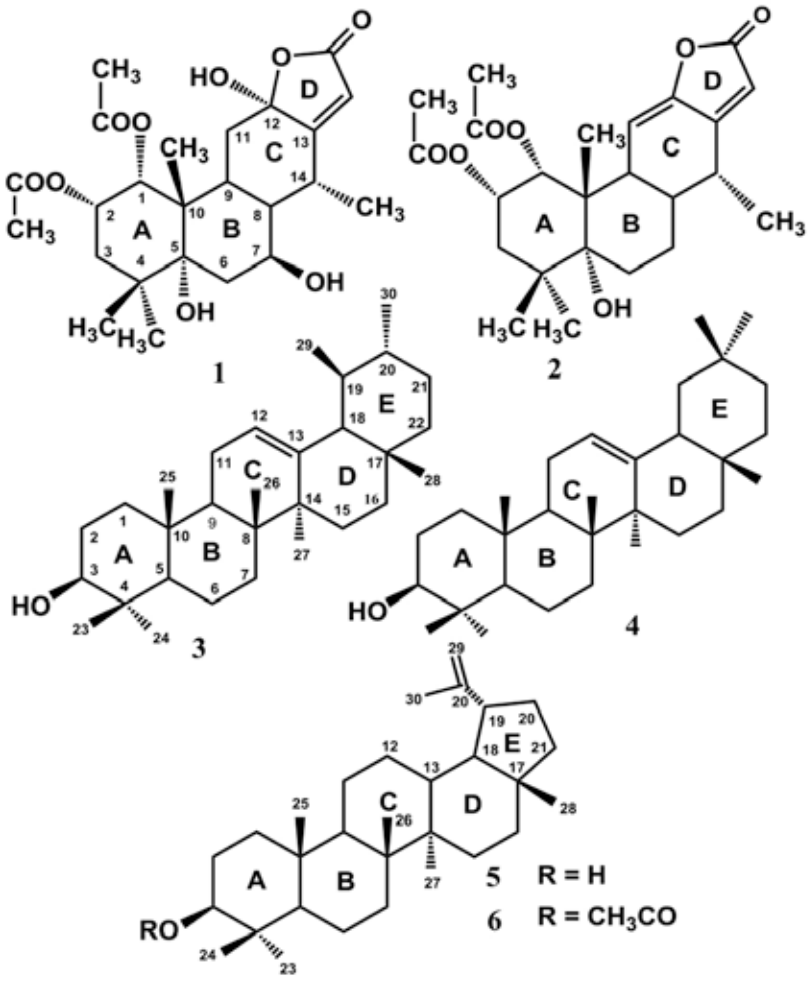

Figure 1: Diterpenoids and triterpenoids isolated from the seeds of *G. bonducella.*

Brine Shrimp (*Artemia salina* Leach) Lethality Bioassay

This assay was adapted from the literature[42-46]. Eggs of brine shrimps (*Artemia salina* Leach) were purchased from a local fish store. A brine shrimp container was filled with artificial sea water (about 3.8%) [44-46]. The seawater was incorporated with three different salts like $MgCl_2.6H_2O$, Na_2SO_4 and $CaCl_2 \cdot 2H_2O$[44]. Sea salt and yeast suspension (3mg dried yeast for each 5ml seawater sample) was also bought from the local fish store. Syringes of 5ml, 1ml, 500µl, 300µl, 200µl, 100µl, 50µl and 10µl capacity and 2 dram vials (9 per sample and 3 for control) were also redeemed from the indigenous market. Sea salt solution was prepared artificially by dissolving 38g sea salt in 1000 ml distilled water. The final solution was filtered. The filtrate was taken in a small plastic tub that was divided by a partition, having holes in it. The brine shrimp's eggs were sprinkled in one portion of the tub and covered with a black carbon paper. Other half of the tub was illuminated with an electric lamp to attract the hatched brine shrimp's larvae. The solution in the tub was constantly supplied with regular air flow with at a normal pressure and suitable light conditions which were essential for the hatching process[42-46]. After 48 hours, the shrimp's eggs were hatched and matured as nauplii. The mature nauplii were then used further in the experiment. 20mg of each of the compound was taken in a small vial and dissolved in 2ml of methanol to serve as stock solution. From the stock solution, 500µl's, 400µl, 300µl, 200µl, 100µl, 80µl, 60µl, 40µl, 20µl, 10µl, 5µl 2.5µl and 1.25µl (corresponding to the 1000, 800, 600, 400, 200, 160, 120, 80, 40, 20, 10, 5 and 2.5µg respectively) were transferred to the vials with three replicates of each concentration of the isolated compound. The vials were placed in an open area for 24 hours for complete evaporation of methanol. 2ml of sea salt solution was then added to each vial. 10 brine shrimp's larvae were reassigned to each vial (30 brine shrimp's larvae per dilution) with the help of a long-tipped dropper. Total volume of liquid in each vial was adjusted to 5ml with sea salt solution. Sluggish or anechoic brine shrimp's larvae were counted for all concentrations of isolated compounds after 24 hours. Colchicine[44-46] in the same concentrations was utilized as positive control. Total number of annihilated brine shrimps per dilution of each compound was tabulated. LC_{50} (lethal concentration in 50% individuals) along with the upper and lower confidence limits of each compounds were calculated by probit analysis[47], using a computer program[48]. The number of obliterated brine shrimp's larvae due to the results of the effects acquired by the six isolated compounds from the ethyl acetate extract of the pulverized seeds of *G. bonducella* and also by colchicine, their LC_{50}, along with the upper and lower confidence limits had been outlined in Table-1.

Dose levels (µg/ml)	Compounds						
	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Colc.
500	*26/30†	25/30	18/30	15/30	21/30	22/30	30/30
400	22/30	23/30	17/30	14/30	20/30	21/30	27/30
300	20/30	21/30	11/30	12/30	18/30	18/30	25/30
200	18/30	20/30	10/30	10/30	17/30	17/30	22/30
100	15/30	14/30	07/30	08/30	12/30	10/30	20/30
80	12/30	10/30	05/30	07/30	10/30	09/30	17/30
60	07/30	08/30	04/30	06/30	08/30	07/30	15/30
40	06/30	05/30	03/30	04/30	06/30	05/30	13/30
20	05/30	04/30	01/30	02/30	03/30	02/30	12/30
10	03/30	02/30	01/30	01/30	02/30	01/30	10/30
5.0	01/30	01/30	01/30	01/30	01/30	01/30	08/30
2.50	01/30	01/30	00/30	0/30	——	——	05/30
1.25	00/30	00/30	00/30	0/30	——	——	02/30
LD_{50} (µg)	26.421	28.329	460.562	532.326	27.342	76.797	15.061
U.C.L.	40.321	41.201	204.932	980.22	208.35	121.623	125.331
L.C.L.	15.320	16.131	115.354	350.31	329.789	45.326	9.732
χ^2	1.712	1.561	1.671	1.621	0.208	1.962	1.80

Where:— **Comp. 1** = Neocaesalpin C; **Comp. 2** = Neocaesalpin D; **Comp. 3** = α-Amyrin; **Comp. 4** = β-Amyrin; **Comp. 5** = Lupeol; **Comp. 6** = Lupeol acetate; **Colc.** = Colchicine.

* = Number of brine shrimp's larvae killed after 24 hours; † = Total number of brine shrimp's larvae used; LD_{50} = Lethal dose where 50% brine shrimps were killed; U.C.L. = Upper confident limit; L.C.L. = Lower confident limit; χ^2 = Chi square.

Table 1: Cytolysin potentials of the compounds isolated from the seeds of *G. bonducella* L. herb on brine shrimps.

RESULTS AND DISCUSSION

It was a common observation that the disturbing feeling of *Guilandina bonducella* L. seeds during harvesting season of the crop, was demonstrated in most of the local farmers who deals with the removal of seeds from the plant. Skins of fingers, specifically the internal skin of index and first finger of their right hands were frequently involved. It often developed inflammatory eruption, after prolong handling seeds of the plants. Such skin eruption appeared to be due to some of the stringy actions induced by some of the materials from the seeds of the plant. This reaction was settled down after five or six days. This type of lubricious

response of the seeds of this species on human skin motivated us to probe into the chemical nature of its hostile active compounds.

During the preliminary cytolysin attempt, it was ascertained that the MeOH extract of *G. bonducella* seeds was not fatal to the of brine shrimp's (*Artemia salina*) larvae (nauplii) than the EtOAc and H_2O extracts. Moreover, EtOAc extract of the seeds appeared to be more assertive towards the brine shrimp's larvae, as compared to the H_2O extract. EtOAc extracts was thus further fractionated through silica gel column, analytical thin-layered and preparatory thin-layered chromatography to isolate its active cytolysin ingredient/s. Six active cytolysin compounds, along with a number of non-active components were isolated from this extract and purified by chromatographic and re-crystallization methods. First two active compounds were identified as diterpenoid while other four were recognized as being triterpenoids by comparative physical and spectroscopic data (Fig. 1). Their spectroscopic data were based on EIMS, FAB-MS,^{1}HNMR and ^{13}CNMR assignments. The structures of both the diterpenoid, compound-1 and compound-2 (i.e., neocaesalpin-C; neocaesalpin-D) were established by comparing their physical and spectroscopic data with previously reported similar compounds[19] (Fig. 1) while the structures of the four triterpenoid compounds, compound-3 to compound-6 (i.e., α-amyrin[33], β-amyrin[33], lupeol[32,34] and lupeol acetate[34]) were established after comparison with previously described compounds (Fig. 1).

Formerly many research workers had made good use of brine shrimp's larvae (i.e., nauplii of *Artemia salina*) assay for assessing the cytotoxicity and cytolysin potential of solvent extracts, fractions and phytochemical compounds from different natural crude drugs[42-46]. It appeared that the brine shrimp lethality bioassay was a simple measure for cytolytic potential of the natural products and their isolated compounds[42-46]. It was thus utilized to assist the bio-active maneuvering fractions which on conclusion lead to the bioactive cytolysin phytochemical compounds from our natural products. It was estimated that the difference between toxicity and efficacy of a drug was its dose. This assay often indicated that the fractionation of solvent extracts of natural products guided towards most-valuable bioactive toxic phytochemical compounds. Cytolysin activities were frequently expressed by the research workers in ppm or in μg as LC_{50} (Lethal dose where 50% of individuals in a population were killed) values with 95% confidence intervals[42-46].

To compare the cytolysin potential of these compounds, the brine shrimp assay was engaged in measuring the LD_{50} at the time, at which the death of the brine shrimp's larvae was ascertained. The input data for a computer program consisted of the dose of testing materials (i.e., MeOH solution, EtOAc extract, column fractions or isolated compounds), the total number of test animal's larvae used

and the number of test larvae responding (i.e., the number of dead larvae) to that dose. The program transformed the dose to the *log* dose and the test animal's larvae reacted to the *probit* of percentage responses. It then make fit a probit regression line to the resulting points and computed the values for LD_{50} along with their upper and lower confident limits[47]. The output data consisted of a listing of LD_{50}, upper and lower confident limits and a value of χ^2. The purpose of the χ^2 test was to detect whether the assay, after transformation, was satisfactorily represented by a probit regression line. If the χ^2 test pointed out a divergence of transformed results from linear shape, these could not be assigned to a random biological variation (i.e. if χ^2 value is not significant at $p > 0.05$, then the results obtained by probit analysis would not be legitimate)[47].

The results indicated that both the EtOAc and H_2O extracts of *G. bonducella* seeds had cytolysin potential against the brine shrimp's larvae but the EtOAc extract was even more violent cytolysin than H_2O extract, when compared with the known cytolysin compound, colchicine[44-46]. Colchicine was employed as positive controlled cytolysin material in this bioassay[44-46]. All the six terpenoid compounds (compound-1 to compound-6) (Fig.1) from this extract revealed a cytolysin potential against brine shrimp's (*Artemia salina*) larvae (nauplii) when compared with colchicine applied in the same concentrations[45] (Table 1). The results also demonstrated that among all the six isolated compounds, the compound-1 (neocaesalpin C) and compound-2 (neocaesalpin D) were the most active cytolysin compounds (with LD_{50} = 26.421 and 28.329). Their LD_{50} values were nearly close to the colchicine (LD_{50} = 15.061). Moreover, the compound-1 (neocaesalpin C) appeared to be more active than compound-2 (neocaesalpin D) and exhibited the highest cytolysin activity (Table-1). Other four compounds i.e., compound-3 (α-amyrin); compound-4 (β-amyrin); compound-5 (lupeol), compound-6 (lupeol acetate) (with LD_{50} = 460.562, 532.326, 27.342 and 76.797 respectively) displayed a lesser cytolysin activity than colchicine (Table-1). The results also indicated that the two compounds i.e., compound-3 (α-amyrin) and compound-4 (β-amyrin) demonstrated the least cytolysin potential (with LD_{50} = 460.562 and 532.326) against the brine shrimp's larvae than colchicine (Table-1).

The potent cytolytic / toxic effect on brine shrimp's larvae, induced by neocaesalpin C, neocaesalpin D, lupeol and lupeol acetate from *G. bonducella* seeds was probably due to a rapid penetration through the larvae's skin and quickly bioavailable to the living tissues of the animals. These compounds perhaps caused a blockage of respiratory centers which ultimately stimulated a quick tissue deterioration in the larvae leading to their death. The comparatively less toxic reaction of α-amyrin and β-amyrin was possibly due to their direct actions, at some of the receptor sites in the animal's larvae.

It was concluded that the EtOAc extract of *G. bonducella* seeds contained related cytolysin di and triterpenes which could be hostile not only to the brine shrimp's larvae but might also be insalubrious to the bodies of higher animals and human beings. Further work was necessitated to amplify this property through the preparation of derivatives of these active molecules, which would perhaps be elaborated for the structure-activity relationship of such important cytolysin molecules, both for *in vivo* and *in vitro* studies. These cytolysin molecules and their derivatives might also be important against animal's and human's cancerous tissues, which could further be tested with the standard processes of WHO[49]. Further work had also been designed to ascertain some cytolysin inhibitor/s from our natural sources, which could overcome the adverse action of such phytochemical compound/s from *G. bonducella* seeds and related species of the family Caesalpiniaceae.

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Fully Investigation of RP- HPLC Analytical Method Validation Parameters for Determination of Cefixime Traces in The Different Pharmaceutical Dosage Forms and Urine Analysis

Mostafa F. Al-Hakkani [1,2*], Gamal. A. Gouda[1], Sedky H.A. Hassan[3], Osman A. Farghaly[1], and Mahmoud M.A. Mohamed[2]

1 Department of Chemistry, Faculty of Science, Al-Azhar University, Assiut Branch, 71524, Assiut, Egypt
2 Department of Chemistry, Faculty of Science, New Valley University, El-Kharja 72511, Egypt
3 Department of Botany & Microbiology, Faculty of Science, New Valley University, El-Kharja 72511, Egypt

ABSTRACT

Cefixime (Cfx) is a member of the third generation of Cephalosporin antibiotics. It used on a wide scale in prescribed antibiotic drugs as anti-infection for *Gram-positive* and *Gram-negative* microorganisms. The present study aimed to develop an HPLC method of Cfx analysis enjoyed highly linearity, repeatability, robustness, ruggedness, selectivity, rapidly, and economical to use. The chromatographic system depends on the RP- BDS column (250 mm x 4.6 mm x 5 μm). The mobile phase was prepared by mixing Methanol: Phosphate buffer (3:7, v/v) at flow rate 1.0 ml/min with wavelength detection at 254 nm, the temperature at 30° C with injection volume 20 μL. The method revealed that satisfied linearity regression R^2 (0.9996) with repeatability (0.94%) with DL and QL; 59.3 ng/ml and 179.8 ng/ml respectively. The method showed successful and satisfying results for Cfx in bulk and pharmaceutical formulations and urine samples at low levels.

Keywords: Validation, Pharmaceuticals, Cefixime, Detection limit, Quantitation limit

INTRODUCTION

The IUPAC name of Cfx is (6R,7R)-7-[[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2(carboxymethoxyimino)acetyl] amino]-3-ethenyl-8-oxo-5-thia-1-azabicyc-

Corresponding author: Mostafa El Hakkani
Authors contacts:
Gamal. A. Gouda: ggouda73@azhar.edu.eg
Sedky H.A. Hassan: sedkyhassan@scinv.au.edu.eg
Osman A. Farghaly: othman15@yahoo.com
Mahmoud M.A. Mohamed: mmhm802004@gmail.com

lo[4.2.0]oct-2ene-2-carboxylic acid [1]. Cfx is a member of the third generation of the Cephalosporin antibiotics. It was derived semi-synthetically from the marine fungus Cephalosporium acremonium. Cfx contains the Cephalosporins β-lactam core ring as shown in Figure 1 A, B.

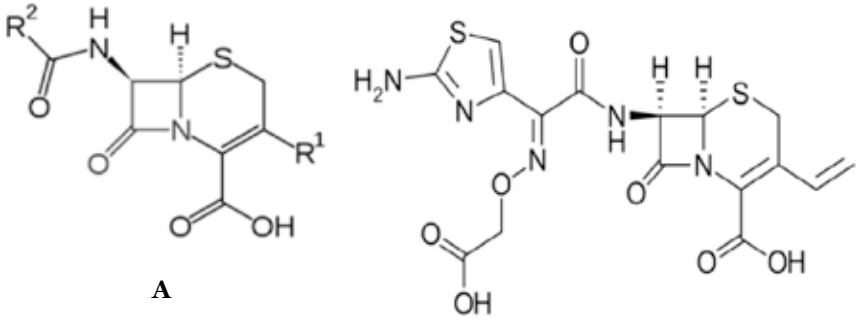

Figure 1. Structure of Cephalosporins *β*-lactam core ring (A) and Cefixime (B)

It used to treat many and various bacterial infections and it has excellent activity against many pathogens as, Enterobacteriaceae, Anaerobes, *Gram-negative* class such as *Haemophilus influenzae*, *Branhamella Catarrhalis*, *Escherichia coli*, *Neisseria gonorrhoeae, klebsiella, Serratia marcescens, Haemophilus, Providencia,* and *Meningococcus* including strains of β -lactamase producing. It is the best oral antibiotic for switch therapy due to its safety profile, high efficacy. Additionally, it has an inexpensive nature [2]. It works by killing bacteria and it has an analytically and clinically significant due to its broad spectrum as stability and antimicrobial activity [3]. Cfx is used for the reduction of the development of drug-resistant bacteria. It is introducing under different finished products; a powder for oral suspension, capsules, and tablets [4].

Several analysis methods have been developed to determine Cfx in different pharmaceutical dosage forms. These methods include different analysis techniques as microbiological methods and high-performance liquid chromatography (HPLC) [5].

Cfx has been quantitatively analyzed in bulk materials and different pharmaceutical dosage forms by Spectrofluorimetric [6-8], Spectrophotometric determination [9-11] Colourimetry, HPLC by capillary electrophoresis [12], Voltammetric determination [13-15], HPLC-MS; mass spectrometric methods may have the highest sensitivity, but the determination process is complicated to use and very expensive [2, 16].

Chromatographic separation technique is one of the most convenient, essential, easiest, and powerful in most qualitative and quantitative analysis. HPLC is currently the most satisfying tool for excellent and optimum separation [5, 17-19].

In the present study, an HPLC method with a photodiode array detector (PDA) was developed for the determination of the lower concentration of Cfx in different pharmaceutical dosage forms. The proposed analytical method of Cfx was found to be precise, repeatable, linear, accurate, rugged, robust, specific, selective, and economic.

METHODOLOGY

Cfx standard (99.7%) was supplied by Covalent laboratories PVT.LTD (India) as a gift sample from Smart pharma (Assuit, Egypt). Methanol HPLC-grade, Sodium dihydrogen phosphate, Hydrochloric acid, Phosphoric acid 85%, Sodium hydroxide, and Hydrogen peroxide (Scharlau, Spain). Deionized water used in the analysis was prepared by reverse osmosis and passed through a 0.45 μm Millipore filter (Millipore Company, USA) before use. Phosphate buffer was prepared by weighing about 16.8 g of sodium dihydrogen phosphate and 0.5 ml of phosphoric acid 85% in 700 ml deionized water.

Chromatographic system configuration

Cfx was measured using the LC-20A HPLC instrument with the PDA (Shimadzu, Japan). The method was conducted using the RP BDS column (250 mm x 4.6 mm x 5 μm) (Thermo Scientific, USA). The mobile phase was prepared at the ratio "Methanol: Phosphate buffer" (3:7, v/v) at flow rate 1.0 ml/min with wavelength detection at 254 nm with column oven 30° C and injection volume 20 μL.

Parameters of method validation

The validation of the HPLC method was carried out according to International Conference on Harmonization (ICH), Food and Drug Administration (FDA), United States of American Pharmacopoeia (USP) and European Pharmacopoeia (EP) guidelines concerning parameters including tuning system and suitability of the system, Range linearity, detection limit, quantification limit, repeatability, recovery and accuracy, robustness, ruggedness, the stability of the solution, specificity and selectivity [20-25].

