ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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

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Aims and Scope of Acta Pharmaceutica Sciencia

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

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

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

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK

Editor

INSTRUCTIONS FOR AUTHORS

Manuscripts must be prepared using the manuscript **template**

Manuscripts should contain the following elements in the following order :

Title Page

Abstract

Keywords

Introduction (Without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

It is best to use the Times New Roman' font, 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 font "Times". Other fonts, particularly those that do not come bundled with the system software, may not translate properly. Ensure that all special characters (e.g., Greek characters, math symbols) are present in the body of the text as characters and not as graphic representations. Be sure that all characters are correctly represented throughout the manuscript—e.g., 1 (one) and l (letter l), 0 (zero) and O (letter o).

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

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

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

Authors may find the following sources useful for recommended nomenclature:

· The ACS Style Guide; Coghill, A. M., Garson, L. R., Eds.; American Chemical Society: Washington DC, 2006.

· Enzyme Nomenclature; Webb, E. C., Ed.; Academic Press: Orlando, 1992.

· IUPHAR database of receptors and ion channels (http://www.guidetopharmacology.org/).

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

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

· Once in the abstract.

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

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

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

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

2.2 Manuscript Organization

2.2.1 Title Page. Title: The title of the manuscript should reflect the purposes and findings of the work in order to provide maximum information in a computerized title search. Minimal use of nonfunctional words is encouraged. Only commonly employed abbreviations (e.g., DNA, RNA, ATP) are acceptable. Code numbers for compounds may be used in a manuscript title when placed in parentheses after the chemical or descriptive name.

Authors' Names and Affiliations: The authors' full first names, middle initials, last names, and affiliations with addresses at time of work completion should be listed below the title. The name of the corresponding author should be marked with an asterisk (*).

2.2.2 Abstract and keywords. Articles of all types must have an abstract following the title page. The maximum length of the Abstract should be 200 words, organized in a findings-oriented format in which the most important results and conclusions are summarized. Code numbers may be used once in the abstract. After the abstract, a section of Keywords not more than five has to be given. Be aware that the keywords, chosen according to the general concept, are very significant during searching and indexing of the manuscripts.

Key words: instructions for authors, template, journal

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

2.2.4. Methodology. Materials, synthetic, biological, demographic, statistical or experimental methods of the research should be given detailed in this section. The authors are free to subdivide this section in the logical flow of the study. For the experimental sections, authors should be as concise as possible in 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.

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

2.3 Specialized Data

2.3.1 Biological Data. Quantitative biological data are required for all tested compounds. Biological test methods must be referenced or described in sufficient

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds.

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

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

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

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

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

3. Submitting the Manuscript

3.1 Communication and log in to Author's Module All submissions to Acta Pharmaceutica Sciencia should be made by using e-Collittera (Online Article Acceptance and Evaluation) system on the journal main page (www. actapharmsci. com)

3.2 Registration to System It is required to register into the e-Collittera system for the first time while entering by clicking "Create Account" button on the registration screen and the fill the opening form with real information. Some of the information required in form is absolutely necessary and the registration will not work if these fields are not completely filled.

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

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

3.3 Submitting A New Article The main page of author module consists of various parts showing the situation of manuscripts in process. By clicking the New Manuscript button, authors create the beginning of new submission, a process with a total of 9 consecutive levels. In first 7 levels, information such as the article's kind, institutions, authors, title, summary, keywords etc. are asked respectively as entered. Authors can move back and forth while the information is saved automatically. If the is transaction is discontinued, the system move the new submission to "Partially Submitted Manuscripts" part and the transaction can be continued from here.

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

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

3.2.2 Institutions Authors should give their institutional information during submission.

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

3.2.4 Title should be English, explaining the significance of the study. If the title includes some special characters such as alpha, beta, pi or gamma, they can easily be added by using the **Title** window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text.

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

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

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

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

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

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

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

The explanation of the files (E.g.. Figure 1, Full Text Word File, supplements etc.) should be added on third step and the last step is submitting the prepared article into the system. Therefore, **Download** button under the **Send your file by clicking on download button** tab is clicked.

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

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

3.3.2 Your Files After adding the article you may find all information related to article under **Your Files** window.

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

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

File Download This window submits two alternatives, one of them is to ensure the file to be opened in valid site and the second one is to ensure to download submitted file into the computer.

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

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

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

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

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

3.3.4.1 Articles Not Completed to be Sent After the sending transaction was started, if article is not able to continue until the ninth step or could not be sent due to technical problems shown at this part. Here you can find the information such as the article's number which is assigned by system, title and formation date. You may delete the articles by using **Delete** button on the right column, if the article is not considered to send into the system.

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

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

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

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

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

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ORIGINAL ARTICLES

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Significance of Crude and Degummed *Citrullus Lanatus* Seed oil on Inflammatory Cytokines in Experimental Infection Induced by *Candida Albicans*

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ABSTRACT

This study evaluated immunomodulatory and candidacidal properties of *Citrullus lanatus* seed oil (CLSO) on *Candida albicans*-infected rat. The immunomodulatory and candidacidal activities of both crude and degummed oil at varied doses were determined using 80 female albino rats, randomised into 10 groups of 8 rats per group based on the days 7 and 14 of study. Delayed hypersensitivity reaction showed a significant increase ($p<0.05$) in the hind paw oedema of rats treated with crude and degummed oil after 24 hours; however after 48 hour, a significant decrease ($p<0.05$) in hind paw oedema was observed. However, the CD4 + T-lymphocytes decreased at day 14 when compared with day 7 counterpart. In the same vein, IFN-γ and TNF-α decreased from day 7 to day 14 of the study; while IL-10 and IgA increased from day 7 to day 14 of the study. CLSO does not boost immune system, rather demonstrated anti-inflammatory activities of the oil.