Tuning and suitability of the system

The performance of the chromatographic system comes first. So, the instrument performance was checked at a standard tuning solution was prepared in the mobile phase at a concentration of 2.0 μg/ml.

Range & linearity

It was said the method is linear if there is a good proportion between the response and working concentration starting from the lowest point in the tested range and the highest point and the R^2 should be ≥ 0.999 [20-24].

Regression linearity equation:

Y = a X+b (1)

Where, Y= Peak area, X= Concentration (%), a is the slope and b is the intercept.

Linearity was conducted using different five concentrations (50%-150%) of the Cfx standard. The working concentrations were prepared as, 1.0, 1.6, 2, 2.4, and 3.0 µg/ml using the mobile phase as a solvent. The later solutions were injected in triplicates.

Detection limit (DL)

It was defined as the minimum concentration of the analyte in the matrix that can be distinguished using the instrument detector. Additionally, it should not be represented in the precision and linearity range [20-24].

Quantitation limit (QL)

It was defined as the minimum concentration of the analyte in the matrix that can be distinguished using the instrument detector. On the contrary to the detection limit, it should be represented in the precision and linearity range [20-24].

DL and QL were calculated according to the linearity of the calibration curve and its standard error according to the following equations:

DL = 3.3 σ / S (2)

QL = 10 σ / S (3)

Where σ: is the standard error and S: is a slope of the linearity calibration curve.

Accuracy and recovery

Recovery and accuracy, each of them are used interchangeably. The accuracy of the measurement is defined as the closeness of the actual concentration (measured value) to the theoretical concentration (true value) [20-24].

Accuracy and recovery were conducted using the addition of three sets of Cfx standard to the in-active ingredient of the drug to give concentration at (1.6 µg/ml), (2.0 µg/ml), and (2.4 µg/ml). recovery estimation was linearity equation dependent:

Recovery %= Act. Conc.% / Th. Conc.% x 100 (4)

Repeatability and precision

Repeatability was conducted using 6 different preparations of the concentration (2.0 µg/ml) of Cfx by the same analyst on the same day using the same equipment [20-24].

Robustness

Robustness was investigated using conscious small changes including the slight diversity in the temperature, composition of the mobile phase, etc [20-24].

Changes were involved in a different organic solvent ratio (Methanol) at (± 10%) and different temperature ± 2^0 C.

Ruggedness

Ruggedness was investigated using conscious and major observable changes including analyst- analyst, column- column, and day- day with maintaining on the rest of experimental parameters and conditions at a constant rate.

Stability of solution

This test was conducted *via* performing the test at the target concentration of (2.0 µg/ml). It was measured over 12 working hours to assess the stability of the solution.

Specificity and selectivity

It can be defined as the measuring of the analyte in the presence of its degradants or interferences interpreted the connotation of specificity [20-24].

- Acid hydrolysis: It was conducted using HCl 0.1 N for 5 minutes.

- Oxidation hydrolysis: It was conducted using H_2O_2 3% *wt/v* for 5 minutes.

Test of the validated method

Cfx analysis in the different commercial dosage forms in the Egyptian local market

Suprax 200 mg capsules, Suprax 100 mg/5ml powder for oral suspension, and Cefipharmart 400 mg dispersible tablet for oral suspension were be tested using the validated method of Cfx.

Cfx traces analysis in the different urine samples

The method was tested for identification and quantitative analysis for 4 different urine samples.

RESULTS AND DISCUSSION

Tuning and suitability of the system

Cefixime peak was stated about at 7.8 minutes as revealed in **Figure 2**. **Table 1** showed a good performance for the selected method parameters where the RSD % < 2.0 % [20-24].

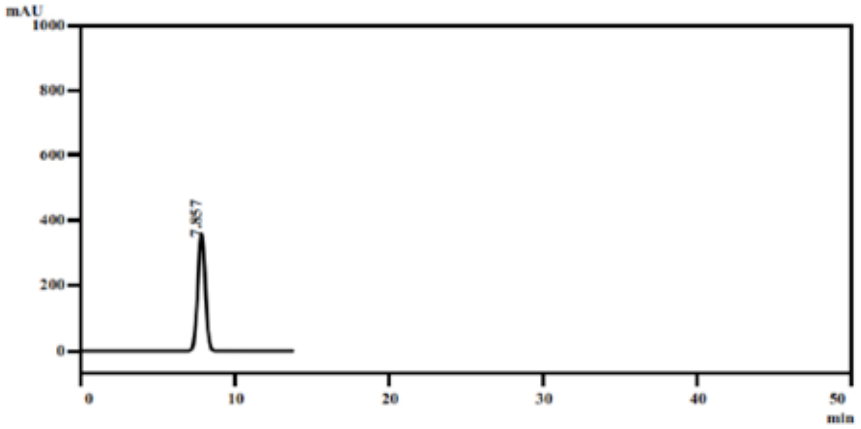

Figure 2. Cfx chromatogram

Table 1. Tuning and suitability of the system

Replicate #	P. A	Tailing	Plates
1	4651	1.221	12521
2	4655	1.223	12565
3	4658	1.218	12476
4	4630	1.216	12515
5	4684	1.218	12542
6	4628	1.218	12548
RSD%	0.44%	0.21%	0.25%

Range and linearity

The results revealed high linearity "R^2 = 0.9996" in between the working concentration range (50 %-150 %) as we can see in Figure 3 and Table 2.

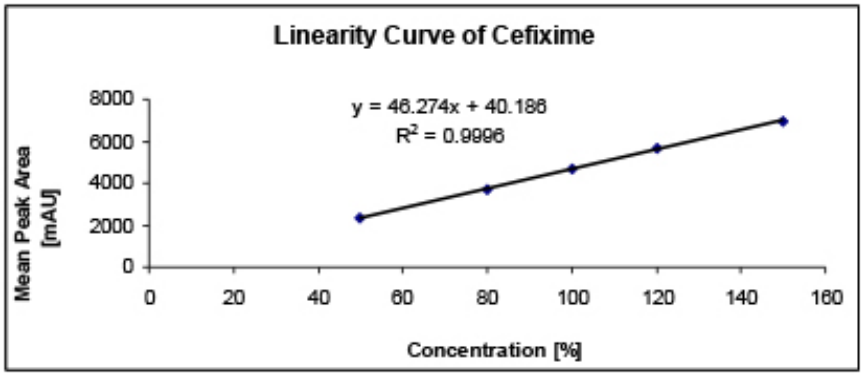

Figure 3. Linearity calibration curve of Cfx

Table 2. Range and linearity

Conc %	Average P. As	Statistical data	
50%	2351	R^2	0.9996
80%	3705	Slope	46.274
100%	4702	Intercept	40.186
120%	5632	Standard error	41.60657
150%	6948		

DL and QL

DL and QL limits were determined simply using the linearity calibration data of Cfx. DL was found to be 59.3 ng/ml where QL was 179.8 ng/ml.

Accuracy and recovery

Table 3. Revealed satisfaction results for recovery and accuracy within the tested range (80-120 % from the target concentration).

Table 3. Accuracy and recovery

Theoretical conc%	Average P. As	Actual conc%	Recovery %
80%	3798	81.2%	101.5%
100%	4715	101.0%	101.0%
120%	5539	118.8%	99.0%

Repeatability and precision

The RSD% of Peak areas was used for judgment on the repeatability of the analyte using six different preparations at the same concentration as in Table 4. It was found to be 0.94 % as it demanded in repeatability requirements [20-24].

Table 4. Repeatability and precision

#	Sample P. A	Statistical data	
1	4605	Average P. As	4622.333
2	4670	STDEV	43.509
3	4574	RSD%	0.94%
4	4590		
5	4614		
6	4681		

Robustness

The results of conscious small changes included temperature ± 2^0 C and organic (±10 %) were determined using RDS %. The RSD% was found to be < 2 % in all cases as shown in Tables 5 and 6.

Table 5. Change in temperature results

Replicate #	Set # 1 30° C	Set # 2 (32° C)	Set # 3 (28° C)
1	4651	4698	4616
2	4655	4691	4605
3	4658	4686	4594
4	4630	4712	4587
5	4644	4674	4621
6	4628	4695	4600
Pooled mean		4646.944	
Pooled RSD%		0.84%	

Table 6. Change in organic ratio results

Replicate #	Set # 1 300 ml	Set # 2 330 ml	Set # 3 270 ml
1	4651	4777	4574
2	4655	4779	4545
3	4658	4786	4596
4	4630	4751	4550
5	4644	4758	4558
6	4628	4735	4555
Pooled mean		4657.222	
Pooled RSD%		1.86%	

Ruggedness

The results of conscious major and observable changes including analyst-analyst, column-column, and day-day. Data were be presented as shown in Tables 7-9. RSD % found to be < 2 % in all cases [20-24].

Table 7. Day-to-day precision results

Replicate #	Set # 1 First day	Set # 2 Second day	Set # 3 Third day
1	4651	4718	4798
2	4655	4733	4718
3	4658	4735	4742
4	4630	4728	4757
5	4684	4749	4811
6	4628	4725	4774
Pooled mean		4716.333	
Pooled RSD%		1.16%	

Table 8. Analyst-to-Analyst precision results

Replicate #	Analyst 1	Analyst 2	Analyst 3
1	4651	4581	4611
2	4655	4580	4625
3	4658	4572	4687
4	4630	4588	4628
5	4684	4510	4601
6	4628	4529	4681
Pooled mean		4616.611	
Pooled RSD%		1.09%	

Table 9. Column-to-Column precision results

Replicate #	Column #1	Column #2
1	4651	4752
2	4655	4758
3	4658	4747
4	4630	4792
5	4684	4772
6	4628	4764
Pooled mean	4707.583	
Pooled RSD%	1.31%	

Stability of solution

The experimental results guided us that the tested solution of Cfx can be given repeatable and precise data over 12 hours at room temperature as in Table 10.

Table 10. Stability of solution

#	0 hour	3 hours	6 hours	12 hours	Average P. As	STDEV	RSD%
Test P. A	4703	4568	4661	4575	4626.8	66.123	1.43%

Specificity and selectivity

The current method supplied us with highly specific information about the resolution and separation performance of the nearest co-eluted peaks with a resolution parameter at least 5.2 as in Figure 4 A, B.

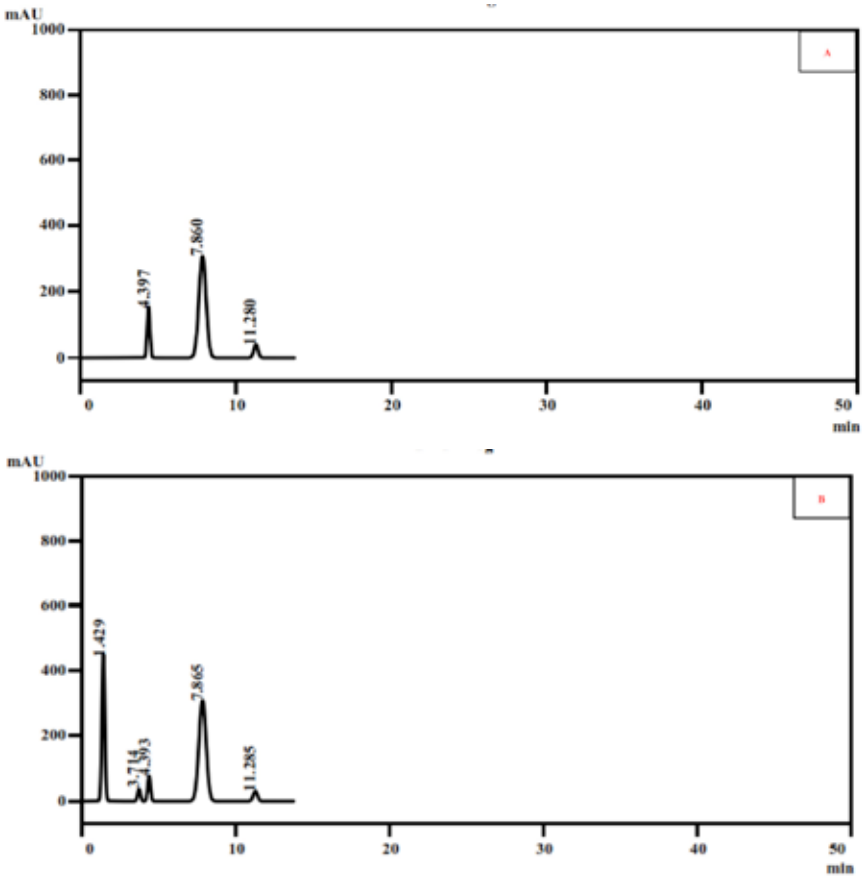

Figure 4. Effect of acid degradation (A) and H_2O_2 degradation (B) in specificity test

Test of the validated method

Cfx analysis in the different commercial dosage forms in the Egyptian local market

The Cfx average assay results of Suprax 200 mg capsules, Suprax 100 mg/5ml powder for oral suspension, and Cefipharmart 400 mg dispersible tablet for oral suspension revealed good results; 103.5 %, 101.8 %, and 104.4 % respectively.

Cfx traces analysis in the different urine samples

The method was succeeded in Cfx traces analysis at low concentrations reached 77.6, 98.1, 199.5, 260.7 ng/ml.

The current method introduces a sensitive, rapid, easy, economical, and accurate method of Cfx analysis. The method revealed a good behavior as linear, precise

(repeatable), robust, rugged, selective, and specific as the resolution factor between Cfx peak and any adjacent peak at least anyway > 1.5. DL and QL also, evaluated and showed an appreciated and satisfying value as 59.3 ng/ ml and 179.8 µg/ ml respectively. So, the analysis method is valid to use for Cfx determination at the minimum level of concentrations with convenient tools of analysis. The validated method gave satisfying results for the practical application of Suprax and Cefipharmart assay determination for three different dosage forms as revealed in the results. Also, the method showed a good result to investigate and quantitative analysis against urine samples at low concentration levels.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided here.

ABBREVIATIONS

Cfx	Cefixime
HPLC	High- performance liquid chromatography.
PDA	Photodiode array detector
UV	Ultraviolet
EP	European Pharmacopeia
USP	United States Pharmacopeia
DL	Detection limit
QL	Quantitation limit
Conc	Concentration
P. A	Peak area
P. As	Peak areas
STDEV	Standard deviation
RSD	Relative standard deviation
Th.	Theoretical

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Formulation, Evaluation and Anti-Hemorroidal Activity of Suppositories Containing *Moringa Oleifera* Lam. Seed Oil

Christianah Y. Isimi[1*], Lucy B. John-Africa[2], Kokonne E. Ekere[1], Olubunmi J. Olayemi[1], Olusola I. Aremu[3], Martins O. Emeje[1,4]

1 Department of Pharmaceutical Technology and Raw Materials Development, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria

2 Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria

3 Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, University of Ilorin, Nigeria

4 Centre for Nanomedicine and Biophysical Drug Delivery, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria

ABSTRACT

The plant *Moringa oleifera* has been reported to have various ethnomedicinal uses, of particular interest is the anti-inflammatory effect of the seed oil. In this study, suppository formulations containing Moringa seed oil (MSO) were developed for the management of inflammatory conditions of the anorectal region. The suppositories were prepared using a water soluble base, macrogol (MG) and a fatty base, dika fat (DF), obtained from *Irvingia gabonensis* seeds; they were evaluated for appearance, hardness, weight variation, melting point, pH, liquefaction time and *in vitro* release according to standard pharmacopoeia procedures. Anti-hemorroidal activity of the formulations in laboratory rats were also evaluated. Results show that all the suppositories prepared had good physicochemical properties. *In vivo* studies revealed that the optimized preparation containing dika fat was effective in reducing hemorrhoids induced in rats. Therefore, this study demonstrates the propensity of Moringa seed oil suppositories in the treatment of anorectal inflammatory conditions.

Keywords: Moringa seed oil; suppositories; Dika fat; Macrogol; Anti-inflammatory.

Corresponding author:
Department of Pharmaceutical Technology and Raw Materials Development, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.
Telephone number: +2348033156606
E-mail address: yetsimi@yahoo.com
ORCID
Christianah Isimi 0000-0002-9066-9984,
Lucy John-Africa 0000-0002-2771-0526,
Kokonne Ekere 0000-0001-8876-9284,
Olubunmi Olayemi 0000-0001-5759-7176,
Olusola Aremu 0000-0002-3890-1256,
Martins Emeje 0000-0002-0202-5426
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INTRODUCTION

The plant *Moringa oleifera* Lam. is a species of the family, Moringaceae; it is native to South Asia (India, Pakistan, Bangladesh and Afghanistan) but has been cultivated in the Philippines and the Sudan, Latin America and Africa (Fahey, 2005). In Nigeria, *Moringa oleifera* has become naturalized and is popularly known as "Okwe-beke" by the Igbos, "Zogale" by the Hausas, and "Ewe igbale" by the Yorubas) (Evbuomwan, Dick, & Chioma, 2017).

The plant is called the "miracle tree" because of its action against a wide range of ailments. Preparations of the various parts of the plant have been reported to have antimicrobial, anti-inflammatory, anticancer, hepatoprotective, antioxidant, cardiovascular, antiepileptic, antidiabetic, diuretic, anthelminthic, antiulcer and wound healing activities (Mishral et al., 2011; Amrutia, Lala, Srinivasa, Shabaraya, & Moses, 2011; Gupta et al., 2012; Rastogi, Bhutda, Moon, & Aswar, 2009).

The seeds particularly possess constituents that make them useful in the treatment or management of anti-inflammatory diseases (Saini, Sivanesan, & Keum, 2016), traditionally, the seed oil has been used for the treatment of rheumatism, warts, arthritis, mineral and vitamin deficiency (Fahey, 2005; Mishral et al., 2011). Moringa seed oil can be obtained from the seed kernels using organic solvents like n-hexane; the extracted oil is pale yellow, sweet, non-sticky, non-drying and resistant to rancidity (Olaleye & Kukwa, 2018; Lalas & Tsaknis, 2002). This fatty oil contains palmitic, behenic, stearic and arachidic acids as major parts of its fatty acid contents in addition to small traces of cerotic, lignoceric, myristic, margaric, erucic and caprylic acids. The oil also contains oleic acid as the predominant fatty acid i.e. 73.57 % of the total fatty acids and about only 1.2 % polyunsaturated fatty acids (Ogunsina et al., 2014).

Studies have shown the anti-inflammatory properties of Moringa seed oil; Suryadevara, Doppalapudi, Sasudhar, Anne and Mudda (2018) developed a cream formulation using Moringa seed oil and found that the cream reduced carrageenan-induced paw edema by 70 %, which was similar to that reported for Ibuprofen. In a similar study by Somnath et al. (2015), microemulsion formulations of Moringa seed oil were also found to reduce carrageenan-induced paw edema for up to 3 h. Another study showed that the hydro-alcoholic extract of *M. oleifera* seeds and its chloroform fraction were able to reduce acetic-acid induced colitis in rats (Minaiyan, Asghari, Taheri, Saeidi, & Nasr-Esfahani, 2014).

Hemorrhoids are in essence a cluster of tissues and muscles that line the anal canal, but they are inappropriately used as such when these tissues and muscles become swollen or inflamed. Symptoms of "hemorrhoids" include rectal bleeding,

pain, protrusion and treatment is often initiated by insertion/application of nonsteroidal anti-inflammatory drug products into the rectal region (Perry, 2019). Herbal remedies have also being exploited in the management and treatment of hemorrhoid inflammation (Eshghi et al., 2010; Hamidpour & Rashan, 2017).

Suppositories are solid bodies of various weights and shapes, adapted for introduction into the rectal, vaginal, or urethral orifice where they melt, soften, or dissolve at body temperature to release the stored drug. They are usually formulated using lipophilic or hydrophilic bases (Goodman, 2001). Incorporation of Moringa oil in this dosage form could be used in managing such diseases like "hemorrhoids".

Therefore, the aim of this study was to formulate oil extracted from Moringa seeds into suppositories using water soluble base; macrogol (MG) and fatty base; dika fat (DF) and to investigate the anti-hemorroidal activity in laboratory rats.

METHODOLOGY

Plant materials used include *Moringa oleifera* and *Irvingia gabonensis* seeds. Other materials used were liquid paraffin, Polyethylene glycol 1000 & 4000 (Emprove EXP, Merck Germany), Petroleum ether (Loba chemie, India), Sodium hydroxide, Hydrochloric acid and Sodium dihydrogen orthophosphate (Analar, Germany), Nutrient Agar (Sigma Life Sciences, USA), Ferric chloride (Sigma Aldrich, USA) and Distilled and Deionised water (National Institute for Pharmaceutical Research and Development Laboratory, NIPRD, Abuja, Nigeria).

Animals

Adult Wistar Albino rats (200 – 240 g) were obtained from the Animal Facility Centre (AFC) of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja. The animals were housed under ambient conditions of temperature 26 ± 1 °C and light approximately 12/12 h light/dark cycle. They were fed on standard rodent diet with free access to clean drinking water from the Municipal water system. The experiments were carried out on animals handled according to the Institutional Animal Ethical committee guideline as reflected in the Institutional SOP No 05:003.

Extraction

Extraction of dika fat from Irvingia gabonensis seeds

Irvingia gabonensis seeds were purchased from Karmo market, Abuja, Nigeria. They were validated at the herbarium section of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja where a voucher sample (NIPRD/H/6983) was obtained. The seeds were milled and 490 g of the pulve-

rized seed was weighed (Mettler Toledo, Switzerland), and then macerated with petroleum ether at a ratio of 1 to 5. The mixture was left for about 72 h after which the supernatant was decanted, filtered and concentrated over a water bath (Karl Kobb, Derieich West Germany) at about 100 °C. The resulting extract was weighed and kept at room temperature until further use.

Extraction of oil from Moringa oleifera seeds

Moringa oleifera seeds were obtained from the medicinal garden of the National Institute for Pharmaceutical Research and Development, NIPRD, Abuja. It was identified at the herbarium and a voucher number NIPRD/H/7078 was obtained. A quantity of 300 g of seed was pulverized using a blender (Qlick, Japan) and used for the continuous soxhlet extraction using petroleum ether in the ratio of 1 to 5 at 60 °C. The oil obtained was heated on a water bath (Karl Kobb, Derieich West Germany) at about 100 °C to evaporate the residual solvent. The extracted oil (MSO) was weighed, packaged in a sterile container and stored at room temperature.

Preparation of MSO suppositories using different bases

Pour moulding method was used for the manufacture of the suppositories in precalibrated mould with different bases. Calculated displacement values were used in defining the various final quantities of the bases used. The suppository mould was properly cleaned and lubricated with liquid paraffin. Appropriate quantities of bases and MSO as presented in Table 1 were put into a beaker and allowed to melt at 60 °C on a water bath (Karl Kobb, Derieich West Germany). In the case of macrogol base, an emulsion was initially formed based on required Hydrophilic-Lipophilic Balance (HLB) of MSO, 12. The mixtures were vigorously stirred together at about 50 °C using a magnetic stirrer (VWR Company, Germany) to allow for homogenous mixture. The mixture was poured into the mould until it overflowed; it was re-filled as the solidifying mixture was shrinking. The mould content was allowed to solidify, the suppositories were thereafter removed and packaged in aluminum foil until further evaluations were conducted. This procedure was repeated for production of placebo suppositories as control formulations.

Preparation of suppositories for animal studies

For the animal studies, 0.3 g of 5 % and 10 % MSO suppositories were prepared, this was done to adjust to the anatomic size of the rats.

Table 1: Composition of suppository formulations

Ingredients	MSD1(g)	MSD2(g)	MSD0(g)	MSM1(g)	MSM2(g)	MSM0(g)
Moringa seed oil	5	10	-	5	10	-
Tween 80	-	-	-	4.22	4.74	-
Span 20	-	-	-	0.78	0.26	-
PEG 1000 80 % + PEG 4000 20 %) to	-	-	-	98.4	98.4	98.4
Dika fat to	80.2	80.2	80.2	-	-	-

MSM0 = macrogol base alone, MSM1 = 5 %w/w Moringa seed oil + macrogol base, MSM2 = 10 %w/w Moringa seed oil + macrogol base, MSD0 = dika fat base alone, MSD1 = 5%w/w Moringa seed oil + dika fat base, MSD2 = 10 %w/w Moringa seed oil + dika fat base.

Evaluation of Suppositories

Appearance

Six suppositories were randomly selected from each group including placebo and they were observed as an intact unit and also after splitting them longitudinally. Colour, odour, shape, the absence of fissuring, pitting, exudation, sedimentation and the migration of the active ingredients were also assessed.

Weight uniformity

The weight uniformity test was carried out as designated in the British Pharmacopoeia (BP, 2013). Twenty suppositories were randomly chosen from each batch of the formulations and weighed independently using an analytical balance (Mettler Toledo, Switzerland). The average weights and standard deviations were calculated.

Determination of pH

The pH of each melted suppository was determined by a pH meter (Jenway, UK). All measurements were an average of three measurements and expressed as mean ± standard deviation.

Hardness/ Crushing Strength

The crushing strength, a measure of mechanical power or hardness of the sup-

pository was determined using the hardness tester (Erweka GmbH, Germany). Six suppositories randomly selected from each batch were used for the measurement. The weight at which each suppository cracked was documented in Kilogram force and converted to Newton.

Liquefaction Time

Six suppositories were indiscriminately chosen from each lot for this test. Thereafter, 60 mL of phosphate buffer with a pH of 7.4 was heated up to 37 ± 1 °C and maintained. Each suppository was dropped inside the buffer and the time taken for the suppository to completely dissolve or melt was noted as the liquefaction time.

Melting point Determination

The melting point of MSO suppositories were determined according to the technique of Adebayo and Akala (2005). A suppository randomly selected from each batch was put in a beaker with a thermometer introduced. The beaker was immersed in a water bath (Karl Kobb, Derieich West Germany) at about 6 cm depth, controlled to a steady temperature rise of 1 °C/2 min. The temperature at which the suppository sample began to melt was taken as the melting point. The outcome was an average of five determinations. The melting point of the placebo was also determined in a similar fashion**.**

***In-vitro* release**

The release of MSO from suppository bases was determined using agar diffusion method (Aremu et al., 2019). A quantity of 0.25 mL of melted suppository was measured into a 25 mL volumetric flask and made up to 25 mL with phosphate buffer, then mixed thoroughly. Sterilized nutrient agar was poured into a plate and left to solidify, the surface of each plate was flooded with a dye and the extra solution was drained off. Two holes were bored in these plates using a 6 mm cork borer, and 0.5 mL of 0 %, 5 % and 10 % w/w of MG and DF formulated suppositories were respectively placed in the holes. The plates were then placed on a laboratory bench for 1 hour for diffusion to occur before being transferred to the incubator (Karl Kobb, Derieich West Germany) at 37 °C. The zones of colour change were measured for each sample at time intervals of 1 h, 2 h, 3 h and 12 h.

Fourier-transform infrared spectroscopy (FTIR)

The method of Kauss et al. (2013) was adopted in preparation of the pellets. The suppositories were ground, triturated with potassium bromide and compressed into pellets. Infra-red spectra were obtained from the impact 410 Nicolet FTIR spectrometer (Thermo fisher Scientific, USA) between frequency range of 4000 and 650 cm^{-1}.

Gas chromatography-mass spectrometry (GC-MS)

The method of Okhale et al. (2018) was adopted. Each component was recognized by matching their mass spectra with known compounds and NIST Mass Spectral Library (NIST 11).

Animals

Adult Wistar Albino rats (200 – 240g) were obtained from the Animal Facility Centre (AFC) of the National Institute for Pharmaceutical Research and Development (NIPRD) Idu, Abuja. The animals were housed under ambient conditions of temperature 26 ± 1 °C and light approximately 12/12 h light/dark cycle. They were fed on standard rodent diet with free access to clean drinking water from the Municipal water system. Ethical permission for the study was obtained from NIPRD Animal Care and Ethics (NIPRD/05:3:05-03) in line with International Guiding Principles for Biomedical Research involving animals (CIOMS/ICLAS, 2012).

Studies on croton oil induced haemorrhoids in Wistar Albino rats

Twenty-five overnight fasted rats were randomly placed into 5 groups of 5 animals each. Group 1 served as the sham group. Hemorrhoids were induced in animals in group 2 – 5. The hemorrhoid inducing agent was prepared using deionized water, pyridine, diethyl ether, and 6 % croton oil in diethylether in the ratio of 1:4:5:10. The inducing agent (0.16 mL) was dropped onto sterile cotton swab of 4 mm diameter and was carefully inserted through the anal opening up to a length of 20 mm. This was held in place for 10 seconds after which the cotton swab was removed. Twenty-four hours after induction, animals were treated as follows: Group 2 served as negative control and received no treatment, group 3 was treated with the suppository (0 % MSO), group 4 received suppository with 5 % Moringa seed oil, while group 5 was administered 10 % Moringa seed oil suppository. The suppositories were administered daily for 5 days. Twenty-four hours after administration of the last dose, the animals were euthanized by diethyl ether inhalation. Thereafter, the distal 2 cm of the anal region was isolated and weighed on a digital balance (Mettler Toledo – SNR 1113092341). The tissues were subsequently preserved in 10 % buffered formaldehyde solution for histological examination (Nishiki, Nishinaga, Kudoh, & Iwai, 1988; Azeemuddin et al., 2014).

Recto-anal Coefficient (RAC) was calculated using the formula:

$$\text{Recto-anal Coefficient} = \frac{\text{Weight of Recto-anal tissue (mg)}}{\text{Weight of animal (g)}}$$

Statistical analysis

Values are presented as Mean ± SEM and analyzed by one-way ANOVA followed by Dunnet's post Hoc test. Level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Table 2. Physicochemical properties of Moringa seed oil suppositories

Parameters	MSM1	MSM2	MSD1	MSD2	MSM0	MSD0
Shape	Torpedo	Torpedo	Torpedo	Torpedo	Torpedo	Torpedo
Colour	White	Off-White	Milky	Cream	White	Light-Yellow
Mean Weight (g)	2.39 ± 0.02	2.32 ±0.02	1.97 ±0.07	2.02 ±0.01	2.39 ±0.02	2.32 ±0.01
Melting Point (°C)	36.17±0.49	36.73±0.59	31.53±0.15	31.80±0.15	37.43±0.45	32.27±0.21
Hardness (N)	19.6±2.0	13.7±2.0	12.3±0.3	10.7 ±1.1	22.2±1.1	16.0±1.1
Liquefaction time (min)	27.3 ±0.89	24.2 ±0.55	40.5±0.46	30.1 ±0.42	32.4±0.45	36.0±0.42
Displacement value	0.65	-	0.74	-	-	-
Ph	6.37±0.01	6.67±0.02	5.12±0.01	5.20±0.15	5.97±0.09	5.08±0.03

MSM0 = macrogol base alone, MSM1 = 5 %w/w Moringa seed oil + macrogol base, MSM2 = 10 %w/w Moringa seed oil + macrogol base, MSD0 = dika fat base alone, MSD1 = 5 %w/w Moringa seed oil + dika fat base, MSD2 =10 %w/w Moringa seed oil + dika fat base.

The rate at which the active ingredient is released from the suppository is shown in Figure 1

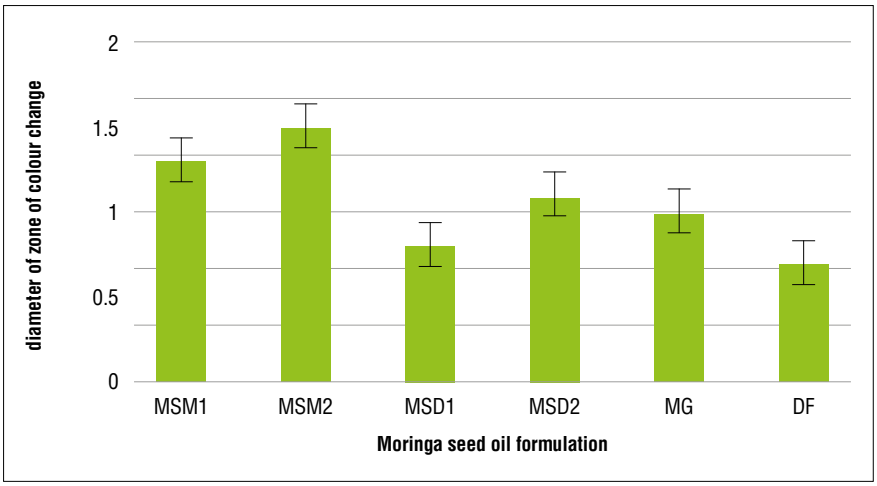


Figure 1. Average diameter of zone of colour change for prepared suppositories

Table 3. Effects of Moringa Oil suppository on Recto-anal Coefficient

Group	Treatment	Recto-anal coefficient
1	Sham	1.94 ± 0.15[c]
2	Control	3.50 ± 0.30
3	MSD0	3.20 ± 0.17
4	MSD1	2.26 ± 0.17[b]
5	MSD2	2.05 ± 0.38[b]

Values are presented as Mean ± SEM (n = 5),

Significance compared to control, [b]$p < 0.01$, [c]0.001 groups (One-way ANOVA, Dunnet's Post Hoc)

The extraction yield of of dika fat from the seeds was 40.16 %w/w while that of Moringa oil was 24 %w/w. This value is lower than already reported (Efeovbokhan, Hymore, Raj, & Sanni, 2015; Eman & Muhamad, 2016; Siyanbola et al., 2015) and could be attributed to the difference in extraction solvents used. Dika fat (DF) was light yellow with its characteristic odour, Moringa seed oil (MSO) was pale yellow but with a characteristic peanut odour.

When the suppositories were split longitudinally, it was observed that there was absence of fissures, the suppositories were stable and had uniform colour. There

was also absence of sedimentation and exudation indicating uniform suppository mix. All the suppositories had uniform weight; not deviating from the average by more than 5 % as specified by the British pharmacopeia (BP, 2013). This indicates that the pouring of the suppository mixture into the mould was accurately done. Uniformity of weight is of importance in the formulation of drugs as it ensures that the required osr specified amount of drug reaches the site of action.

Hardness/crushing strength is a key parameter assessed in pharmaceutical formulations as it indicates the degree to which a particular formulation resists mechanical wear and tear during handling and transportation. The results of the crushing strength of the placebo in order of their strengths were MSM0 (22.2±1.1) > MSD0 (16.0 ± 1.1 N) while those containing medicaments were MSM1 (19.60 ± 2.0) > MSM2 (13.70 ± 2.0 N) > MSD1 (12.30 ± 0.3) > MSD2 (10.70 ± 1.1 N). Generally, hardness/crushing strength of suppositories should be at least 1.8-2 kg pressure; it was observed that suppositories without Moringa seed oil (MSO) were stronger than those containing the incorporation of the MSO. Based on this, the suppositories with the macrogol base can be said to stand a better chance of withstanding rigorous handling and other mechanical conditions.

The pH of a pharmaceutical preparation is not to be neglected as it indicates compatibility of the preparation with the site of action. The pH of the macrogol based suppositories was similar to that of the rectum which is between 6 and 8, while the dika fat based suppositories had a slightly acidic pH (5.12- 5.20), which may likely irritate the rectal mucosa.

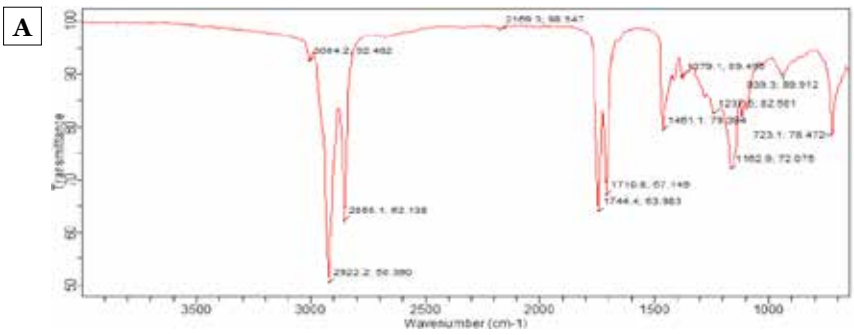
One of the most important characteristics of suppository bases is stability at room temperature, that is, it should not melt at room temperature but melt or dissolve at body temperature in order to release the active ingredient. Generally, the melting point should be less than or equal to 37 °C. It can be observed that the inclusion of the Moringa seed oil reduced the melting point of the suppositories. The melting point of the suppositories in increasing order are MSM2 (36.73±0.59) > MSM1 (36.17±0.49) > MSD2 (31.80±0.15) > MSD1 (31.53±0.15). These values were observed to be lower than the melting point of MSM0 and MSD0 (suppositories without Moringa seed oil).

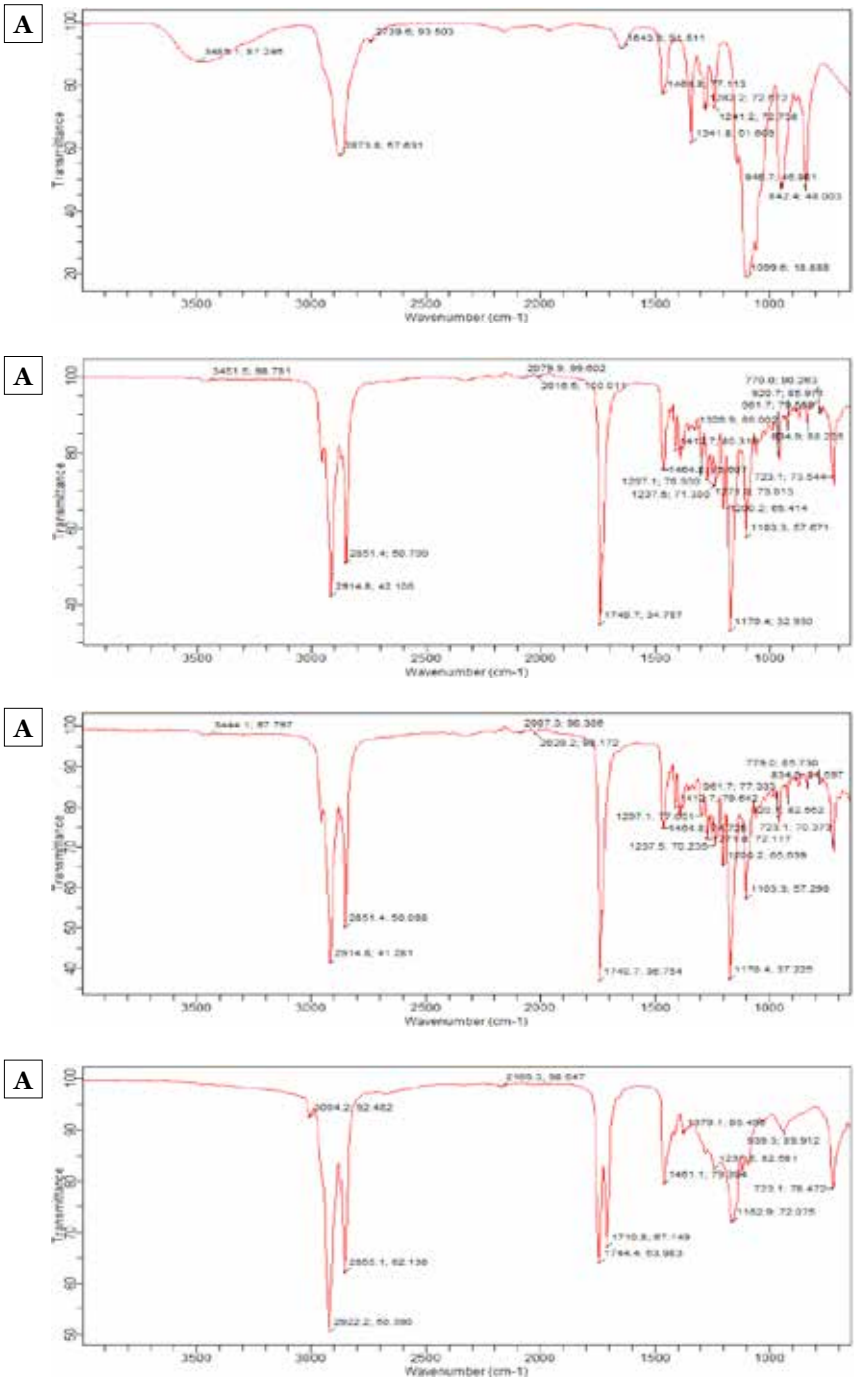
The liquefaction time is the time taken for a suppository to melt or dissolve at body temperature. The liquefaction time is a function of the melting point. It is important to note that the liquefaction time of suppositories should take no longer than 30mins (Mosbah & Mokhtar, 2016). The liquefaction time exhibited by the suppositories was satisfactory except for the MSD1 which had a liquefaction time above 30mins. A suppository which does not melt or dissolve within 30mins

would take a longer time to elicit its action (Taha, Zaghloul, & Kassem, 2003).

In the release study carried out, it was observed that the zone of colour change increased with time. The suppositories with macrogol base had a wider zone of colour change compared to suppositories with dika fat. Generally, lipophilic drugs formulated with hydrophilic bases tend to release faster than those formulated with lipophilic bases, which is due to less affinity for the base as seen in this study.

FTIR spectrum of Moringa seed oil (Figure 2) shows prominent peaks at 2922 and 2855 cm^{-1} which correspond to the assymmetric and symmetric C-H bond in the CH_2 functional group. These sharp peaks could also be attributed to high lipid contents of the seed. The broadband at around 3004.2 cm^{-1} can be due to the O-H stretching, in addition, the presence of N-H due to amides as a result of high protein content of Moringa seed could also be responsible for the peak observed. Peaks observed at 1744.4 and 1710.8 cm^{-1} could be attributed to the carbonyl group (C=O) which is due the lipid portion of the seed. The spectrum for macrogol shows characteristic peaks at 3485.1, 28738, 1464.8, 1341.8, 1099.6 cm^{-1} indicating the presence of O-H and C-O functional groups. Dika fat spectrum shows major peaks at 3485.1, 2873.8, 1341.8, 1099.6 cm^{-1}; the broad peak at 3485.1 cm^{-1} connotes the presence of O-H stretch while the others connote the presence of C-H group as is consistent with materials containing high fatty acids content. Incorporation of macrogol into Moringa seed oil revealed loss of the seed oil characteristic sharp peaks at 2922 and 2855 cm^{-1} which could be an indication of interaction. On the other hand, the major peaks present in Moringa seed oil were observed to be retained when dika fat was incorporated into the seed oil implying the absence of interaction. This justifies the use of dika fat in optimized suppository formulations of Moringa seed oil.