Keywords: Candidiasis; CD4 count; cytokines; degummed oil; immunosuppression.

INTRODUCTION

Interest in the effects of fatty acids upon the immunity and inflammation dates back many years, but this interest intensified with the elucidation of the roles of eicosanoids derived from arachidonic acid (ARA; 20:4n-6) in modulating immunity and inflammation [1], and with the knowledge that the metabolism of ARA to yield these mediators can be inhibited by the long polyunsaturated fatty acids (PUFA).[1] Free fatty acids may modify the immune response by modulating immunological parameters such as phagocytosis or cytokine production.[2] Dietary

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lipids or free fatty acids not only affect the immune systems directly, but also interfere in the production of other substances through modification of activity of enzymes involved in the synthesis of these substances.[3] Disorders of the immune system can result in autoimmune diseases, inflammatory diseases and cancer [4], and immunodeficiency. Immunodeficiency occurs when the immune system is less active than normal, resulting in recurring and life-threatening infections.

The currently available drugs that are used for the treatment of inflammatory and immunodeficiency diseases are often ineffective and require high doses or prolonged periods of treatment, thus causing adverse effects on the gastrointestinal tract, kidneys, and liver.[5] Thus, the use of natural products or traditional medicines that have favorable therapeutic effects with fewer adverse effects has gained interest in the treatment of immunomodulatory and inflammatory diseases.[6] Some of the medicinal plants are believed to enhance the natural resistance of the body to infections. [7]

Watermelon (*Citrullus lanatus*) is taxonomically classified as a member of the Cucurbitaceae family, which is also known as the gourd family. Other gourds include pumpkins, cucumbers, squash, and other melons. It has been investigated for its potential as significant diuretic agents Gul *et al.*[8] In addition, the antioxidant activity, total phenolics and flavonoid contents of fermented and unfermented water melon rind (outer layer) have been investigated.[9] A study has concluded that *Citrullus lanatus* seed extracts possess antioxidant activity and the potency of antioxidant activity depends on the type of extract.[10] The n-hexane extract of *Citrullus lanatus* seed oil has shown a promising role as an anti-inflammatory agent.[11] There is no documented evidence of immunomodulatory effect of degummed and crude *Citrullus lanatus* seed oil. As a consequence, experimental oropharyngeal candidiasis infection was induced in rats and treated with both degummed and crude *Citrullus lanatus* seed oil in order to determine the ability of the oil to modulate of the immune response.

METHODOLOGY

Materials

The plant material used in this study was the fresh *Citrullus lanatus* seeds. The seed of *Citrullus lanatus* (water melon) was procured from the dealers within Zuba in Bwari area council of FCT Abuja, the identity of the plant was confirmed and Voucher specimens UNN/PSB/Consult/2017/2721-03 were deposited at the herbarium at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Fungal strain

Candida albicans SC5314 used in this study was obtained from Department of Microbiology University of Nigeria, Nsukka.

Animals and experimental design

A total of 80 female albino rats weighing 120-150g were obtained from the Animal House of the Faculty of Biological Sciences, University of Nigeria, Nsukka. They were acclimatized for one week under standard environmental conditions and maintained on a regular feed (Grand Cereals Ltd, Enugu, Nigeria) and water *ad libitum*. The animals were handled according to the guidelines of the Ethical Committee on the use and care of experimental animals of the Department of Biochemistry, University of Nigeria, Nsukka (UNN) and approved by the Departmental Animal Ethics Committee (DAEC), UNN (Approval No. UNN/DAEC/2017/B79). They were grouped in ten (10) cages of eight (8) rats per cage and orally fed with crude and degummed *Citrullus lanatus* seed oil (CLSO) for a total of 14 days. Controls were treated with 2% Tween 80 while fluconazole and levamisol as standard drugs. The rats were sacrificed on the 14th day, blood samples were collected through ocular puncture for biochemical and haematological analyses.

Methods

Collection and processing of *Citrullus lanatus*.

The fruits were washed, cut and the seeds selected manually from the pulp. Tap water was used to wash off pulp on the seeds before sun-drying for 72 hours. Samples of watermelon seeds were crushed using a commercial blender (TSK-949, WestPoint, France), put in air tight container and stored in a desiccators for analyses.