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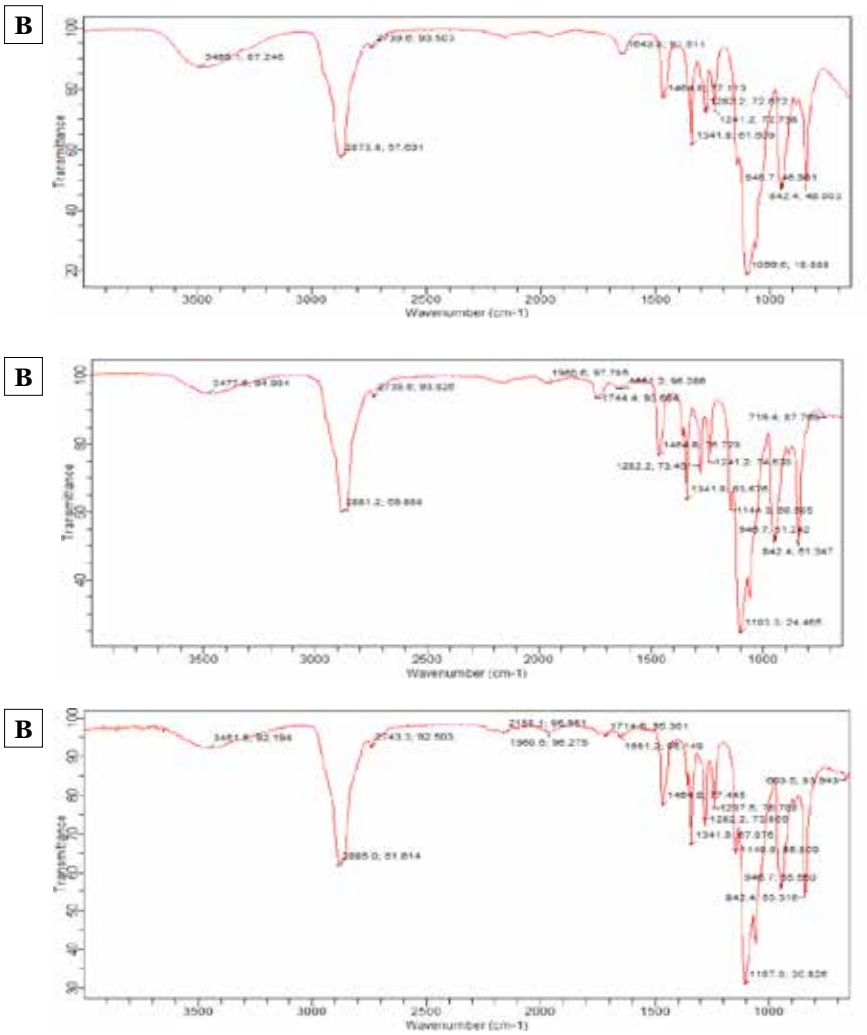

Figure 2. FTIR spectrum of moringa seed oil (A1), dika fat (A2), 5 %w/w moringa seed oil+dika fat (A3), 10 %w/w moringa seed oil+dika fat (A4), macrogol (B2), 5 %w/w moringa seed oil+macrogol (B3), 10 %w/w moringa seed oil+macrogol (B4).

GC-MS analysis of Moringa seed oil showed the presence of various compounds with 9-Octadecenoic acid (56.98 %) being more abundant compared to other compounds. 9-Octadecenoic acid has been found to inhibit production of inflammatory agents in RAW 264.7 cells. The compound had an inhibitory effect on nitric oxide and other inflammatory cytokines such as TNF-α, IL-6 (Kang et al., 2018). Another fatty acid found in the GC-MS analysis of seed oil is n-Hexadecanoic acid which through enzyme kinetics study is known to inhibit Phospholipase A(2) which is involved in initiating inflammation (Aparna et al., 2012).

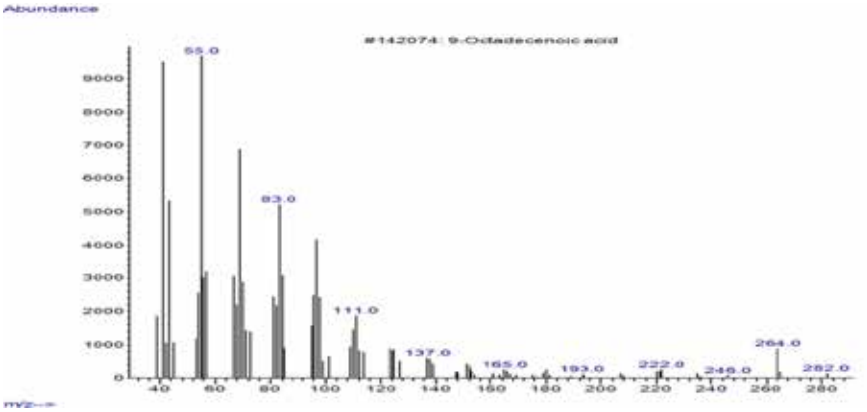

Figure 3. Gas chromatography spectrum of Moringa seed oil

Application of the inducing agent to the recto-anal region of rats caused significant increase in the recto-anal coefficient when compared to the sham group. Administration of suppositories prepared with dika fat (MSD1 and MSD2) caused a significant ($p<0.05$), dose dependent reduction of the RAC at 2.26 ± 0.17 and 2.05 ± 0.38 when compared with control of 3.50 ± 0.30. (Table 3). Symptomatic hemorrhoids occur when the integrity of the supporting tissues of the recto-anal region deteriorates; the condition is characterized by vasodilatation, inflammation reaction, haemorrhage, thrombosis and necrosis of vascular tissues in the recto-anal region (Sun & Migaly, 2016; Faujdar, Sati, Sharma, Pathak, & Paliwal, 2019). Croton-oil is widely used to induce experimental hemorrhoids in laboratory animals. Treatment with suppositories prepared with Moringa seed oil caused a reduction of the RAC which is an indication of the reduction of inflammation**.** In other studies, Moringa seed extract has also been shown to demonstrate anti-inflammatory activity in gastric and other tissues (Suryadevara et al., 2018; Minaiyan et al., 2014).

Histological examination showed the tissues of the recto-anal region in control rats presented with haemorrhage, infiltration of inflammatory cells, glandular hardening and necrosis. However, treatment with the suppositories caused an amelioration of tissue injury caused by croton oil. This is observed as reduction of the severity of damage caused to the tissues. Tissues of the recto-anal region in the sham group (group 1) showed normal features, whereas the control (group 2) presented with moderate haemorrhage with infiltration of inflammatory cells and glandular necrosis. Slight glandular hardening necrosis was observed with MSD0 containing no Moringa seed oil (group 3), while the groups treated with MSD1 showed infiltration of inflammatory cells with secretory glands hypertrophy (group 4) and those treated with MSD2 (group 5) presented slight hae-

morrhage and infiltration of inflammatory cells (Figure 4). The biological activity of plant products may be attributed to the component phytochemical compounds as reported by Azeemuddin et al. (2014) and Shivani, Vjayabhaskar, Rao, Kumar, & Yadav (2019) who recorded reduction of RAC and repair of gastric tissues on administration of plant products on croton oil induced hemorrhoids.

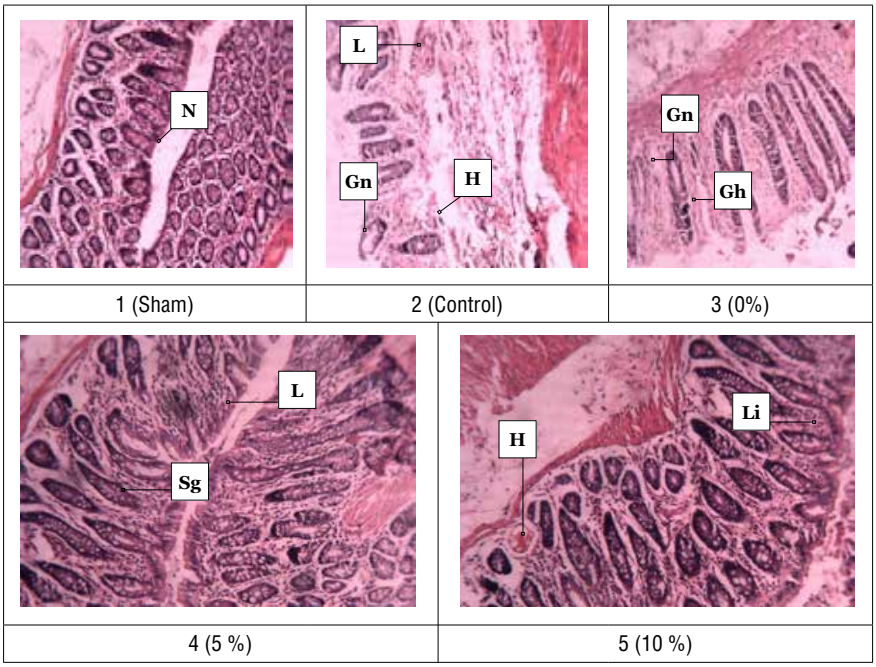

Figure 4. Effects of Moringa oil suppository on croton oil induced haemorrhoids in Wistar rats.

Li - lymphocyte infiltration, N - normal, H - hemorrhage, SG - normal gland, Gn - Glandular necrosis, GH - glandular hardening

In this study, Moringa seed oil suppositories formulated with macrogol and dika fat exhibited good physicochemical characteristics. *In vivo* anti-inflammatory activity of the optimized formulation show the potential of dika fat as a suppository base for the delivery of Moringa seed oil in the treatment/management of anorectal conditions like hemorrhoids.

AUTHOR CONTRIBUTIONS

Design- Christianah Y. Isimi

Acquisition of data- Lucy B. John-Africa, Kokonne E. Ekere, Olubunmi J. Olayemi

Analysis of data- Christianah Y. Isimi, Lucy B. John-Africa, Kokonne E. Ekere, Olubunmi J. Olayemi

Drafting of the manuscript-Christianah Y. Isimi, Lucy B. John-Africa, Kokonne E. Ekere

Critical revision of the manuscript- Olubunmi J. Olayemi, Olusola I. Aremu, Martins O. Emeje

Statistical analysis- Lucy B. John-Africa

Supervision- Christianah Y. Isimi

CONFLICT OF INTEREST

Authors declare that there is no actual or potential conflict of interest with respect to this article.

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ABBREVIATIONS USED

MSO-Moringa seed oil

DF-dika fat

MG-macrogol

FTIR-Fourier transform infrared spectroscopy

GC-MS-Gas chromatography-mass spectrometry

NIPRD-National Institute for Pharmaceutical Research and Development

HLB-Hydrophilic-Lipophilic Balance

MSM0-macrogol base alone

MSM1-5 %w/w Moringa seed oil + macrogol base

MSM2-10 %w/w Moringa seed oil + macrogol base

MSD0- dika fat base alone

MSD1-5%w/w Moringa seed oil + dika fat base

MSD2-10 %w/w Moringa seed oil + dika fat base

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Anticonvulsant, Anxiolytic and Sedative Activities Of The Methanol Extract of *Abrus Precatorius* (Linn.) Leaves

Jacob Olayemi[a], Adewale Bakre[b*], Esther Oshireku[a], Segun Olowoparija[b,c]

a Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Nigeria

b Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Nigeria

c Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Science, University of Medical Sciences, Ondo, Nigeria

ABSTRACT

Abrus precatorius L. is a woody twinning climber, a member of the Fabaceae family indigenous to parts of Asia and Africa. It has been used widely in traditional medicine for curing many conditions such as malaria, sores, chest pain, cough, inflammation and many others. It was also reported that the hot water extract of the leaves and the roots have been used in treating persons suffering from episodes of convulsions in Tanzania. The aim of this present study was to evaluate the anti-convulsant, anxiolytic and sedative activities of the methanol extract of *A. precatorius* (Linn.) leaves in-vivo using murine models of convulsion, anxiety and sedation.

The anticonvulsant activity of ethanol extract of *A. precatorius* leaves was investigated using pentylene tetrazole, strychnine and picotoxin-induced convulsion. Anxiety tests used included head dips and elevated plus maze. Phenobarbitone-induced sleeping test was used to assess the sedative effect of *A. precatorius*. Safety of the extract was determined using the brine shrimp lethality assay.

Preliminary phytochemical screening on the dried and powdered leaves indicated that alkaloids, tannins, flavonoids and saponins, with a yield of 17.40%are present in the leaves. The brine shrimp lethality assay showed a LC50 of 8.189µg/ml.Results from the *in-vivo* neuropharmacological profile of the crude methanol extract of the leaves at doses of 200,400 and 800mg/kg body weight had central effects although not sig-

Corresponding author:
Adewale G. Bakre,
Department of Pharmacology & Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria.
Telephone: +2348081062365
E-mail: bakreeyitemi@live.com
ORCID
Jacob Olayemi 0000-0002-5504-7960,
Adewale Bakre 0000-0001-9769-8510,
Esther Oshireku 0000-0001-6276-6077,
Segun Olowoparija 0000-0003-3232-1901
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nificant ones compared with diazepam which was used as the standard**.** The results from the convulsion studies expressed as percentage protection indicates that 400mg/kg and 800mg/kg had 25% protection against pentylenetetrazol induced convulsions. The methanol extract of *A. precatorius* leaves might possess anticonvulsant and sedative activities, thus justifying its use in the management of epilepsy.

Keywords: Anticonvulsant, Anxiolytic, sedative, *Abrus precatorius,* epilepsy.

INTRODUCTION

Abrus precatorius L. is a woody twinning climber, a member of the Fabaceae family indigenous to parts of Asia and Africa. It has been used widely in traditional medicine for curing many conditions such as malaria, sores, chest pain, cough, inflammation and many others. It was also reported that the hot water extract of the leaves and the roots have been used in treating persons suffering from episodes of convulsions in Tanzania.

Over 50 million people are estimated to have epilepsy, making it one of the most common non-communicable neurological diseases, with a very large proportion of affected persons living in developing countries of the world, especially in Asia and Africa. A vast majority of these patients rely on traditional medicine almost exclusively because therapy is symptomatic, controlling seizures in only about 25%, and neither effective prophylaxis nor cure is available[1].The characteristics of the disease are periodic and unpredictable seizures due to disordered, synchronous and rhythmic firing of population of the brain's neurons[2]. A study in south western Nigeria identified the cause of epilepsy as psychological stress, head injury, birth or head injury, and supernatural or due to a contagion[3].

Over the years, various mechanisms to alleviate the condition have led to the development/production of a wide variety of antiepileptic drugs (AEDs). AEDs are classified majorly into first, second and third generations, with 14 new AEDs licensed for clinical use between 1989 and 2009. Majority of the AEDs have very potent adverse effects and drug interactions, but the third-generation drugs exhibit less interaction compared with the first- and second-generation AEDs [4]. The ideal AED would suppress all seizures without causing any unwanted effects. Unfortunately, none of the current drugs used control seizure activity without frequent unwanted effects which include impairment of the CNS, drowsiness, lethargy, euphoria, dizziness, headache, and convulsion. Anxiety is a common psychological disorder in epileptics[5]. It is described as an unpleasant emotional state with an unidentified, uncontrollable and unavoidable cause.

The hot water extract of the leaves of *Abrus precatorius* (Linn.) is used in traditional medicine of various neurological conditions including convulsion[6]. How-

ever, there is paucity of detailed scientific study of *Abrus precatorius* leaf extract applied on the central nervous system to control convulsions. The aim of this present study was to evaluate the anti-convulsant anxiolytic and sedative activities of the methanol extract of *A. precatorius* (Linn.) leaves in-vivo using murine models of convulsion, anxiety and sedation.

METHODOLOGY

Plant material and preparation of extract

The plant material was collected in May 2016 from the University of Ibadan Botanical Gardens. It was identified and authenticated at the Forest Herbarium Ibadan, Oyo State, Nigeria in the Forestry Research Institute of Nigeria, Ibadan, where a voucher specimen (FHI. 110694.) was obtained and deposited. The leaves of flowering *A. precatorius* were dried properly in air and then pulverized using a blender to obtain a coarsely ground powder which was extracted using cold extraction method with distilled methanol. 178 g of the coarse powder was macerated in a macerating tank for 48 hours exhaustively. The crude extract was decanted off and filtered. It was thereafter concentrated at 20 ^{o}C using the rotary evaporator (BUCHI Rota vapor R-205).

Animal

Young male albino Swiss mice (18–25 g) were obtained from the Animal Centre, College of Medicine, University of Ibadan, Nigeria, and were housed in plastic cages at room temperature with a 12:12 h light–dark cycle. They were fed with balanced rodent pellet diet and water ad libitum. The animals were acclimatized for at least 1 week before being used for experiments. The experimental procedures were following the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Drugs and chemicals

Diazepam (Hoffman-La Roche, Switzerland), pentylenetetrazol (Sigma, USA), picrotoxin (Sigma, USA), and strychnine (Shaanxi Xin Sheng Long Industrial Co., Ltd. China).

Experimental design

Thirty mice randomly divided into six groups (n=5) were used for all the tests except the anticonvulsant assays. The groups included two controls (vehicle and standard drug) and four treatment groups for doses 100, 200, 400 and 800 mg/Kg. A similar grouping was used for the anticonvulsant test except that there were 10 animals per group (n=10)

Brine shrimp lethality assay

The stock solution of the crude methanol extract of *A. precatorius* with concentration of 1000µg/ml was prepared by dissolving 50 mg of the extract in 5ml of natural sea water. Concentrations of 1000 µg/ml, 500 µg/ml, 100 µg/ml, 10 µg/ml and 1µg/ml were used for the assay by appropriate dilution with sea water in sample bottles. The experiment was carried out in triplicates. Ten viable *Artemia salina* were introduced into each sample bottle and made up to 5ml using natural sea water. After a period of 24 hours, the number of dead shrimps was counted and recorded[7].

Novelty induced behavior (NIB)

NIB was assessed using the method described by Ajayi and Ukponmwan (1994)[8] with some modifications. The mice were allowed 6–10 mins epochs during which locomotion, rearing and grooming were observed and scored to allow for characterization of drug-induced alterations. The mice were then returned to their home cages. Each test session involved allowing the mice to acclimatize to the testing environment (a quiet well-ventilated room) for 30 mins. All behavioral testing was carried out between 9 am and 2 pm. Six groups of five mice each were given 10 mL/Kg vehicle; 100, 200, 400 and 800 mg/Kg MEAP; and 3 mg/kg diazepam orally, before placement in the open field arena. The open field is a rectangular arena composed of a hardboard floor (36×36 cm^2) with a surrounding wall (30 cm high) made of white painted wood. The extract dosages were chosen guided by brine shrimp lethality assay and literature. (Similar doses were used in other protocols in this study.)

The floor was divided into squares of 9 cm^2. One hour after administration, each mouse was introduced into the arena; the frequency of grooming (the number of body cleaning with paws picking of the body and pubis with mouth and face washing actions) and rearing frequency (number of times each mouse stands on its hind legs or with its forearms against the wall of the cage or in free air) were scored for 30 mins. The procedure was repeated for all the mice in the different groups. After each session, the floor of the apparatus was wiped with 70% ethanol and dried thoroughly to remove traces of previous path.

Exploratory activity

To determine potential sedative effects, the hole-board test was used. The hole-board is a wooden board (40×40 cm^2) with 16 holes (diameter 3 cm) evenly spaced on the floor [9]. Immediately after the NIB test for each mouse, it was placed at the center of the hole-board and the number of head dips into the holes was scored over a 5 mins period. Results obtained were expressed as mean total number

of head dips (Lister, 1987). The procedure was repeated for all the mice in the different groups. After each trial, the floor of the apparatus was wiped with 70% ethanol and dried thoroughly to remove traces of previous path.

Learning and memory

Y-maze was used to assess the effect of the extract on short term memory. The Y-maze is composed of three equally spaced arms (120°; 41×15×5 cm). The parameters assessed were arm entries (locomotor activity) and spontaneous alternation performance (memory). Immediately after the hole-board test for each mouse, it was placed in one of the arm compartments and allowed to move freely for 5 mins. Entry was defined as when the body (excepting the tail) of a mouse completely enters into an arm compartment. The sequence of entry was manually recorded. Alternation is defined as entry into all three arms consecutively. The arms were labeled A, B, and C, and consecutive entries ABC, BCA, and CAB. The maximum number of spontaneous alternations was then calculated as:

[*Total number of arms entered*]- 2;

Percentage alternations was calculated as

$$\left[\frac{\textit{Actual alternation}}{\textit{Maximum alternation}}\right] (X100) \quad (10)$$

The procedure was repeated for all the mice in the different groups. The apparatus was cleaned after each session to eliminate the odor left off the immediately preceding mouse[11].

Anxiolytic test

The elevated plus maze model [12, 13]was used to assess anti-anxiety effect. Lister (1987) [14]validated the use of the elevated plus maze in testing anxiolytic effect in mice. First, the mice were assessed for the aversion of the open space and height. For this aspect, the elevated plus maze with two open and two closed arms was used. The plus maze used is made of wood with open arms (30×5×15 cm) and closed arms (30×5×15 cm). The arms extend from the central platform (5×5 cm). The open arms, the central platform, and the floor of the closed arms were painted black. Next, the apparatus was mounted on a wooden base at an elevation of 38.5 cm above floor level. For the open arms, a slight ledge 4 mm high was erected to prevent the mice from slipping and falling off the edge. Immediately after the learning and memory test, each mouse was placed one after another at the center facing one of the closed arms and assessed for 5 mins. The following behavior was scored: open arm entries, closed arm entries, time spent in open arm and time spent in closed arm. The procedure was repeated for all the mice in the different

groups except the group that was given diazepam; the group was replaced with another set of mice that was given 1 mg/Kg diazepam. After the assessment of each mouse, the lingering olfactory cues were cleansed using 70% ethyl alcohol. The doses used fell in the range that did not affect motor coordination [15].

Activity cage

The locomotor activity of the mice was measured as horizontal and vertical movement using the multiple activity cage apparatus (Ugo Basile 47420) complete with two sets of emitter/sensor arrays for horizontal and vertical activity. A total of six rodents per group was placed in pairs of 2 mice per session in the activity cage for aduration of 5 minutes. The apparatus was preset for 2 mice per session of 5 minutes on two trials according to the manufacturer's manual. Thereafter, the rodents were returned to their home cages.

Anticonvulsant tests

Pentylenetetrazol-induced convulsion: PTZ (85 mg/kg; s.c.) was used to induce clonic–tonic convulsion in mice [16]. The mice were divided into six groups of ten rodents each. The groups were 10 mL/Kg vehicle; 100, 200, 400 and 800 mg/Kg extract; and 40 mg/Kg phenobarbitone. One hour after the administration (p.o.), the convulsant was used to challenge the animals. The percentage of survival was recorded for each group.

Picrotoxin-induced convulsion: Picrotoxin (14 mg/kg; i.p.) [17]was used to induce limbic seizures followed by status epilepticus in mice. Again, the mice were placed in six groups of ten rodents each. The groups were 10mL/Kg vehicle; 100, 200, 400 and 800 mg/Kg extract; and 40 mg/Kg phenobarbitone. Sixty minutes after initial administration (p.o.) the rodents were challenged using the convulsant. The survival percentage for each group was then recorded.

Strychnine-induced convulsion: seizures were induced using Strychnine (2 mg/kg; i.p.) [17]. Once more, every mouse was placed in a group of six with ten rodents each. Group onewas 10mL/Kg vehicle; groups two to five got 100, 200, 400 and 800 mg/Kg extract; while group six received 40 mg/Kg phenobarbitone. An hour from administration (p.o.) the convulsant was used to induce the rodents. Finally, the survival rate was recorded for each group.

Statistical analysis

Results of the experiments and observations were expressed as mean± standard error of mean (SEM). The significance of differences between groups was determined using one-way analysis of variance (ANOVA) followed by at least one of the following posthoc test: Dunnet's multiple comparison tests, Tukey's

t-test and Student Neuman Keuls test. A level of significance $p<0.05$ or 0.01 was considered for each test.

RESULTS AND DISCUSSION

Secondary metabolites are the most beneficial active principles derived from various parts of plants and over time, scientific evidence that they possess the pharmacological activities for which the plant is known are being provided. The therapeutic or prophylactic effects of plant materials typically result through additive or synergistic action of the secondary metabolites present in the plant acting at single or multiple target sites associated with a physiological process[18]. The combination of secondary metabolites in a particular plant is often taxonomically distinct[19]. This could be the basis for the specificity and uniqueness of medicinal actions of plant. Preliminary phytochemical screenings of the crude methanol extract of the leaves of *A. precatorius* (MEAP) show the presence of alkaloids, tannins, saponins and flavonoids which are responsible for the various pharmacological actions of the leaves and extracts from the leaves of the plant. Alkaloids e.g. piperine, raubasine have been shown specifically to possess anticonvulsant activities as well as flavonoids, terpenoids and saponins. Adedapo *et al.*, [20]reported that intraperitoneal LD_{50} of *A. precartoris* in ethanol in mice is less than 0.1 µg/kg, while Ogbuehi *et al.*, 2015[21] gave oral LD_{50} in methanol to be 3942 mg/kg. The study revealed that the extract might be cytotoxic with LC_{50} of 8.189 µg/ml. Compounds or extract with LC_{50}value less than 1000µg/ml are considered to be cytotoxic while those giving LC_{50} values greater than 1000 µg/ml are not considered to be[22].

Table 1: Table of results from Brine shrimp lethality assay.

Concentrations (µg/ml)	Death in 1st vial (x/10)	Death in 2nd vial (x/10)	Death in 3rd vial (x/10)	Total death (x/30)	% mortality
1000	9	10	10	29	96.7
500	9	7	10	26	86.7
100	6	3	9	18	60
10	6	4	3	13	43.3
1	4	2	6	12	40

Different pharmacological techniques were used to investigate the anxiolytic, anticonvulsant, and sedative effects of MEAP. In activity cage, open field and hole board tests, decreased number of horizontal and vertical movements, rearing and grooming, and head dips respectively reveal that MEAP might possess sedative effect. Increased activities (movements, novelty induced behavior and head dips) of rodents are indicative of their explorative capability, which are also considered to be central excitatory behavior [8]. The reduction of these activities by MEAP could be due to their central inhibitory action on excitatory neural systems such as glutamatergic and dopaminergic systems among others or their possible potentiation of the central inhibitory systems such as γ-aminobutyric acid (GABA).

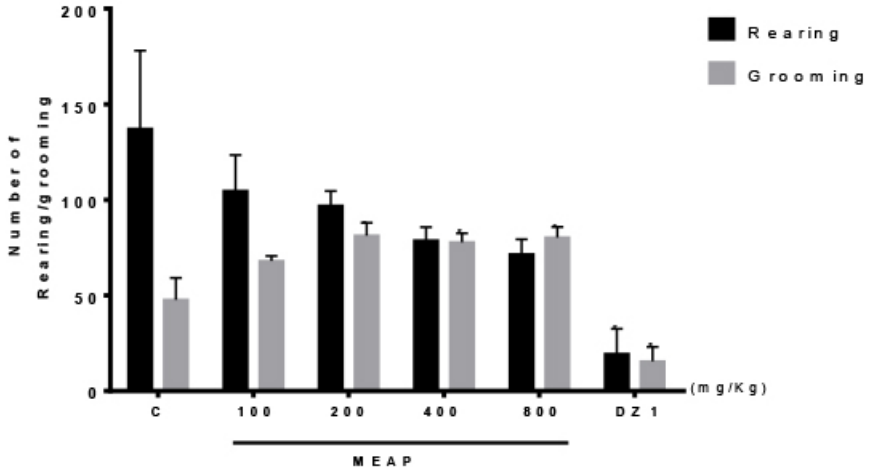

Figure 1: Effects of the methanol extract of the leaves of *A. precatorius* on Novelty-induced rearing and grooming in mice. Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * $p< 0.05$, indicate significant difference from the control (distilled water).

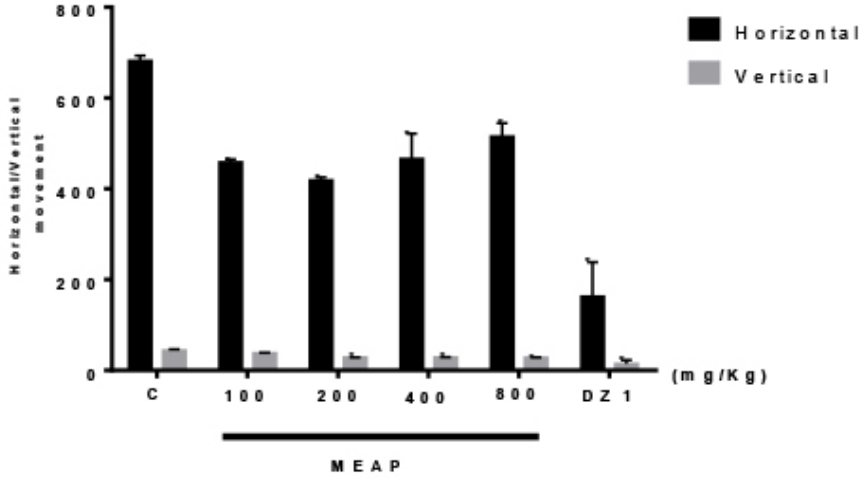

Figure 2: Effects of the methanol extract of the leaves of *A. precatorius* on horizontal and vertical locomotion in mice. Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * p< 0.05, indicate significant difference from the control (distilled water).

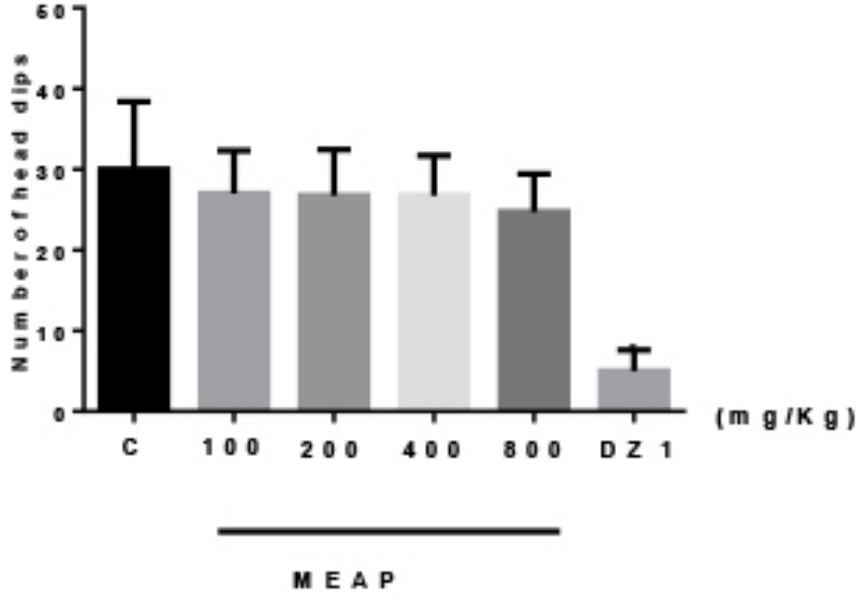

Figure 7: Effects of the methanol extract of *A. precatorius* leaves on exploratory behavior in mice Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * p< 0.05, indicate significant difference from the control (distilled water).

Also, the results from spontaneous alternation test using the Y-Maze showed that MEAP has no significant effect on spatial working memory at doses used in this study.

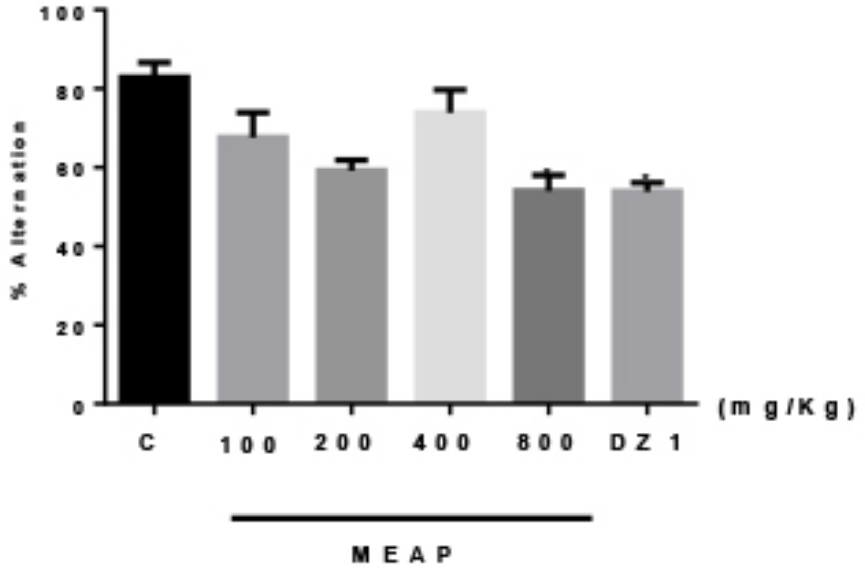

Figure 3: Effects of the methanol extract of the leaves of Abrus precatorius on the percentage alternation of mice in the Y-maze.Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * $p< 0.05$, indicate significant difference from the control (distilled water).

EPM favorable for testing of $GABA_A$-receptors linked anxiolytic drugs [29, 30]. Agents, which increase animals' time spent and number of entries into open arms and/or reduce time spent and number of entries in closed arms of EPM, are considered to possess anxiolytic effects [31]. MEAP significantly decreased time spent in open arms (Figure 4), and increased time spent in closed arms (Figure 5) suggesting that it lacks anxiolytic activity. Avoidance of the open arm portrays a manifestation of fear and anxiety. MEAP increased the index of open arm avoidance (Figure 6). Standard benzodiazepine anxiolytic like diazepam increased time spent in open arm and reduced time in closed arm at 1 mg/kg [32].

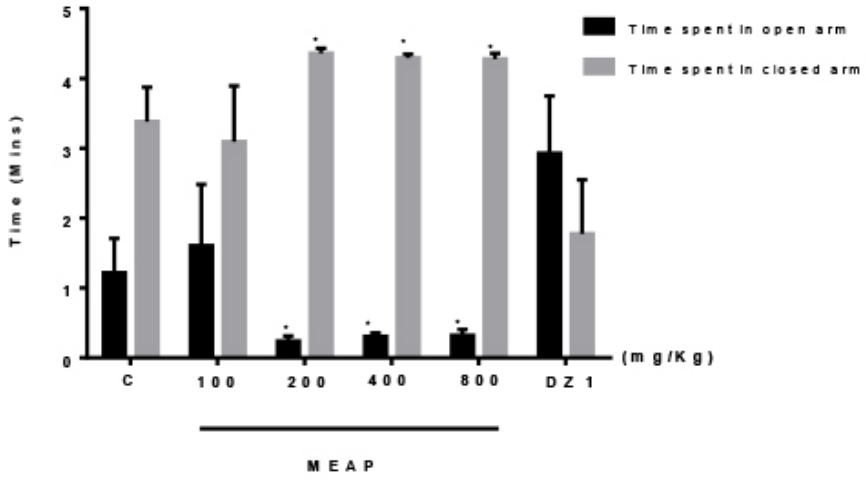

Figure 4: Effects of the methanol extract of the leaves of *A. precatorius* on the time spent in the open and closed arms respectively in the Elevated plus maze test in mice. Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. *< 0.05, indicate significant difference from the control (distilled water).

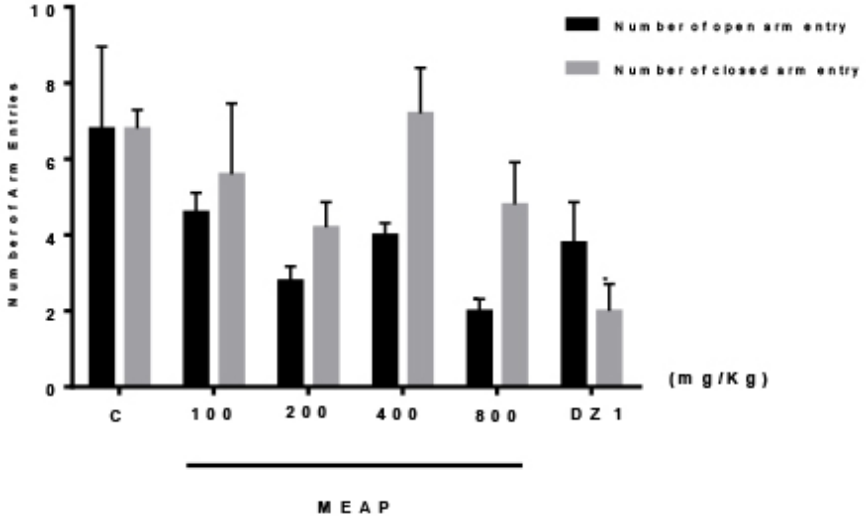

Figure 5: Effects of the methanol extract of the leaves of *A. precatorius* on the number of open and closed arm entries in the elevated plus maze in mice Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * p< 0.05, indicate significant difference from the control (distilled water)

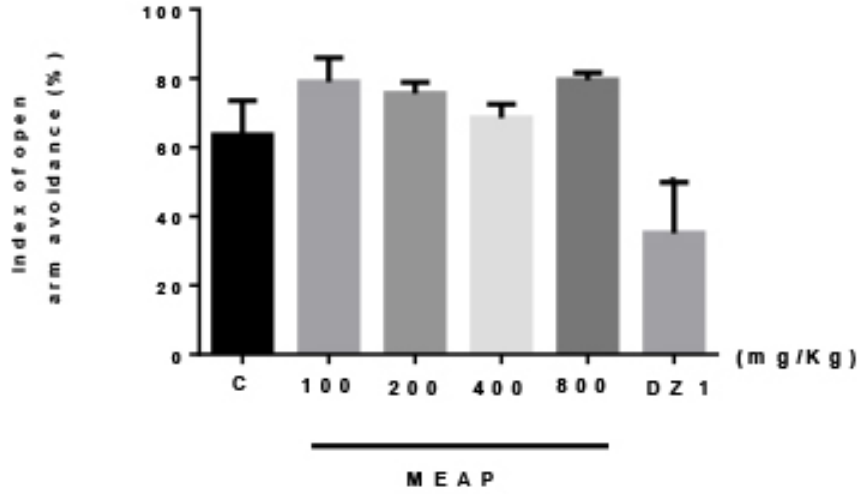

Figure 6: Effects of the methanol extract of *A. precatorius* leaves on the index of open arm avoidance Bars represent mean values with error bars. One-way ANOVA followed by Dunnet's multiple comparison test. * $p< 0.05$, indicate significant difference from the control (distilled water).

Commonly,anti-seizure drugs are screened using PTZ-induced convulsion [23, 24]. MEAP protected 25% at 400 and 800 mg/kg in PTZ-induced convulsion, but showed no protection in picrotoxin and strychnine-induced convulsion (Figure 8). Abolishment or increase in the seizure threshold is associated to anticonvulsant activity. This observation suggests that MEAP might possess anti-epileptic effect which is probably mediated by the chloride channel of GABA/benzodiazepine receptor complex and not by the chloride channel of glycine receptors. PTZ evokes convulsions via inhibition of GABAergic neurotransmissions by interfering with $GABA_A$ receptors[25]. The enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion respectively [26, 27]. Phenobarbitone and diazepam exert their antiepileptic effects by enhancing the GABA-mediated inhibition in the brain [28]. MEAP might possibly antagonize pentylenetetrazol convulsion by interfering with GABA aminergic mechanism(s) to exert its anticonvulsant effect.

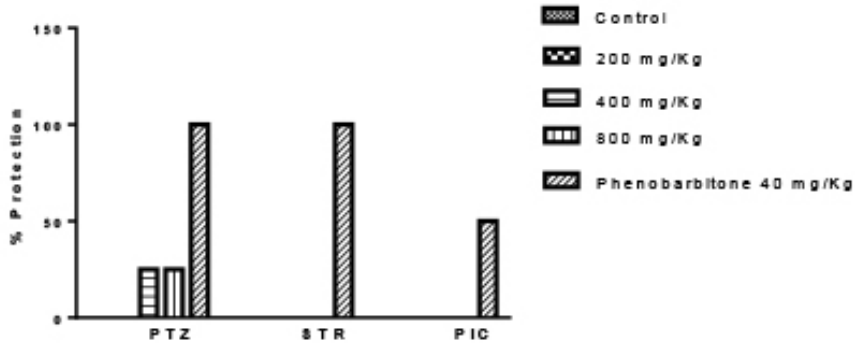

Figure 8: Effects of methanol extract of *A. precatorius* leaves on PTZ, Strychnine and Picrotoxin induced convulsions. Notes: PTZ Pentylenetetrazol 85mg/Kg; STR Strychnine 2 mg/Kg; PIC Picrotoxin 14 mg/Kg

Increasing evidence from several studies indicates that antiepileptic drugs therapy may play a role in intellectual and behavioral impairment in children with epilepsy [33, 34].

The findings of this study have shown that the methanol extract of *Abrus precatorios* has anticonvulsant and sedative actions ('activity' seems most suitable). These identified activities justify the proposition that the herbal (plant) extract should be subject to further experimental test conditions with human agents to properly determine how effective its use can be in the management of epilepsy.