Oil extraction and determination of percentage yield

The pulverized samples weighing 2480 g were extracted for 6 h using n-haxane and soxhlet extractor equipped with thimble (chem glass). The extract was then concentrated using rotary evaporator (EYELA, N-N Series; Rikakikai Co Ltd., Tokyo, Japan) to obtain n-hexane-free crude *Citrullus lanatus* seed oil. After concentration, crude *Citrullus lanatus* seed oil was immediately placed in a hot air oven maintained at 60°C for 24 h to ensure complete removal of solvents. The concentrated oil (642 ml) sample was then stored in a bottle in a cool dry place until degumming.

$$\textit{Percentage yield} = \frac{\text{Mass of the oil extracted}}{\text{Mass of the seed before extraction}} \times 100$$

Degumming of *Citrullus lanatus* Seed Oil

The *Citrullus lanatus* seed oil contains phosphatides, gums and other complex compounds which can promote hydrolysis (increase in free fatty acid) of oil during storage. The crude oil sample was heated to 80°C, mixed with water (5% vol) and stirred for 15 minutes by magnetic stirrer. Then the mixture was centrifuged (7000 rpm) for 20 minutes.[12] Precautions were taken in order to prevent the introduction of air and subsequent oxidation of oil. Due to this, phosphatides and other impurities were settled down and were centrifuged out from the degummed oil.

Preparation of Sheep Red Blood Cell (SRBC) as an Antigen

Fresh blood was collected from sheep sacrificed in local slaughter house. Sheep red blood cells (SRBC) were washed three times in normal saline and RBC of this suspension was adjusted to a concentration of 5 x 10^9 cells per ml for immunization and challenge.[13]

SRBC-induced Delayed Type Hypersensitivity Reaction (DTH response)

The animals were grouped in eight (8) cages of four (4) rats per cage. DTH response was induced in rats using SRBC as an antigen according to Doherty method.[14]

Humoural Antibody Response (HA response)

A total of forty four (44) rats were divided into eleven (11) groups of four (4) rats per cage was used in this study. Groups 1 and 2 acted as control groups treated with 100 mg/kg b.w. of crude and degummed oil without being challenged with 0.05 ml of 5 x 10^9 SRBC, Group- 3 was standard control treated with 50mg/kg b.w. of levamisole after being challenged with SRBC, group 4 was treated with 5% DMSO after SRBC challenge, group 5 received 5% DMSO without SRBC challenge, groups 6 and 7 received 50mg/kg b.w of degummed and crude oil respectively, groups 8 and 9 received 100mg/kg b.w. of degummed and crude oil respectively while groups 10 and 11 received 150mg/kg b.w. respectively. Crude and degummed oil was administered at different doses (50, 100 and 150 mg/kg b.w.) and the rats were immunized with 10% suspension of SRBC on day 0 intraperitonially (i.p.) and treatment continued till the 7^{th} day of the experiment, they were challenged, blood samples were collected from the retro orbital plexus of individual animals for primary antibody response and on day 14 for secondary antibody response and their antibody titre from serum was determined according to Puri *et al.* [15] Serum was diluted in 50µl of phosphate buffer

saline (PBS) of pH 7.2 with two fold serial dilution in 96-well microtire plate and mixed with 50µl 1% SRBC suspension in PBS. Plates were kept at room temperature for 2 hour. The value of antibody titre was considered at the highest serum dilution showing visible haemaglutination and expressed as HA titre.

Preparation of *Candida albicans* for animal study.

Stock cultures of *C. albicans* SC5413 cells were kept at 4°C and passaged once a month to maintain viability. Before each experiment, *C. albicans* SC5413 cells were grown in Sabouraud's dextrose agar at 37°C for 48 h. Cells were harvested by centrifugation at 3500 × *g* for 10 min. Cells were washed three times with 50 ml of sterile non pyrogenic phosphate buffered-saline (PBS), counted with a haemacytometer, and resuspended in PBS to the required concentrations ($1x10^7$CFU/ml).

Induction of oropharyngeal infection with *Candida albicans* in rat

After seven (7) consecutive days of immunosupression with 50mg/kg body weight of pyrogallol. Disseminated candidiasis infection was induced by oral (*p.o.*) administration of 0.2 mL of $1x10^7$ CFU/ml. Also 1 g/ml of antibiotic (tetracycline) was administered *po* for three days (to eliminate competitive normal flora) during inoculation of *Candida albicans* SC5413 cells. Twenty-four (24), forty-eight (48) and seventy-two (72) hours after infection, nine animals were euthanised to check the effectiveness of the infection by assessing the fungal load in the kidneys. After which 48 h was chosen for the study.

Full blood count assays

Full blood counts including packed cell volume (PCV), Haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), differential WBC (lymphocytes and mixed) were estimated using the Sysmsex® Automated Haematology Analyzer (KX-21N, Sysmex Corporation, Kobe-Japan). The whole sample method was used where blood was mixed manually, and then fed into the transducers. The transducer chamber has a minute hole called the aperture. On both sides of the aperture, there are electrodes between which flows direct current. Blood cells pass through the aperture, causing direct current resistance to change between the electrodes. As direct current changes, the blood cell size is detected as electric pulses. Blood cell count is then calculated by counting the pulses, and a histogram of blood cell sizes is plotted by determining the pulse sizes. Also, analyzing a histogram makes it possible to obtain various analysis data including differential whole blood count, red cell indices and derived values.

CD4+ T-lymphocyte count

The CD4+ lymphocytes were determined using the CyFlow® Automated Cell Counter (Partec, Germany). The Counter uses a 'no lyse, no wash' procedure for CD4 counting.[16] EDTA-anticoagulated blood (fifty microliters) were added to 10 µl of monoclonal antibodies. After 15 min of incubation, 1ml of no lyse dilution buffer was added and the sample tube was attached to the Counter for automated counting. Results were available in 2 minutes and were expressed in a histogram (CD4+ cells/µl).

Cytokine assays

Enzyme-linked immunosorbent assay. Quantification of each cytokine in the serum was determined using commercially available ELISA kits (Vector Best, Russia). One hundred microlitre (100 µl) of Solution for Sample Dilution (SSD) (Vector Best, Russia) was added to each well, then 100 µl of calibration, sample and control per well. It was sealed with adhesive film and incubated for two (2) hours at 37°C. The wells were aspirated and washed (5 times) with PBS-T×25 and blotted with a clean cotton gauze. Conjugate No.1 (100 µl) (Vector Best, Russia) was added to each well, covered with new adhesive and incubated for an hour at 37°C. After incubation, the wells were washed (5 times) as described before, then Conjugate No.2 (100 µl) was added to each well and covered with new adhesive strip and incubated for 30 minutes and washed as stated above. Tetramethylbenzidine (TMB) solution (100 µl) (Vector Best, Russia) was added to each well and incubated at 25°C for 25 minutes and protected from light. Stop solution was added to each well. Optical density of IL-10 was determined within 10 minutes using microplate reader set at 450nm. Same was carried out for IgA, TNF-α, and IFN-γ. All cytokines and IgA concentrations (in picograms per milliliter) were determined by comparison with the standard curve.

Data analysis

The data obtained from this study were expressed as means ± standard deviation (SD). Statistical comparisms between the groups were done using one way analysis of variance (ANOVA). Whereas the means were separated using Duncan's Multiple Range Test of $p < 0.05$.

RESULTS

Percentage Yield: The percentage yield of the oil was 26.40%.

Delayed-type hypersensitivity (DTH) test

The result of DTH in Figure 1 shows that there was significant increase ($p < 0.05$) in the hind paw oedema of rats treated with crude and degummed oil (CO and DO respectively) after 24 hours when compared with the control. There was no significant increase ($p>0.05$) in hind paw oedema of rats treated with both 25 (5.17 ± 0.29 mm) and 50 mg/kg b.w. (5.23 ± 0.25 mm) of degummed oil when compared with the standard control (4.87 ± 0.21 mm). But there was a significant ($p < 0.05$) increase in hind paw oedema of rats treated with 100 mg/kg b.w. (6.10 ± 0.66) of degummed oil when compared to the standard control (4.87 ± 0.21 mm). For the CO, paw oedema of all the treated groups were found to be significantly ($p < 0.05$) higher when compared with both normal and standard control. After 48 hours, the paw oedema of the normal control (6.10 ± 0.46 mm) and standard control (4.50 ± 0.40 mm) increased significantly ($p<0.05$) compared to their 24 hours values. A significant ($p < 0.05$) decrease in hind paw oedema of rats treated with 100 mg/kg b.w. (3.90 ± 0.53mm) after 48 hours was observed when compared with 6.10 ± 0.66 mm obtained after 24 hours. There was also a significant ($p < 0.05$) decrease in hind paw oedema of groups 2 (4.867 ± 0.57 mm), 3 (3.900 ± 0.53 mm), 4 (5.200 ± 0.27 mm) and 6 (3.133 ± 0.321 mm) after 48 hours when compared with the normal control (6.100 ± 0.458 mm).

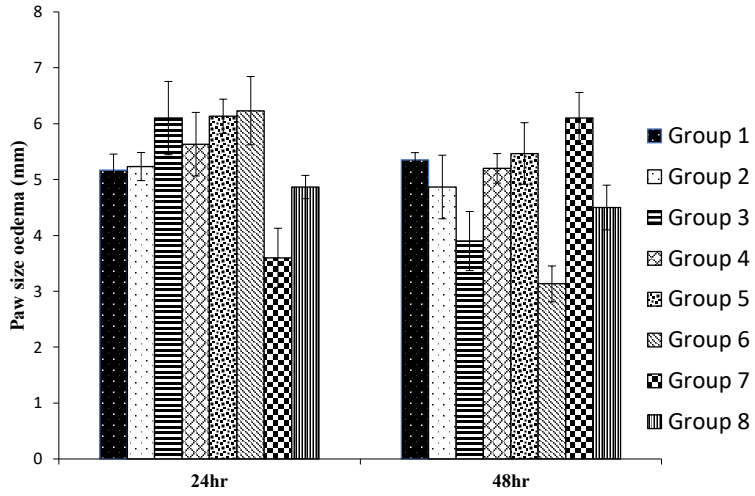

Figure 1: Paw size oedema of different group challenged with SRBC

Results expressed as means ± SD

Key:

Group 1 (25 mg/kg b.w.) {Degummed}
Group2 (50mg/kg b.w.) {Degummed}
Group 3 (100mg/kg b.w.) {Degummed)
Group 4 (25 mg/kg b.w.) {Crude}
Group 5 (50 mg/kg b.w.) {Crude)
Group 6 (100 mg/kg b.w.) {Crude}
Group 7 (normal control) (DMSO)
Group 8 (Standard Control) {25mg/kg b.w. Levamisole}

Humoural antibody titre using SRBC as an antigen

Figure 2 shows that there was non-significant ($p > 0.05$) decrease in secondary antibody titre values when compared with the primary antibody titre values. In the primary antibody titre, there was a significant increase ($p<0.05$) in group 1 when compared with that of group 2. However, there was no significant increase ($p>0.05$) in group 2 when compared with group 3. Similarly, a significant increase ($p<0.05$) in group 4 when compared with group. In the same vein, group 7 exhibited a significant increase ($p<0.05$) when compared with group 6. But there was no significant increase ($p>0.05$) in group 9 when compared with group 8. There was no significant increase ($p>0.05$) in group 10 when compared with group 11.