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Determination of Phenytoin in Human Plasma by a Validated HPLC Method: Application to Therapeutic Drug Monitoring Study

Running title: Measurement of phenytoin in plasma by a novel HPLC method

Emrah Dural [1*], Aslı Bolayır[2] , Burhanettin Çiğdem[2]

1 Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Turkey
2 Department of Neurology, Faculty of Medicine, Sivas Cumhuriyet University, Sivas, Turkey

ABSTRACT

The aim of this study was to develop a simple and reliable HPLC method for the determination of phenytoin (PHT) in human plasma. Accuracy (RE%) were determined between (-0.93%) to 2.49% and precision (RSD%) values was ≤7.94. The quantitation limit was 3.54 μg/mL and recovery was found between 82.15% and 101.06%. The method was applied to real plasma samples (n = 7). Plasma-PHT levels were found between 1.12 and 18.76 μg/mL (9.52 ± 7.78, mean±SD). Both the plasma and dose-rated plasma results of PHT showed so high RSD% which were between 81.74% and 89.61%. In addition plasma-PHT levels were outside the recommended treatment range in 4 of the 7 patients (57.14%) examined, and also surprisingly PHT could not be detected in a patient's plasma. This procedure is relatively simple, precise, and applicable for routine therapeutic drug monitorization of PHT in neurology clinics or toxicological analyses in reference laboratories.

Keywords: Phenytoin, Human plasma, Therapeutic drug monitoring, Method validation, HPLC-UV.

Corresponding author:
Emrah Dural, Associate Professor
Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology 58140, Sivas, Turkey
Telephone number: +90 346 2191010 (#3894)
Fax: +90 346 219 1634
E-mail: emrahdural@cumhuriyet.edu.tr
ORCID
Emrah Dural 0000-0002-9320-8008,
Aslı Bolayır 0000-0001-6566-3751,
Burhanettin Çiğdem 0000-0003-4941-9497.
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INTRODUCTION

Epilepsy which affecting people of all ages is the most common serious neurological disease that statistic studies have shown that, it affects 1% of the world population [1] and may cause permanent deformation and reduce quality of life and necessitate expensive treatment [2]. It is a serious, potentially life-threatening brain disease that can successfully treat symptoms in most patients with one or more antiepileptic drugs [3].

PHT, 5,5-diphenyl-imidazoline-2-4-dione, (Figure 1.a) is one of the most widely used anticonvulsant drug for the treatment of many epileptic-type seizures (grand-mal complex partial seizures), and is usually given orally in doses ranging from 100 to 600 mg/day [4]. Either it can be use to treatment of epilepsy seizures or use for prevent the seizures in patients under the risk [5]. PHT, following the oral ingestion, precipitates in stomach. A good correlation is showed between its plasma concentration and clinical effect [6]. Although peak plasma levels occur 3-12 hours after a single dose, its absorption can be extended up to 2 weeks, especially at large overdose. PHT is highly bounding to plasma proteins (90%) and free (unbound) PHT is the component that responsible to its pharmacological effect, so any factor that modulates protein binding of PHT is expected to alter free drug levels. The interaction of PHT with other drugs or diseases (e.g., renal failure, uremia) and critical diseases can lead to the observation of plasma levels in which its toxicity may occur by altering its pharmacokinetics and/or efficacy. PHT has poor water solubility and a narrow therapeutic index, so it is very important to achieve equilibrium to avoid pharmacodynamic activity as well as dose-dependent side effects [7]. It is also reported that the enzyme system including PHT metabolism becomes gradually saturated, in which the increase in the PHT dose caused to a decrease in its elimination. That means after the enzyme system becomes saturated with PHT, even a small change in dose could be cause to a big change its plasma levels. PHT concentrations leading to enzyme saturation are highly variable among individuals, therefore, a 50-fold difference in plasma PHT concentration may be observed between patients receiving the same dose. In addition, it stated that PHT may demonstrate non-linear pharmacokinetics even with the therapeutic range [5]. It is excreted by bile as inactive metabolites, which are reabsorbed from the intestinal tract and by urine [8]. Monitoring of PHT plasma levels is clinically so important to achieve and maintain its therapeutic efficacy. Recommended PHT therapeutic serum/plasma concentrations are reported between 10 to 20 μg/mL. Oral exposures are associated predominantly with CNS symptoms, so its common dose-related adverse effects are somnolence, fatigue, dizziness,

confusion, visual disturbances, nystagmus and ataxia, addditionally, nausea, vomiting and anorexia which are gastrointestinal side effects are also seen. The effects of PHT overdose between 20 μg/mL and 40 μg/mL are far lateral nystagmus, 45° lateral nystagmus, ataxia and decreased mentation, respectively. When PHT plasma value is reached greater than 100 μg/mL, it lethal effect [8].

Therapeutic drug monitoring (TDM) is a well-established procedure that helps maximize the effectiveness of antiepileptic treatment and minimizes its adverse effects and also increases clinical efficacy. Currently, monitoring of PHT plasma concentrations like to most of other anti epileptic drugs, carried out by commercial immunochemical determination methods. In addition to that imminochemical kits are mostly designed for just an individual drug. Although they can be very sensitive in some applications, unfourtunately they have the cross-positive reaction risk. So they can give false-positive result. For these reasons, the results obtained from drug monitoring of immunochemical methods need to be confirmed by any chromatographic method.

HPLC is a simple, sensitive, accurate and cost-effective method [9,10] and it gives a good recovery with high precision and also easily accessible in many laboratories since cheaper compare to other techniques [11]. This chromatographic technique used to separate the components in a mixture, to identify each ingredient, and to measure each component. The method includes a liquid sample being passed over a solid adsorbent packed into a column using a flow of liquid solvent known as the mobile phase. Each analyte in the sample interacts with the adsorbent at a different level, which causes a change in the flow of the analytes in the column. If the interaction is strong, the analytes flow off the column in a long amount of time and if the interaction is poor, then the elution time is short [11]. This technique could give excellent results at the identification of many pharmaceuticals. For these reasons, high-performance liquid chromatography (HPLC) has been using as reliable techniques for the determination of PHT and other anticonvulsant drugs.

HPLC coupled with ultraviolet (UV) detection [12–18], flourescence detection [19] , tandem mass spectrometry (MS-MS) [20–23]; gas chromatography (GC) coupled with mass spectrometry (MS) [18,24] and capillary electrophoresis (CE) coupled with UV [6] are the reported methods that were used for determination of PTH in biological samples. In addition to that, colouremetric based determination methods [25], radioimmunoassay [18], enzyme immunoassay (EMIT) [18,26], fluorescence polarization immunoassay (FPIA) [27] and spectrophotometric assay [18] have been often used monitorization of PHT.

Some extraction tecniques which based on protein precipitation (PPT) [12], liquid-liquid extraction (LLE) [13,16,22,23], solid-phase extraction (SPE) [12,14,16,24], solid-phase micro extraction (SPME) [28,29] has been used for preparation of the biological samples to the PTH analyses. Protein precipitation method is a simple, easier and fast method compare to all of these applications. It needs moderate amount solvent and short spin-down step and generally after these steps, supernatant/resulting extract can be apply to the HPLC that desired amount. In addition to this, some apllications needs to filtration of the samples. However, there is a risk that the extract obtained may block the column if the method is not well established. Also, obtained chromatograms applied this tecnique usually shows very high backround that is exactly so important for selectivity and specificity of the method. LLE is often preferred in the monitoring of many pharmaceuticals. It is a technique in which the matrix pH of the solvents to be used in the application can be adjusted according to analytes and application [30]. After evaporation of the solvent obtained at the end of the application, the residue can be analyzed by reconstitution in the appropriate solvent. Extraction recovery values are sufficient for drug analysis from biological samples. Also, resulting extract product may be transferred, evaporated to dryness and reconsituted with a suitable solvent prior the analysis. But in this technique usually, a high volume of solvents consume which has toxic properties. In addition, the LLE application has multiple steps, each of which takes a long time. SPME was introduced as a solvent-free process that may use different types liquid samples. Although, it has important properties, techniques need to expensive fibers that rather sensitive complex matrix like to plasma. However, protein precipitation method is a simple and fast method compare to all of these applications. It needs moderate amount solvent and short spin-down step and generally after these two steps, supernatant/resulting extract can be apply to the HPLC that desired amount. SPE is a selective sample preparation method that uses a packet solid sorbent (silica or polymer) to isolate the desire analyte, that compare to LLE, less amount solvent which may toxic uses in SPE. However, the price of adsorbents needed in this technique is expensive, leading to an increase in the cost per analysis. Although the price of commercial adsorbents needed in this technique is expensive, the recovery efficiency obtained is quite successful. In the samples obtained using this method, validation test results, especially accuracy and precision, are generally quite successful. In addition, many natural-plant materials and synthetic products produced from natural materials can be used as solid phase absorbant [30].

Aim of this study is to develop a simple, rapid and reliable chromatographic method and to validate it in terms of linearity, repeatability, sensitivity, recovery, and robustness according to ICH guideline [31]. This simple, reliable and

precise extraction method allowed the determination of human plasma PHT without any process of the deproteinization and derivatization. The method has a wide linear range that could be used efficiently in cases of therapeutic, subtherapeutic and overdose poisoning. It was used in a therapeutic drug monitoring study to determine the PHT levels in a plasma sample of 7 patients treated with PHT. Blood PHT concentrations of volunteers treated with PHT were determined by this method and the relationship between drug doses and blood results were statistically analyzed.

METHODOLOGY

Chemicals and reagents

The pharmaceutical standards of PHT (Figure 1-a) and DZP (Figure 1-b) were kindly donated by VEM Pharmaceuticals Company (Istanbul, Turkey) and Forensic Science Institute of Ankara University (Ankara, Turkey), respectively. NovaPack® solid-phase cartridge was obtained from Waters (Milford, MA-USA). HPLC grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade potassium dihydrogen phosphate, dipotassium hydrogen phosphate and orthophosphoric acid were bought from Merck (Darmstadt, Germany). Membrane filters (0.45 μm pore size) obtained from Millipore (Massachusetts, USA). Elga Purelab Water Purification System (Lane End, Buckinghamshire, UK) was used to obtain ultra-pure water.

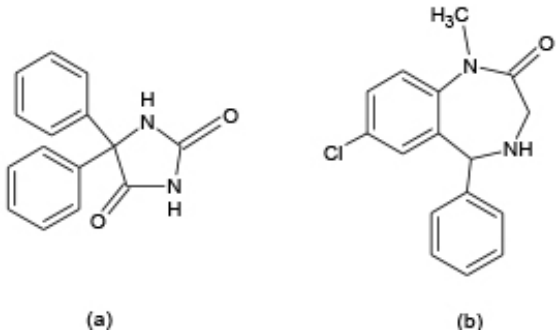


Figure 1. Chemical structures of PHT (a) and DZP (b).

Selection of internal standard chemical to be used in analysis

Fluphenazine, carbamazepine, opipramol, imipramine, sildenafil, and DZP were tested for use as an ISTD. Although, carbamazepine and sildenafil showed good ultraviolet chromatographic characteristics, their retention times (tR) were unsuitable for the chromatographic analysis. Opipramol and imipramine didn't show good intensity at these chromatographic conditions. DZP's chromatographic characterics were so fine for using as ISTD. It showed very good intensity in the low concentrations. The obtained extraction recovery values were shown to be acceptable and reproducible. Also, it's separation sharpness and retention time has acceptable in the chromatogram. Furthermore, the co-administration of PHT and DZP in treatment is not frequently observed due to the different pharmacological effects. Thus, it was decided that the DZP should be selected as ISTD.

Instrumentation and chromatographic conditions

A Hewlett-Packard Agilent 1100 series (California, USA) high-performance liquid chromatography (HPLC) system which equipped a degasser (G1322A, Degasser), a gradient pump (G1311A, QuadPump), a column oven (G1316A, Colcom), a manual injector (Rheodyne 7725i) has 20 μL loop volume, and an ultraviolet detector (G1314A, VWD) was used for separation and quantification. The integration of chromatographic data and system control (Palo Alto, USA) were achieved by Agilent Chemstation 08.03 software was used as. Analytical simultaneous separation was performed by a stainless steel Zorbax RP analytical column (4.6 mm x 250 mm) packed with C_{18} filling material has 5 μm particle size (USA).

Optimum chromatographic conditions were set after performed for column, separation temperature, mobile phase content and detector wavelength. The better analytical seperation results obtained from the C18 RP Zorbax column (4.6 x 250 m, 5 μm particle size) than the C18 RP Waters column (3.9 x 150 mm, 5 μm particle size). The minimum back pressure and enough peak resolution for column were reached at 40°C in oven temperature. Although different wavelengths (246, 254, 270 and 290 nm) were also used to determine PTH and DZP, the highest peak sharpness and the lowest interference were obtained from 220 and 230 nm, respectively. The ultraviolet spectrums of PTH and DZP are given in Figure 2.

The mobile phase, composed with 10 mM KH_2PO_4 and 10 mM K_2HPO_4 that contain 0.1 % triethylamine and acetonitrile (60:40, v/v), was filtered through a 0.45 μm membrane (Illinois, USA) and before the using it was degassed by an ultrasonic bath, took at 30 minutes. Mobile phase's phosphate buffer was prepared with fresh ultrapure water and after adding triethylamine pH was ad-

justed to 3.0 with 100 mM ortophosphoric acid and mixed with acetonitrile. The mobile phase was isocratically applied to the column set at 40°C with 1.0 mL/min constant flow. Determination of PTH concentration in the quality control and human blood samples were carried out to using linear regression of response (drug/ISTD peak area) versus DZP concentrations.

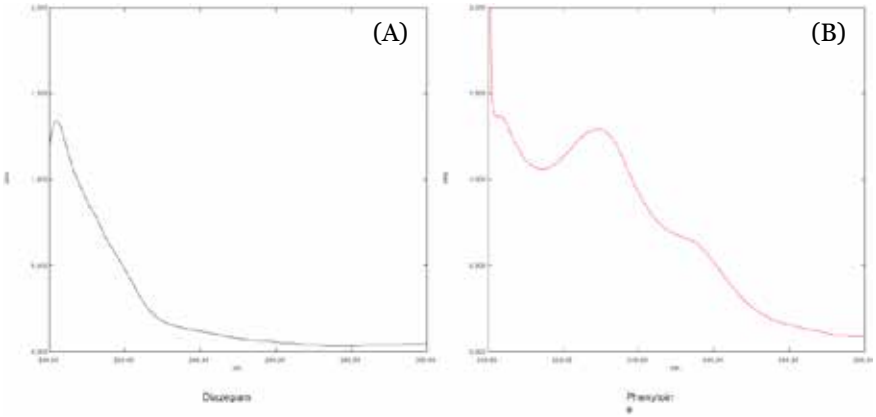


Figure 2. a. The ultraviolet spectrum of PHT. **b.** The ultraviolet spectrum of DZP used as an internal standard.

Preparation of stock standard solutions and working standards

Stock solution of PTH was prepared in methanol as 10 mg/mL and stored at -20 °C until use. It had been observed that stable at least 1 months. Working solutions of PHT were prepared weekly from the main stock solution in methanol as 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50 mg/mL concentrations. Working standards were prepared weekly, and used to spike blank plasma samples daily, prior to analysis. PHT dilutions were freshly prepared into the drug-free human plasma to provide concentrations of 5, 10, 15, 20, 30, 40 and 50 μg/mL.

DZP main stock solution (1 mg/mL) was prepared and used weekly with methanol to yield 20 μg/mL of DZP, in plasma. Likewise, plasma quality control standards spiked with 5, 20 and 40 μg/mL of PHT were prepared to measure the repeatability values of the method. Also same protocol was used in preparation of limit of detection (LOD), quantification (LOQ), recovery and robustness test samples.

Preparation of quality control samples and real plasma samples

Fresh the pool of human blood were prepared that collected from Sivas Cumhuriyet University, Blood Center of Medical School. These plasma samples have been used the forming of the quality control samples used during the development and validation process of this method. Also, the collected real patient blood centrifuged at 4000 rpm for 5 min to separate the plasma. Quality control plasma and real patient plasma samples were stored at -20˚C until the analyses were carried out. Working solutions were checked chromatographically for purity before experiments, were utilized as quality control specimens and were checked for the stability before and after the injections of every sample set.

Waters (Milford, MA-USA) NovaPack® cartridges (100 mg, 1 ml) were used for sample pretreatment. The SPE procedure was carried out on a glass SPE apparatus according to the following steps:

i. Cartridge adsorbent was conditioned with 1 mL acetonitrile;

ii. Equilibration was done with 1 mL water;

iii. Applying sample constituted in 500 µL plasma with 10 µL ISTD (1 mg/mL) standard solution and 10 µL STD (for quality control samples) to cardridge;

iv. Washing (2x) with 1 mL water;

v. Eluting with acetonitrile for 2 min at 60 kPa (to elute acetonitrile as completely as possible);

vi. Evaporating of the collecting extraction solvent under nitrogen;

vii. Injecting into the analysis system as a volume of 20 µL after reconstitute of the residue in 500 µL of the mobile phase.

Method validation

The developed analytical method was validated in terms of the specificity and selectivity, linearity, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), recovery and robustness. Intraday and inter-day validation protocol were applied considering reproducibility of the method to obtain accurate and precise measurements in accordance with International Conference on Harmonization (ICH) Q2R1 guideline [31].

Specificity and selectivity

The method showed excellent chromatographic specificity without endogenous interference at the retention times of PHT and DZP (5.8 and 10.0 min) in plasma. Representative chromatograms, which are blank (Figure 3-a), spiked (Fig-

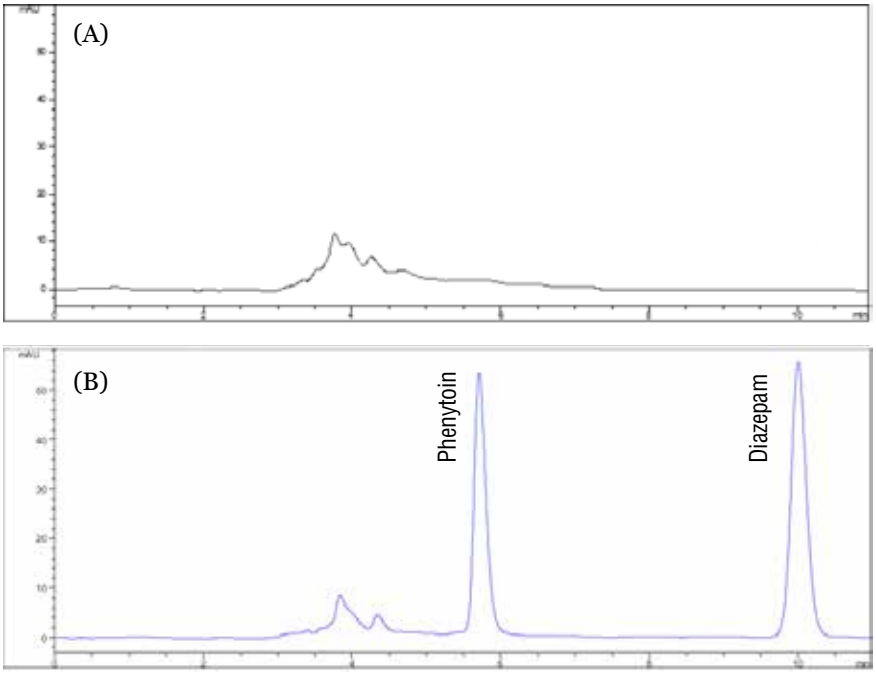
ure 3-b) and real patient samples (Figure 3-c), were illustrate the high chromatographic resolution that conducted in 11 minutes.

Linearity

After chromatographic conditions were established, matrix-based calibration curves of PHT was plotted concentrations over the range 5 - 50 μg/mL versus peak-area ratios to the ISTD. The calibration points (n=7), which were 5, 10, 15, 20, 30, 40 and 50 μg/mL composed 3 individual replicates were prepared by standard addition method in plasma and injected to HPLC.

Accuracy and precision

The accuracy, defined as the relative error (RE%) was calculated as the percentage difference between the added and found PHT quantity by 5 individual replicates both intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by five separate replicates of PHT both intraday and inter-day. Five replicate spiked samples were assayed intraday and inter-day at the three different concentrations (5, 20 and 40 μg/mL).



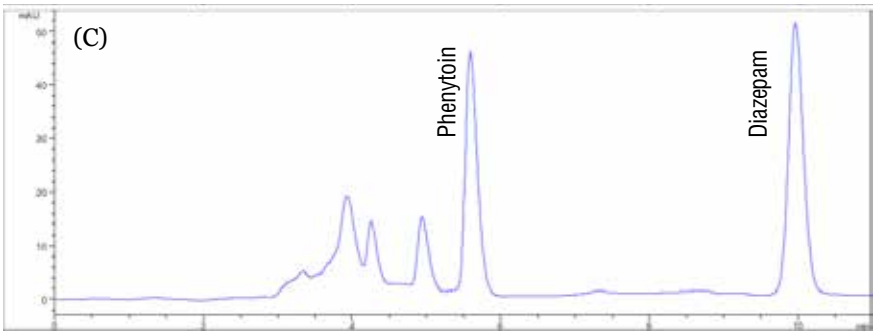


Figure 3. a. A chromatogram sample which belongs to blank plasma used to establish the quality control standards in validation studies. **b.** A chromatogram sample that contained PHT as 20 μg/mL which is prepared by standard addition method used as quality control sample. **c.** A chromatogram sample obtained form the real patient plasma

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH recommendations based on standard deviation of the response and the slope of the calibration graph. 10 μg/mL was used as the lowest calibration point in sensitivity test of PHT.

$$LOD = 3.3 \frac{\sigma}{s} \quad ; \quad LOQ = 10 \frac{\sigma}{s}$$

(σ: The standard deviation of the response; S: The slope of the calibration curve).