In the secondary immune response, there was a decrease in the titre when compared with the primary antibody response. There was a significant increase ($p<0.05$) in group 1 when compared with group 2 but the titres are quite lower than the primary titres. There was also a significant increase ($p<0.05$) in group 3 when compared with group 4. Also group had a significant increase ($p<0.05$) when compared with group 5 but there was no significant increase ($p>0.05$) in group 2 when compared with group 5. In the same vein, there was no significant difference ($p>0.05$) between groups 6 and 7, just as there was no significant increase ($p>0.05$) in group 10 when compared with group 11; however group 9 shows a significant increase ($p<0.05$) in humoural antibody titre when compared with group 8.

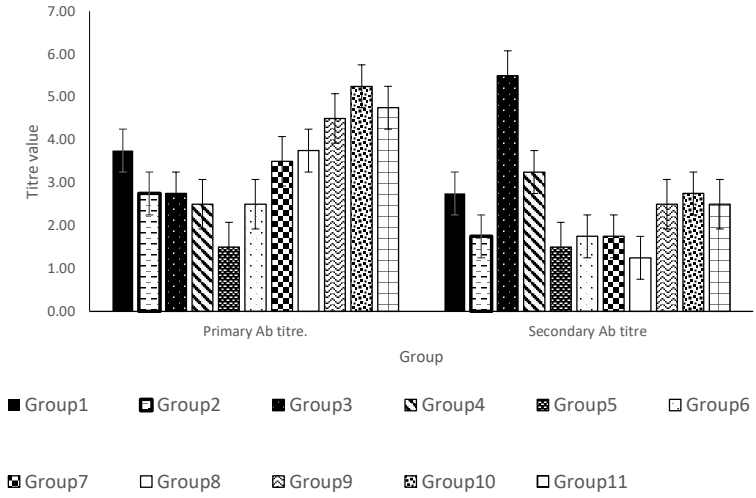

Figure 2: Humoural antibody titre of rats challenge and unchallenged with SRBC Means with different superscript within rows are significantly different (P<0.05)

Results expressed as means ± SD

Key:

Group 1 (100mg/kg b.w. of crude oil)
Group 2 (100mg/kg b.w. of degummed oil)
Group 3 (challenged and treated with 50mg/kg b.w. of levamisole)
Group 4 (challenged and treated with DMSO)
Group 5 (unchallenged and treated with DMSO)
Group 6 (50mg/kg b.w. of degummed oil)
Group 7 (50mg/kg b.w. of crude oil)
Group 8 (100mg/kg b.w. of degummed oil)
Group 9 (100mg/kg b.w. of crude oil)
Group 10 (150mg/kg b.w. of degummed oil)
Group 11 (150mg/kg b.w. of crude oil)

Effect of *Citrullus lanatus* seed oil on complete blood and CD_4 count at 7th and 14th days of treatment

The result shows no significant increase ($p > 0.05$) in PCV, Hb, WBC, lymphocyte, neutrophil, eosinophil, monocyte, platelet and RBC among the groups treated with the oil except groups 4 and 9 which showed a significant increase ($p < 0.05$) in PCV values while Hb showed a significance increase ($p<0.05$) in groups 4, 5 and 9 when compared with group 10. WBC had significant increase ($p<0.05$) in groups 3 and 6 when compared with group 10. Also neutrophil significantly increased ($p < 0.05$) in group 5 when compared with group 10. Red

blood cell (RBC) showed a significant increase ($p < 0.05$) in groups 5 and 6 when compared with group 10. The CD4 significantly increased in groups 4, 7 and 9 when compared with group 10.

After 14 days of treatment, there was significant increase ($p < 0.05$) in PCV and Hb conc. of groups 4, 5, 6, 7, 8 and 9, while group 6 had a significant increase ($p < 0.05$) in WBC, groups 5 and 7 also had a significant increase ($p < 0.05$) in platelet values when compared with group 10. However, lymphocyte, neutrophil, monocyte and eosinophils had a no significant increases ($p > 0.05$) in their values when compared with group 10. But the CD4 count values decreased ($p > 0.05$) below group 10 (normal control).

Table 1A: Effect of Citrullus lanatus Seed Oil on Complete Blood Count at 7th day of Treatment

Groups	PCV (%)	Hb (g/dl)	WBC(x 10^9/L)	Lymph (%)	Neut (%)	Platelet(x 10^9)	RBC (10^{12})	Mono (%)
1	40.33±4.51^{abc}	12.17±1.00^{abc}	8.93±1.25^{b}	66.67±2.08^{abc}	27.00±3.46^{bc}	437.00±14.00	6.90±1.00^{abc}	4.33±0.58^{ab}
2	36.33±3.06^{a}	11.10±0.50^{a}	14.00±1.35^{de}	74.33±2.08^{bcd}	19.67±1.53^{ab}	595.33±19.50	5.77±0.35^{a}	4.00±1.00^{ab}
3	35.67±5.51^{a}	11.07±1.01^{a}	13.87±1.07^{de}	72.67±3.06^{bcd}	21.00±1.73^{ab}	570.67±83.12	6.83±0.50^{abc}	3.67±1.53^{ab}
4	46.33±2.52^{c}	13.30±0.70^{c}	10.12±1.38^{bc}	67.33±2.52^{abc}	27.33±3.06^{bc}	605.33±230.66	7.27±0.58^{bc}	3.67±1.53^{ab}
5	41.67±2.08^{abc}	12.77±0.59^{bc}	12.40±0.80^{cde}	57.33±18.9^{a}	35.00±15.13^{c}	568.67±115.45	7.80±0.70^{c}	6.33±1.53^{b}
6	42.00±4.58^{abc}	12.23±0.72^{abc}	14.57±1.40^{e}	71.33±3.21^{bcd}	23.00±3.00^{ab}	597.33±57.14	7.17±0.42^{ab}	4.00±2.00^{ab}
7	40.67±4.04^{abc}	12.43±0.91^{abc}	12.63±0.67^{cde}	64.33±8.02^{ab}	28.00±8.54^{bc}	531.33±131.44	7.27±0.70^{bc}	5.67±1.15^{b}
8	39.33±2.08^{ab}	11.80±0.44^{ab}	10.87±1.94^{bcd}	71.00±1.00^{bcd}	23.00±2.65^{ab}	597.33±117.75	6.67±0.61^{abc}	5.33±2.52^{ab}
9	44.33±1.53^{bc}	13.03±0.40^{bc}	13.53±3.16^{de}	79.67±3.79^{cd}	17.33±4.04^{ab}	525.33±129.68	7.50±0.62^{c}	2.67±0.58^{a}
10	38.67±2.52^{ab}	11.00±0.85^{a}	5.77±2.29^{a}	82.67±5.86^{cd}	12.67±5.51^{a}	462.67±31.64	6.20±0.26^{ab}	3.67±0.58^{ab}
P-value	0.031	0.006	0.000	0.014	0.019	0.591	0.020	0.133

Means with different superscript within rows are significantly different ($P<0.05$)

Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppresses without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisol
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Normal control

Table 1B: *Effect of Citrullus lanatus seed oil on complete blood count at 14th day of treatment*

Groups	PCV(%)	Hb (g/dl)	WBC (x 109/L	Lymph (%)	Neut (%)	Mono (%)	Platelet (x 10^9)	RBC (10^{12})
1	46.00±3.00^{c}	13.17±0.31^{c}	9.60±0.20^{abc}	78.33±7.64	22.33±4.16	2.67±0.58	666.33±140.5^{cd}	7.70±0.10^{e}
2	30.00±4.00^{a}	9.93±1.04^{b}	6.57±0.86^{ab}	71.33±8.08	24.33±9.29	3.00±1.00	478.00±42.67^{abc}	5.50±0.56^{a}
3	37.67±6.51^{b}	8.77±0.72^{a}	7.33±0.83^{ab}	77.33±6.81	17.67±7.77	3.33±0.58	542.00±60.56^{abcd}	5.73±1.06^{ab}
4	49.75±2.36^{c}	13.33±0.40^{c}	9.68±1.86^{abc}	73.25±14.86	23.00±14.90	2.50±0.58	544.75±36.67^{abcd}	7.28±0.53^{de}
5	50.50±3.70^{c}	13.05±1.00^{c}	10.50±0.43^{bc}	80.50±6.95	15.25±6.95	3.00±0.82	678.50±66.98^{d}	7.05±0.37^{cde}
6	48.25±1.50^{c}	13.10±0.45^{c}	11.75±4.07^{c}	75.00±0.00	20.50±1.29	3.50±0.58	645.25±115.35^{bcd}	7.35±0.17^{e}
7	46.50±1.29^{c}	12.53±0.17^{c}	8.68±2.59^{abc}	73.00±6.06	21.25±2.99	3.50±0.58	725.50±226.69^{d}	7.00±0.61^{cde}
8	50.67±7.02^{c}	13.33±1.00^{c}	9.03±3.00^{abc}	77.00±2.00	19.00±1.00	3.33±0.58	601.33±12.22^{abcd}	7.27±0.32^{de}
9	45.33±4.04^{c}	12.33±0.59^{c}	8.50±2.23^{abc}	79.00±1.00	16.33±2.08	3.33±1.15	408.33±102.05^{a}	6.43±0.29^{bcd}
10	38.67±2.52^{b}	11.00±0.85^{b}	5.77±2.29^{a}	82.67±5.86	12.67±5.51	3.67±0.58	462.67±31.64^{ab}	6.20±0.26^{abc}
P-value	0.000	0.000	0.060	0.638	0.553	0.469	0.012	0.000

Means with different superscript within rows are significantly different (P<0.05)

Results expressed as means ± SD

Means with different superscript within rows are significantly different (P<0.05)

Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisol
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control

Table 2: *Effect of Citrullus lanatus Seed Oil on CD_4 Count at 7th and 14th days of Treatment*

Groups	CD4 cells/ul at day-7	CD4 cells/ul at day-14
1	2206.33±285.50^{a}	2070.67±72.50^{abc}
2	1961.33±84.50^{a}	1387.00±438.63^{a}
3	4602.33±2094.77^{abc}	1206.00±202.52^{a}
4	6710.67±1788.6^{c}	1682.00±415.98^{ab}
5	4434.67±464.95^{abc}	1906.50±236.21^{abc}
6	3759.67±1007.46^{ab}	1662.00±197.58^{ab}
7	6375.00±1507.84^{bc}	2627.00±822.40^{bc}
8	4585.00±706.62^{abc}	1945.00±284.45^{abc}
9	12952.67±2767.94^{d}	3980.33±1082.47^{d}
10	2837.33±1002.07^{a}	2946.33±1002.07^{c}
p-value	0.000	0.000

Means with different superscript within rows are significantly different (P<0.05)

Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisol
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control

Effect of *Citrullus lanatus* seed oil on cytokines at 7th and 14th days of treatment

Figures 3A - 3D showed that at day 7, TNF-α significantly ($p < 0.05$) increased in groups 4 (56.20±23.86), 5 (43.70±11.75) and 8 (43.17±4.00) when compared with group 10 (16.43±15.36). There was no significant ($p > 0.05$) decrease in group 2 when compared with that of groups 1 (24.43±101.95) and 3 (22.03±60). At day 14, there was a significant ($p < 0.05$) increase in TNF-α

in groups 4 (41.63±9.52) and 8 (52.00±7.44) when compared with groups 2 (14.03±1.72) and 10 (21.17±10.32). IFN-γ significantly (p<0.05) increased in group 4 (352.60±138.36) at day 7 when compared with groups 2 (135.83±11.10) and 10 (118.67±18.75) while at day 14, there was a significant (p < 0.05) decrease in groups 4 (209.93±13.42), 5 (204.70±26.73), 8 (198.50±18.23) and 9 (190.90±18.23) compared with that of group 3 (83.73±23.02). Between days 7 and 14, the expression of IFN-γ was decreased just like that of TNF-α.

However, the concentration of IL-10 and IgA increased in response to *C. albicans* infection between days 7 and 14. There was a significant (p < 0.05) increase in IL-10 concentrations in all the groups when compared with that of group 2 (5.56±0.77) rats. At day 7, IgA significantly (p < 0.05) increased in groups 5 (27.20±2.28) and 7 (27.00±5.72) when compared with groups 2 (7.83±4.96) and 10 (21.67±6.15).

At day 14, there was a significant (p < 0.05) increase IgA in groups 4 (54.30±14.63), 5 (51.10±3.83), 6 (55.96±1.59), 7 (49.03±1.60), 8 (49.36±7.16) and 9 (63.37±13.91) when compared with that of groups 2 (8.20±1.76) and 10 (18.40±5.99).

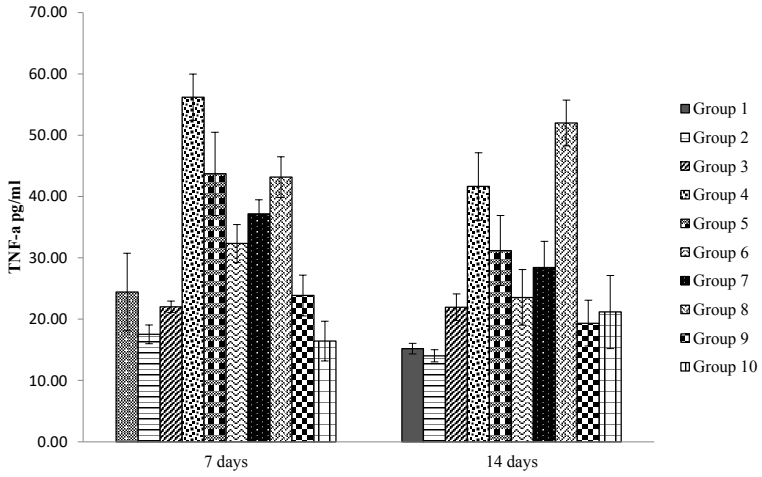

Figure 3A: *Tumour Necrosis Factor-α concentration at days 7 and 14 of treatment*

Means with different superscript within rows are significantly different (P<0.05). Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisole
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control

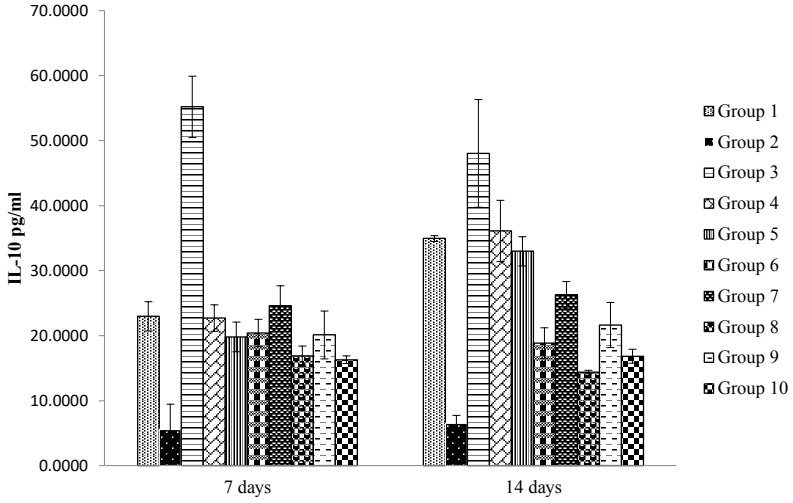

Figure 3B: *IL-10 expression at 7th and 14th day of treatment.*

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisole
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control