Recovery

The recovery of extraction procedures from human plasma was determined by comparing pre-extraction spikes with the post-extraction spiked ISTD. Five individual replicates of spiked samples at low, middle and high concentrations (5, 20 and, 40 μg/mL, respectively) of PHT were prepared with and without ISTD. Extraction procedure was carried out as described before in sample preparation step.

Robustness

The robustness test was performed with 20 μg/mL of PHT, which is the approximate medium concentration of the calibration interval. The response of the method of changes in ultraviolet wavelength (± 3 nm), mobile phase flow rate (± 0.1 mL/min), mobile phase solvent content (± 5%) and column temperature (± 5 °C) was observed.

Collection of plasma samples

Approximately 1 mL whole blood samples were taken from patients who had formed the steady-state concentration of the PHT in their plasma. As a rule, blood samples were taken from the patients after 12 hours the last drug administration. After it was centrifuged at 4000 rpm for 5 minutes, obtained individual 0.5 mL plasma samples stored in -18 °C until analyzed. The ethical permission of the this research was approved by the Clinical Research Ethics Committee which belong to Sivas Cumhuriyet University Medical School, with 2018-01/22 decision number on 10 January 2018 and was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Prior to inclusion in the research, the informed consent was obtained from all volunteers. Blood samples were obtained from 7 patients who were under the PHT treatment at the Sivas Cumhuriyet University, Medical School Department of Neurology. Blood samples collected in vacuum tubes containing Na_2EDTA were then centrifuged at 4000 rpm for 5 minutes on the same day. The supernatant (plasma) was transferred to the micro experiment tubes and it has been stored at the -20 °C freezer until the analysis. Plasma PHT levels in patients were measured in less than one months.

Statistical analysis: All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 22.0. Statistical analyses were performed using Student's t test for two independent means and correlation analysis- bivariate with a p value <0.05 considered to be statistically significant.

RESULTS AND DISCUSSION

Method validation

Validation procedures were conducted according to ICH-Q2R1 guideline during the whole test steps. [31]

Linearity

Calibration curves of PHT drawn at 7 points (n=3) between 5 - 50 μg/mL concentration versus the area of DZP as ISTD by the standard addition method showing excellent correlation with $r^2 = 0.9939$, respectively (Table 1). The correlation of values obtained at the individual different 7 points was quite good. The linearity study was designed to covered sub-therapeutic, therapeutic and toxic drug levels of the drug. The wide linear range has also had a positive effect on the use of the method. Since, the obtained real blood results was shown very high standard deviation.

System suitability parameters show that it has a good resolution (Rs) and selectivity (α). Capacity factor and theoretical plate number (*N*) show enough chro-

matographic properties for a successful determination of PHT from plasma as it can follow in Table I.

Table I. Chromatographic characteristics and system suitability parameters of the method.

Analyte	Retention time (min) tR	Linear range (µg/mL)	Calibration equation	Correlation coefficient (r^2)	Capacity factor (k')	Theoretical plate number (N)	Selecitivity (Separation) factor (α)	Resolution (Rs)	LOD (µg/mL)	LOQ (µg/mL)
PHT	5.8	5 - 50	y=0.0345+ 0.0581	0.9939	1.0	1893	1.7	2.2	0.12	0.35
DZP	10.0	-	-	-	2.5	10519	-	-	-	-

Capacity factor (k') = $\frac{tR\text{-}tO}{tO}$; Theoretical plate number (N) = 16 $(\frac{tR}{Wt})^2$; Resolution (R_s) = $\frac{\sqrt{N}}{4}\ \frac{\alpha\text{-}1}{\alpha}\ \frac{k}{k+1}$; Specificity factor (α) = $\frac{k2}{k1}$. Abbreviations: t_R: retention time of the analyte peak; to: retention time of mobile phase peak ; WT: peak width

Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve [31] (LOD= 3.3σ/S, LOQ= 10σ/S where σ is the standard deviation of the response and S is the slope of the calibration curve). The results of LOD and LOQ values, which were obtained by the measurement of individual 10 quality control (QC) samples, demonstrated in Table I.

Precision and accuray

The data obtained from the accuracy and precision tests, performed in intraday and inter-day with quality control standards established in the blank plasma samples by standard addition method, showed low RSD% values ≤7.5% and ≤7.94% for interday and intraday respectively and also low RE % average values between (-0.90) – 2.49% for inter-day and (-0.93) – 0.22% for intraday test values (Table II). The obtained repeatability results were show that, the method has excellent precision and accuracy values not only intraday but also inter-day analyses.

Recovery

Recovery test results which at done at 10, 20 and 40 µg/mL were between 82.15% and 101.06% and the results were given in Table II. The method has a highly successful analytical result with the average recovery of 90.36%. Recov-

ery values were obtained in the extraction procedure has demonstrated excellent efficiency. It was observed that extraction procedure was not complicated and also was not need to a sophisticated instruments.

Robustness

No significant changes in the analytical signals were observed upon changing ultraviolet wavelength value (± 3 nm), mobile phase flow rate (± 0.1 mL/min), mobile phase organic solvent ingredient (± 5%), and column temperature (± 5 °C). As well as, change of analysts, columns, sources of chemicals and/or solvents did not lead to significant changes in chromatographic signals and results, too. As it can follow in Table III, robustness experiment results demonstrated that the method has a high ability that created data of acceptable precision and accuracy.

Table II. Confidence parameters that including intraday, inter-day precision and accuracy and recovery values. These results were obtained from individual samples (n=5) prepared as quality control samples in real plasma.

Expected conc. (µg/mL)	Intraday repeatability			Inter-day repeatability			Recovery (%)
	Observed conc. $\bar{x}$±SD (µg/mL)	Precision (RSD %)	Accuracy (RE %)	Observed conc. $\bar{x}$±SD (µg/mL)	Precision (RSD %)	Accuracy (RE %)	
10	10.38 ± 0.82	7.94	0.22	10.25 ± 0.27	2.59	2.49	82.15
20	19.99 ± 0.92	4.48	- 0.05	20.47 ± 1.53	7.50	- 0.90	87.87
40	38.75 ± 2.43	6.27	- 0.93	39.69 ± 0.92	2.33	- 0.78	101.06

Table III. Robustness data of the described method representing as the RSD% value. These results were obtained in the analysis of three variable points calculated by independent (n=5) analyzes.

Analytes	Mobile phases solvent content (± 5%)	Ultraviolet wavelength (± 3 nm)	Flow rate (± 0.1 mL/min)	Column temperature (± 5 °C)
PHT (20 µg/mL)	4.5	2.8	1.8	2.1

Stability

The stability of QC plasma samples (10, 20 and 40 µg/mL) and analytes in stock solutions under several conditions were assessed. Stability of the stock solutions at room temperature was evaluated with 1, 2, 3 and 4 week periods. The stability test of freeze-thaw was executed by three QC samples after operating five repeated freeze-thaw period. The stability test of long-term was carried out for 1, 2 and 3 months using QC samples maintained at -20°C. Neither significant decrease nor degradation were observed in the concentration of VPA in three different conditions. The relative standard deviation in all samples was less than 5.3 %.

Measurement of PHT levels in patient plasma samples

The developed HPLC method was used to monitoring of the PHT levels in plasma samples taken from 7 patients who receiving PHT orally between 100 and 300 mg/day. Plasma samples were prepared according to the extraction method described previously. None of these samples showed any problem for the quantification of the analytes, additionally, peak purity showed that no analytical interference was encountered from endogenous substances. The daily used PHT amounts, PHT plasma levels, its dose-proportional levels and the descriptive statistical analysis for the obtained data are given in Table IV.

Table IV. The samples included to the analysis, daily PHT doses, plasma and dose proportionally plasma PHT concentrations.

Patient sample number	PHT Dose (mg/day)	Method-1st		Method-2nd	
		Plasma PHT concentration (µg/mL)	Plasma PHT concentration/ Dose (µg/mL/g)	Plasma PHT concentration/ Dose (µg/mL/g)	Plasma PHT concentration/ Dose (µg/mL/g)
PHT-01	300	6.87	22.89	3.88	12.93
PHT-02	100	14.30	143.00	<2.5	<25.00
PHT-03	300	18.50	61.66	10.6	35.33
PHT-04	-	7.07	-	-	-
PHT-05	300	18.76	62.52	11.0	36.67
PHT-06	200	1.12	5.59	<2.5	<12.50
PHT-07	-	ND	-	-	-
Average		9.52	59.13	8.49	28.31
SD		7.78	52.99	4.00	13.34
RSD%		81.74	89.61	47.10	47.11

Note: Method–1st refer to our developed and validated analysis method and Method–2nd refers to the analysis method used routinely from Hospital.

Same plasma samples were analyzed with the homogeneous enzym immunoassay (EMIT) tecnique. The method used for routine therapeutic drug monitoring analysis of PTH was established by Sivas Cumhuriyet University, Faculty of Medicine. According to obtained patient results, LOQ of this method was given 2.5 µg/mL. However, this method does not give definite PTH plasma levels which may be observed in low levels due to polymorphism and compliance problem to PTH. The correlation between our method and the other immunoassay based method was 1.00 ($p<0.01$) and a very strong correlation was observed. On the other hand, when the obtained results which got from two different methods were evaluated, it was observed that plasma results showed that the different PTH values. The difference between plasma PTH results obtained by two methods was found to be statistically not significant ($p>0.05$).

As a sample, a volunteer patient chromatogram was given in Figure 3-c which a representative of the real plasma sample separation. As can be clearly seen, no interference was observed that could affect the analysis. All blood samples were successfully pretrated to the analyses and their owned PTH quantities were measured properly. Although these patients undergoing plasma drug monitoring were in multidrug treatment, as can be clearly seen from the sample chromatogram, no interference was observed that could affect the analysis not only as pharmaceutical but also endogenous from plasma.

There was 7 voluntary patients' blood samples, who have been treated orally PTH between 100 and 300 mg/day, and the average dose/day of PTH was 240 mg/day. The detected average blood PTH level was 9.57 μg/mL (7.71 ± 80.58, SD ± RSD%). SD and RSD values were higher than expected due to the deviation between plasma PTH results. Since the therapeutic range of the PTH is so narrow and its have serious toxicological risks, the investigation of blood values is of great importance. For this reason, the drug level monitorization is suggested during treatment with this drug. More interesting results were observed in the dose-proportional results of plasma level of PTH with values of 59.13 μg/mL/g ± 52.99 (89.61) (mean ± SD (RSD%)). The unexpected high levels standard deviation (SD%) and also RSD% emerged in the result. The metabolic differences of enzymes who responsible for the metabolism of this drug between individuals are thought to play an important role in the emergence of this difference. In addition, the bioavailability of this drug may negatively be affected by the first pass effect. This situation leads to a reduction in the pharmacological and pharmacodynamic effect expected from the PTH treatment.

Recommended blood plasma values for the treatment with PTH are between 10 and 20 μg/mL. The result of the study showed that out of 4 in 7 patients treated with PTH (57.14%) had plasma concentrations below these values. However, this is very important because the patients show that they continue to undergo treatment for PTH at a lower level than they need. This means that, despite drug intake, serious symptoms of the disease cannot be prevented. In addition, it is toxicologically important that no overdose was observed for PTH in any of the plasma of the 7 patients monitored. This situation is very important in terms of public health because the treatment has serious toxicological risks.

It was thought that the effect of biotransformation enzymes on PTH was an important factor in the emergence of this unexpected result. On the other hand, multidrug therapy is thought to be another important cause of the outcome. Consequently, this result showed that the importance and necessity during the treatment of the TDM.

The study published by Bugamelli et al. (2002) phenytoine and totaly six antieplieptic and two sellected metabolites were pretreated with two different procedures which are protein precipitation and solid-phase extraction were tested in this study and then obtained extracts were determined by HPLC-DAD method [12]. In this research, 250 µL plasma sample was used in the analysis and instrument was calibrated with quality control samples which were prepared in real plasma. Seperation was achieved with the mixture of methanol, acetonitrile and 15 mM phosphate buffer containing 0.63% (pH 3.0) triethylamine (19.2:16.8:64.0, (v/v/v)) by a C18 column (150 x 4.0 mm, i.d. 4.5 µm). Altrough, LOQ was detected as 0.2 µg/mL, correlatlion coefficiency was foud 0.998. Linearity study was applied between 4 and 40 µg/mL. PTH retention time (Rt) was approximately 11.0. Also, althought recovery was determined between 94 to 101%, precision was detected as ≤ 10.3.

Another HPLC-DAD based study was carried out by Dalmora et al. (2009) [13]. In study, human plasma samples (300 µL) were prepared with liquid-liquid extraction method and isocreatic seperation was achieved with a reverse phase C12 column (150 x 4.6 mm i.d.) with a mobile phase constituted with water: acetonitrile: methanol (58.8:15.2:26, v/v/v). Detector was set at 205 nm. Phenobarbital was used as an internal standard. Chromatographic run time was 12 min. Instrument calibrated with quality control samples prepared between 50 and 2500 ng/mL PTH. Precision was ≤4.48 RSD% and accuracy was between 98.71 to 100.17%. This developed method was succesfully applied to 22 volunteers' blood for the bioequvalance study for evaluation of two tablets formulation.

Khedr et al. 2008 [14] was developed a new HPLC-DAD method in rabbit plasma. Sildenafil was used as an internal standard. Extraction was achieved with a based on solid-phase method and detector set at 220 nm. Isocreatic seperation was achieved with Agilent Zorbax Extended C18 column (150 mm × 4.6 mm internal diameter) and mobile phase which consist of 29% acetonitrile and 71% sodium acetate solution (0.02 M, pH 4.6). Method was linear between 0.15 to 39 µg/mL and LOD was found as 0.15 µg/mL. Recovery was found 101.88%. The mobile phase consist of 29% acetonitrile and 71% sodium acetate solution (0.02 M).

In another study, Maya et al. (1992), a new method was developed was described for determination of PTH in plasma and urine and its metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin, in urine [15]. Seperation of extracts a Nova-Pak RP-C18 column was achieved in the chromatographic seperation using a mobile phase consisting of methanol-water-tetrahydrofuran (40:60:4, v/v/v) with UV detection at 230 nm. The method established to determination of PHT in plasma and urine was linear between 0.4-4.0 µg/mL and 0.1-1.0 µg/mL. Precision showed a good value which is RSD% ≤4.49%.

Guan et al. (2000), [16] is described a reliable and sensitive method for the extraction and quantification of phenytoin (5,5'-diphenylhydantoin), its major metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) and minor metabolite, 5-(m-hydroxyphenyl)-5-phenylhydantoin (m-HPPH) in equine urine and plasma. Solid-phase extraction (SPE), liquid–liquid extraction (LLE), enzyme hydrolysis (EH) methods were used in the sample preparation step and high-performance liquid chromatography (HPLC) was used for determination of extracts.

Bahal and Nahata (1993) [17] was described a new method for determination of both phenytoin and its major metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) in urine. In this method, a Water 10 μm μBondapak C-18 column was used in the sample extraction step, a mobile phase containing 45% acetonitrile and 55% distilled water was used in the seperation, and the ultraviolet detector set at 230 nm was used for the quantitative determination. Analitical run time was ≤9.0 min. As an internal standard, 5-(4-methylphenyl)-5-phenyl-hydantoin was used. The correlation coefficients was 0.999. Accuracy was between 94.3 and 108.8 RE%. Precision (RSD%) was ≤5.0%.

The precision (≤7.94 RSD%) and accuracy ((-0.93) and 2.49 RE%) result of the method showed that it has enough repeatability values. Exactly, the observed values from the accuracy tests were excellent. The obtained results from the method repeatability increase the reliability of the performed analyses. The sample extraction, has single step, was simple and rapid, which provided the excellent reliable recovery values between 82.15% and 101.06% (90.36±9.70, $\bar{x}$±SD). This method has some attractive properties which are the simplicity of sample preparation protocol although it was a solid phase method; relatively short analysis time (11 min) and favourable LOQ value. Furthermore, our analysis method stands out from published methods with remarkable accuracy and recovery values. In addition, the robustness and stability tests performed demonstrated the strength of the method against changing conditions which ability may effect seriously the analysis. Almost all of the patients included in this study were using at least one other drug in addition to PHT, some of which are known to be under heavy drug treatment with multidrug therapy. On the other hand, this did not cause a chromatographic problem in any blood sample and no problem in quantification. Thus, the test results clearly demonstrated that this developed assay method is fast, precise and reliable for plasma PHT analyzes.

We strongly recommended this validated method to be used in routine therapeutic drug analysis of PHT and also it can be adapted for monitoring of overdose/poisoning with this drug. Furthermore, since the method is established in

the range of 5 to 50 μg/mL, it can be used in overdose and suicide cases with PHT as well as in the detection of compliance problems. The proposed method can be easily applied in routine TDM studies of PHT, also it can be preferred in bioequivalence studies, pharmacovigilance and pharmacokinetics studies.

In our study, it was observed that both plasma-PHT levels and plasma-PHT recorrected according to daily drug doses (μg/mL/g) were observed very high RSD% results which are 80.58 and 89.61%, respectively. These results are both pharmacological and toxicologically significant and have the potential to cause serious health problems.

Since these observed unexpected plasma PHT concentrations are thought to be related to the polymorphism of the enzymes CYP2C9 and CYP2C19 responsible for the biotransformation of PHT, it is planned to investigate the polymorphisms of the respective enzymes in the collected blood samples and investigate its relationship with the plasma results obtained.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article have a financial or personal relationship with each other or organizations that may inappropriately affect or bias the content of the paper. All authors declare that, there is no conflict of interest.

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Protective Effect of Exendin-4 Treatment On Oxidative Status Of Liver In Rats Exposed To Chronic Methylglyoxal

Running Title: Exendin-4 treatment in rats

Esra Akcabağ[1*], Selvinaz Dalaklıoğlu[1], İkbal Özen Küçükçetin[2], Sebahat Özdem[2], Arda Taşatargil[1], Sadi Satılmış Özdem[1]

1 Akdeniz University, Medical Faculty, Department of Medical Pharmacology, 07070, Antalya, Turkey
2 Akdeniz University, Medical Faculty, Department of Medical Biochemistry, 07070, Antalya, Turkey

ABSTRACT

The protective effects of Glucagon-Like Peptide-1 (GLP-1) agonists against oxidative stress-induced cellular injury have been well established by previous experimental and clinical studies. Male Wistar rats (200-250 g weight, n=24) were used in this study. First group of rats were not treated with Methylglyoxal (MGO) and served as control group (C group). Second group of rats (MGO group) received MGO (75 mg/kg/day in drinking water) for 12 weeks. Third group of rats (MGO+Ex-4) received Exendin-4 (Ex-4) (1 μg/kg twice daily subcutaneously) concomitant with MGO for 12 weeks. At the end of the 12th week, total oxidant status (TOS), total antioxidant capacity (TAC), sulfhydryl groups (SH), myeloperoxidase (MPO), and advanced oxidation protein products (AOPP) in the liver tissues of all groups were measured spectrophotometrically. In MGO-administered rats, TOS, MPO and AOPP levels were significantly increased. Treatment with Ex-4 for 12 weeks caused a significant decline in the levels of these markers in rats exposed to MGO. Also, levels of TAC and SH were decreased significantly after the 12 weeks of MGO administration. 12

Corresponding Author:
Esra Akcabağ
University of Akdeniz School of Medicine Department of Medical Pharmacology, 07070, Antalya, TURKEY
Telephone: +90 242 2493483
Fax: +90 242 2274482
E-mail: esraakcabag@akdeniz.edu.tr
ORCID
Esra Akcabag 0000-0002-6238-8173,
Selvinaz Dalaklioglu 0000-0001-5612-676X,
Ikbal Ozen Kucukcetin 0000-0002-4541-013X,
Sebahat Ozdem 0000-0002-0619-1405,
Arda Tasatargil 0000-0002-1077-3029,
Sadi Satilmis Ozdem 0000-0003-4325-5209.
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weeks treatment with Ex-4 also increased the levels of TAC and SH in liver tissues of MGO-administered rats. Ex-4 treatment improves oxidative parameters of liver tissue in MGO-administered rats by improving oxidant-antioxidant balance.

Keywords: Exendin-4, Methylglyoxal, Oxidative stress, Live

INTRODUCTION

Methylglyoxal (MGO), as a dicarbonyl metabolite of glucose, is an intermediate product formed during glycation of proteins by glucose and its formation involves many pathways consisting of enzymatic and non-enzymatic reactions in all mammalian cells (Lo et al., 2006; L. Wu, 2005; Yim, Kang, Hah, Chock, & Yim, 1995). Increase in the plasma level of MGO has been reported in various metabolic diseases, including diabetes, obesity and fatty liver (Hanssen et al., 2017; Kong et al., 2014; Lapolla et al., 2003; Rabbani & Thornalley, 2011; Tappy & Lê, 2012). Excess production and/or decreased degradation of MGO give way to its high levels, generating cellular toxicity (Rabbani & Thornalley, 2015). Although it is critical to maintain normal liver function under metabolic stress, metabolic disorders, including diabetes and obesity, are associated with hepatic dysfunction because of the high levels of nutrients and metabolites (Marceau et al., 1999; Rabbani & Thornalley, 2011). Several studies have indicated the pathophysiological roles of MGO in the liver (Cheng, Cheng, Chiou, & Chang, 2012; Choudhary, Chandra, & Kale, 1997)glutathione (GSH. MGO and advanced glycation end products (AGEs) produced through MGO contribute to the pathophysiology of liver toxicity (Seo, Ki, & Shin, 2014; Yılmaz et al., 2018). Besides, in an early-stage liver damage model, the levels of MGO and its metabolite d-lactate were elevated suggesting that d-lactate could be useful as a reference marker for the early stage of hepatitis (W.-C. Wang, Chou, Chuang, Li, & Lee, 2018).

The cellular injury induced by MGO is provoked through the production of oxidative stress (Rabbani & Thornalley, 2015). Reactive oxygen species (ROS) that are generated and accumulated during the metabolism of excess MGO aggravate the oxidative stress (Desai et al., 2010; Rabbani & Thornalley, 2015). The role of MGO as activating and increasing oxidative stress, mitochondrial dysfunction and apoptosis have been shown in some previous *in vitro* studies (Maruf, Lip, Wong, & O'Brien, 2015; Seo et al., 2014). MGO has also been shown to induce mitochondrial dysfunction and cell death in liver by production of ROS (Seo et al., 2014). Moreover, exposure of mice to MGO induced significant changes in redox-homeostasis in the liver (Choudhary et al., 1997). MGO has been shown to decrease the glutathione (GSH) content and increase the lipid peroxidation. It could be concluded that the activities of the enzymes involved in the pro-

tective mechanism as well as GSH levels were altered in the liver of mice by administration of MGO which in turn may disturb the antioxidant status in the animals. Enhancement of lipid peroxidation in liver indicated the possibility of involvement of free radicals in the toxic effect of MGO. All of these studies suggest that oxidative stress is involved in liver toxicity induced by MGO. Thus, usage of antioxidant agents could be an important option for the prevention of liver toxicity induced by MGO. Exendin-4 (Ex-4), a long acting Glucagon-Like Peptide-1 (GLP-1) receptor agonist, was approved as a treatment, called "exenatide", for type 2 diabetes (Buse et al., 2004)"ISSN":"0149-5992","PMID":"15504997","abstract":"OBJECTIVE This study evaluated the ability of the incretin mimetic exenatide (exendin-4 by inducing pancreatic β-cell proliferation and inhibiting glucagon (Baggio & Drucker, 2007). In addition, studies in animal models have demonstrated that Ex-4 displays antioxidant properties in both *in vitro* and *in vivo* conditions (Oeseburg et al., 2010; Z. Wang, Hou, Huang, Guo, & Zhou, 2017; Zeng et al., 2016). Therefore, the antioxidant properties Ex-4 could potentially be of value in the treatment of liver toxicity induced by MGO.

To our knowledge, the in vivo role of Ex-4 treatment on hepatic oxidative stress induced by chronic MGO administration has not been presented in the literature. The goal of the present study was to determine if Ex-4 had an antioxidant activity in the liver tissues of rats exposed to chronic MGO. In light of the aforementioned studies, in the present study, we aimed to investigate the oxidative changes that occur in liver of rats that were chronically treated with MGO along with Ex-4.

METHODOLOGY

Experimental procedures

All animal experiments were carried out with the approval of the Animal Ethics Committee of Akdeniz University Medical Faculty, Antalya, Turkey (Document no: 65-2013.09.10). Totally 24 male rats were randomly assigned into three groups at the beginning of study. For each group, 8 animals were used. First group of rats were treated with neither MGO nor Ex-4 and served as control group (C group). Second group of rats (MGO group) received MGO (75 mg/kg/day in drinking water) for 12 weeks. Third group of rats (MGO+Ex-4) received Ex-4 (1 μg/kg twice daily subcutaneously) concomitant with MGO for 12 weeks. The dosage of Ex-4 was chosen according to our previous study showing that Ex-4 at a dose of 1 μg/kg twice daily did not alter blood glucose levels (Dalakklioglu et al., 2018).

Measurement of oxidative parameters in liver tissue

At the end of the 12th week, all rats were weighed and blood samples obtained from the abdominal vein were collected into test tubes following anesthesia with a cocktail of intramuscular ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) before sacrifice of each rat. Thereafter, serum was separated by centrifugation at 4000x g for 10 min at 4°C. HbA1c levels were measured using commercial kits from Roche Diagnostics according to the manufacturer's specifications. The analyzer was calibrated using Roche calibrators, and quality control sera from the manufacturer were tested alongside the serum samples. To determine the oxidative stress condition in the liver tissues, total oxidant status (TOS), total antioxidant capacity (TAC), sulfhydryl groups (SH), myeloperoxidase (MPO), and advanced oxidation protein products (AOPP) were measured spectrophotometrically.

TOS assay was performed according to the principle that oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/l). Intra and interassay CVs were 1.4% and 1.6%, respectively (Erel, 2005).

TAC was determined using a novel automated measurement method, developed by Erel (Erel, 2004)more stable, colored 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS(*+. Briefly, the reduced 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) molecule is oxidized to $ABTS^+$ using hydrogen peroxide alone in acidic medium (the acetate buffer 30 mmol/l pH 3.6). In the acetate buffer solution, the concentrate (deep green) $ABTS^+$ molecules stay more stable for a long time. While it is diluted with a more concentrated acetate buffer solution at high pH values (the acetate buffer 0.4 mol/l pH 5.8), the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically, and the bleaching rate is inversely related with the TAC of the sample. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays, and the assay results are expressed in mmol Trolox equivalent/l. Intra and interassay CV were 2.6% and 2.9%, respectively.

SH levels were measured spectrophotometrically, using Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with the thiol-disulfide interchange reaction between DTNB and thiol providing the basis of the spectrophotometric assay (Koster, Biemond, & Swaak, 1986).

The liver samples taken were washed in saline in an ice bath and homogenized in the ratio 1:10 (w:v) with ice-cold 150 mM KCl for MPO and protein determination. The rest of the homogenates were stored at -70°C until tissue MPO and protein levels of homogenates assays were performed. The samples were centrifuged at 12,000 g at 4 °C for 20 min. Liver extract MPO levels were estimated by a spectrophotometric method using O–Dianisidine Dihydrochloride as a substrate. MPO was assayed as follows: 0.1 ml of the liver extract supernatant was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, and containing 0.167 mg/ml O-dianisidine dihydrochloride and 1% hydrogen peroxide (Bradley, Priebat, Christensen, & Rothstein, 1982)a plentiful constituent of neutrophils, might serve as a marker for tissue neutrophil content. To completely extract MPO from either neutrophils or skin, hexadecyltrimethylammonium bromide (HTAB. The change in absorbance was measured at 450 nm using a spectrophotometer. One unit of MPO activity was defined as that degrading one μmole of peroxidase per minute at 25°C (*Worthington Enzyme Manual*, 1972). The total protein content of the homogenates was determined by the method of Lowry (LOWRY, ROSEBROUGH, FARR, & RANDALL, 1951).

AOPP levels were measured for only the supernatant fraction, using a spectrophotometric method (Witko-Sarsat et al., 1996). The values are expressed as μmol/g of protein in liver tissue.

Materials

MGO and Ex-4 were purchased from Sigma Chemical (St. Louis, MO, USA). All drugs were prepared fresh daily during experiments and were dissolved in distilled water before use.

Statistical analysis

All values were expressed as mean ± SEM. Statistical analysis of the results were performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value lower than 0.05 was considered significant.

RESULTS AND DISCUSSION

Rats treated with MGO showed significant increases in TOS and AOPP compared to control group rats (Figs.1 and 2). The treatment with Ex-4 for 12 weeks resulted in significant decreases in the levels of TOS and AOPP (Figs.1 and 2).

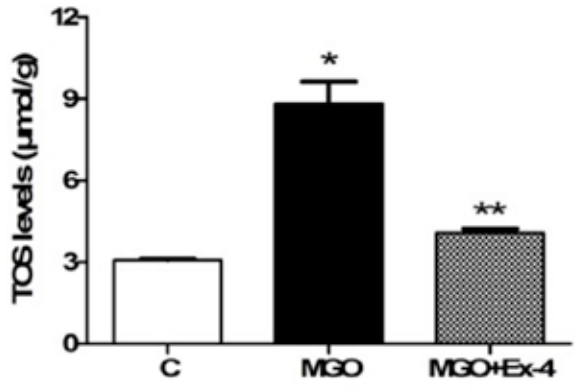

Figure 1: Levels of total oxidant status (TOS) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

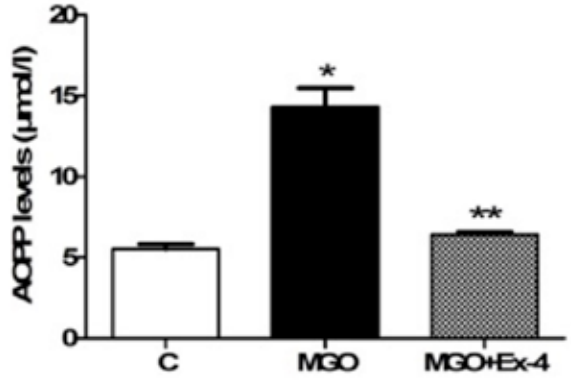

Figure 2: Levels of advanced oxidation protein products (AOPP) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

MGO-treatment also caused a significant reduction in hepatic TAC levels (Fig.3). Decreased hepatic TAC levels in MGO-administered rats were significantly increased with chronic Ex-4 treatment (Fig.3).

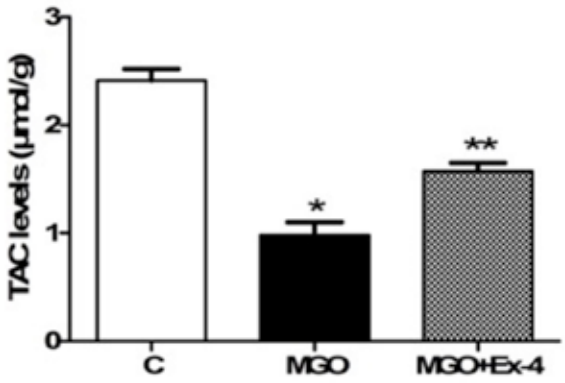

Figure 3: Levels of total antioxidant capacity (TAC) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

Fig.4 shows the levels of sulfhydryl groups (SH) in liver tissues obtained from all groups. After the chronic MGO administration, SH levels were decreased significantly (Fig.4). The treatment with Ex-4 for 12 weeks resulted in a significant increase in SH levels (Fig.4).

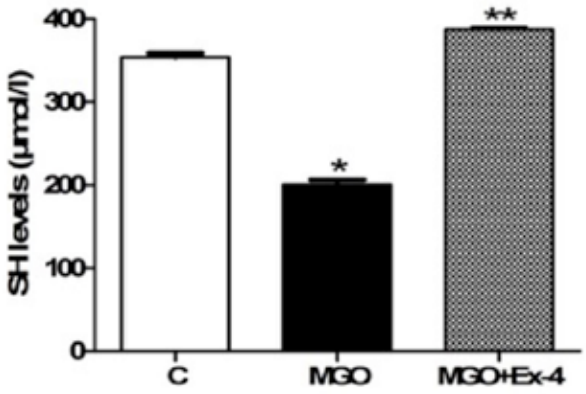

Figure 4: Levels of sulfhydryl groups (SH) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

As seen in Fig.5, MPO levels in liver tissues were higher in MGO-administered rats compared to control rats (Fig.5). Ex-4 treatment also caused a significant reduction in MPO levels in MGO-administered rats (Fig.5).

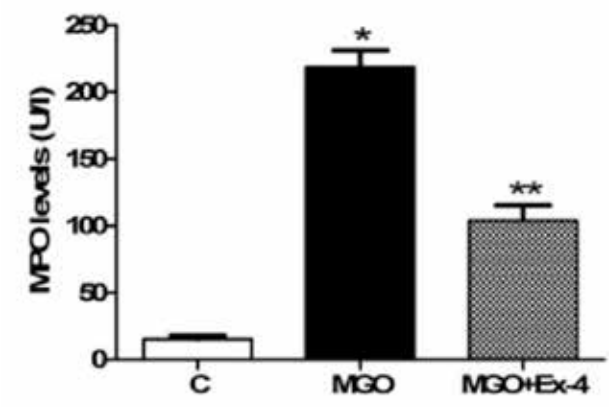

Figure 5: Levels of myeloperoxidase (MPO) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

In addition, percentage of HbA1c were not significantly different between percentage of HbA1c (Fig.6).

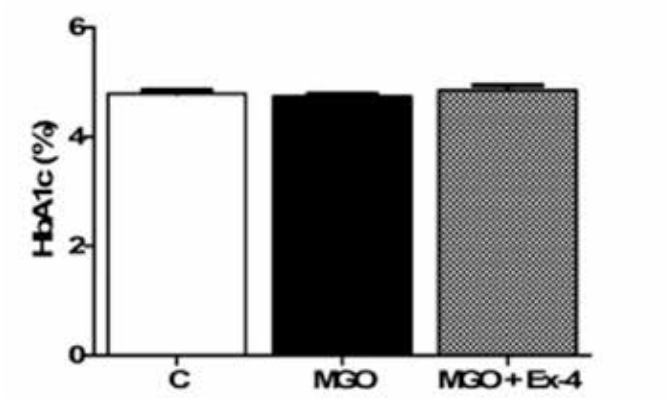

Figure 6: Comparison of HbA1c levels in all group rats. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM.

The present study is the first to describe a protective effect of chronic Ex-4 treatment on MGO-induced oxidative stress in liver tissue of rats, suggesting a novel role for Ex-4 in protecting against MGO-related liver damage. The results of the present study indicate that chronic Ex-4 treatment ameliorates MGO-induced hepatic oxidative stress as confirmed by biochemical assays.

The liver is among the primary organs susceptible to oxidative stress because it is involved in metabolism and detoxifying processes in the body, which may lead to liver tissue injury (Palsamy, Sivakumar, & Subramanian, 2010). However, the liver is also equipped with cellular antioxidant defense such as SH and MPO as not only to neutralize free radicals but also to protect the liver cells from oxidative damage. TOS and TAC are usually measured to determine the toxicity level in damaged tissues (Oguz et al., 2015). The results of Seo et al. showed that MGO increases cell death and induces liver toxicity, which results from ROS-mediated mitochondrial dysfunction and oxidative stress (Seo et al., 2014). The decrease in total antioxidant defense system and the increase in oxidative parameters in liver tissue were also reported in MGO-administered rats (Choudhary et al., 1997). In agreement, our results showed that levels of TOS in the liver was increased in MGO group of rats. Otherwise, protein oxidation is also often studied alongside oxidative stress status (Kalousová, Skrha, & Zima, 2002). Oxidant-mediated protein damage can be determined by the level

of AOPP. In the present study, significantly increased AOPP levels in the liver tissues were found after MGO administration. Moreover, one of the principal molecules released after recruitment and activation of phagocytes is MPO, an important enzyme involved in the generation ROS (Klebanoff, 2005)the phagocytosis and destruction of microorganisms. When coated with opsonins (generally complement and/or antibody. The measurement of MPO may serve as a reliable marker to estimate the degree of oxidative stress (C.-C. Wu et al., 2005)little is known of how different dialysis membranes contribute to the oxidative stress induced by the dialysis procedure per se. We therefore studied the influence of two different dialysis membranes on oxidative stress during HD. METHODS Eight patients undergoing HD three times per week were enrolled in this cross-controlled study. Patients sequentially received HD using polysulphone (PS. Importantly, liver MPO levels also significantly increased in MGO-administered rats as compared with controls. All these findings clearly indicated to an increased oxidative stress in the liver tissues of rats exposed to chronic MGO.

MGO administration can also reduce the hepatic antioxidant defense leading to accumulation of free radicals in hepatocytes. MGO may inhibit several antioxidant enzymes and thereby, increased oxidative stress may be due to reduction in the activities of antioxidant enzymes. One of them, thiol groups are important members of the antioxidant team and have been shown to destroy ROS and other free radicals by enzymatic and non-enzymatic mechanisms (Jones et al., 2000)the redox potential of the GSSG/2GSH pool (-137 +/- 9 mV. Total thiol groups of proteins are mainly responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress (Halliwell & Gutteridge, 1990; Soszyński & Bartosz, 1997). MGO exposure was reported to decrease protein-SH and reduce GSH levels in different cell types and in the liver (Leoncini, Maresca, & Buzzi, 1989; Ray & Ray, 1984)methylglyoxal was found to be the best substrate. The pH optimum of the enzyme was found to be 6.5, and Km for methylglyoxal was 0.4 mM. The molecular weight of the enzyme was found to be 89000 by gel filtration on a Sephadex G-200 column. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel revealed that the enzyme is composed of two subunits. The enzyme is highly sensitive to sulfhydryl group reagents. The inactivation by p-chloromercuribenzoate could be substantially protected by methylglyoxal in combination with NADH, indicating a possible involvement of one or more sulfhydryl group(s. In the present study, we observed decreased levels of SH groups in liver tissues of MGO-administered rats which further supported the involvement of SH in MGO-induced liver damage. Our results also showed that levels of TAC in liver tissues were significantly de-

creased in MGO group rats compared to controls. Hence, it might be suggested that in addition to increased TOS levels due to excessive release of free radicals, reduction in TAC and SH groups in hepatic tissue may also contribute to the MGO-induced hepatotoxicity in rats.

In a previous study, we have demonstrated that Ex-4 attenuated MGO-induced erectile dysfunction through inhibition of oxidative stress (Dalaklioglu et al., 2018). In the present study, we have showed that MGO-induced oxidative stress in liver was also significantly improved by Ex-4 treatment for 12 weeks. The increased oxidative stress in livers from MGO-treated rats as indicated by TOS, AOPP and MPO levels was significantly reversed by Ex-4 treatment. Antioxidant therapy is a potential future therapeutic strategy; increasing antioxidant levels in patients with diabetes mellitus-induced liver damage may hopefully counter the effects of oxidative stress, thereby reducing the severity of diabetic complications. Besides its ability to scavenge free radicals, Ex-4 may also have indirect antioxidant actions. Ex-4 has been shown to enhance several antioxidant enzymes (Ahangarpour, Oroojan, & Badavi, 2018). In accordance with the previous studies, the results of the current study also demonstrated that treatment of rats with Ex-4 for 12 weeks slightly increased the activities of SH and TAC in the liver tissue. Therefore, the finding of increased activities of antioxidant enzymes in the liver tissues of rats treated with Ex-4 alone suggest that Ex-4 not only exhibits a direct scavenging effect on free radicals but also partly stimulates intracellular antioxidant defense mechanisms.

Importantly, it is also to be mentioned that preventive effect of Ex-4 occur by a mechanism independent from the glucose-lowering effects of this drug. Importantly, in agreement with previous results (Cardoso et al., 2014; Dalaklioglu et al., 2018), when MGO (75 mg/kg b.w./day; in drinking water) was given to animals for a period of 12 weeks, no significant change in serum HbA1c levels was found as compared to controls. Moreover, in the present study, we demonstrated that levels of HbA1c did not change with chronic Ex-4 treatment at a dose of 1 μg/kg in MGO-administered rats. Thus, the protective effect of Ex-4 against MGO-induced oxidative stress in liver does not seem to be associated with well-known glucose lowering effect of this drug.

In conclusion, the results of present study provide first evidence for the combined effect of decreased oxidative stress and increased antioxidant defense mechanisms contributing to therapeutic effect of Ex-4 against MGO-induced oxidative stress in liver tissue. Based on the results of the present study, it is possible to suggest that Ex-4 treatment may offer a novel therapeutic approach for the prevention of hepatotoxicity induced by MGO, especially in diabetic adults.

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. Levels of total oxidant status (TOS) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

Figure 2. Levels of advanced oxidation protein products (AOPP) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

Figure 3. Levels of total antioxidant capacity (TAC) in liver tissues obtained from all groups. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM. *P<0.05 as compared with control rats; **P<0.05 as compared with MGO group rats.

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Figure 6. Comparison of HbA1c levels in all group rats. C: Control, MGO: Methylglyoxal, Ex-4: Exendin-4. All values are expressed as mean ± SEM.

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