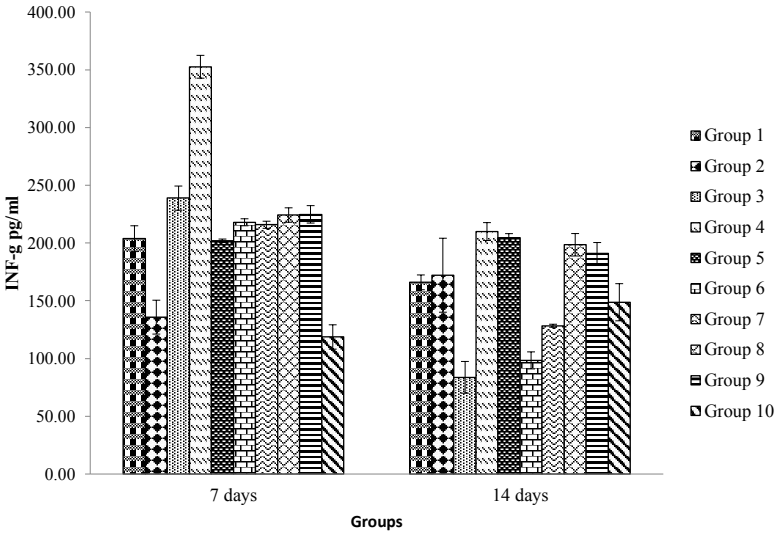

Figure 3C: *Interferon gamma expression at 7th and 14th days of treatment.*

Means with different superscript within rows are significantly different (P<0.05)

Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisole
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control

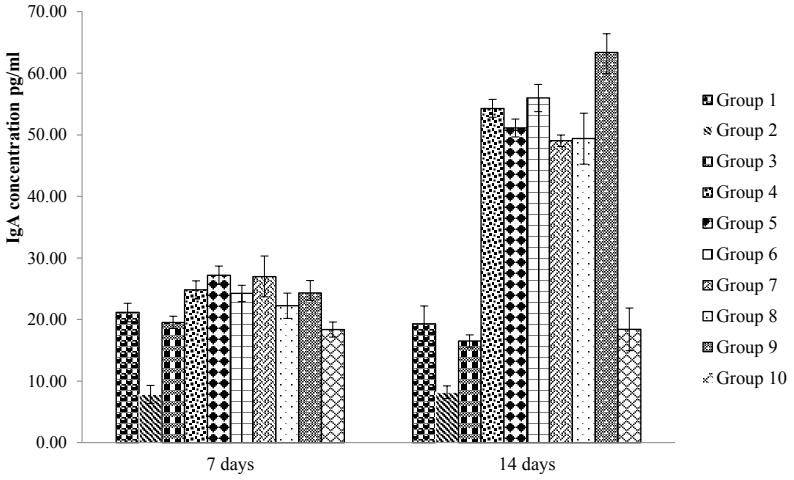

Figure 3D: *Immunoglobulin A production at 7th and 14th days treatment.*

Means with different superscript within rows are significantly different ($P<0.05$)

Results expressed as means ± SD

Key:

Group 1= inoculated without immunosuppression
Group 2=suppressed without inoculation
Group 3=inoculated + suppressed without treatment
Group 4=inoculated + suppressed + treated with 300mg/kg body weight of DO
Group 5=inoculated + suppressed + treated with 500 mg/kg bw DO
Group 6= inoculated + suppressed + treated with 300 mg/kg bw CO
Group 7= inoculated + suppressed + treated with 500 mg/kg bw CO
Group 8= inoculated + suppressed + treated with 25 mg/kg bw levamisole
Group 9= inoculated + suppressed + treated with 5 mg/kg bw Fluconazole
Group 10=Control.

DISCUSSION

Delayed type-hypersensitivity (DTH) response is used as a reflection of the development of immunity that is antigen specific. DTH response is also used to correlate T-cell response by lymphocyte proliferation which causes inflammation. Increase in DTH reaction in response to SRBC is an indication of normal inflammatory response. But after 48 hours, there was a significant decrease ($p<0.05$) in the paw-sized oedema of crude and degummed oil treated groups (groups 1-3) in a dose dependent manner. These results suggest that both deg-

ummed and crude oil which is rich in n-6 poly unsaturated fatty acids exhibited modification of inflammatory responses by the stimulating Th1 cell to effectively respond to inflammation. Previous studies showed that inclusion of n-3 PUFAs significantly decreased DTH response when compared to diets high in n-6 PUFAs.[17, 18] This study has shown that the crude and degummed oil exhibited anti-inflammatory activity but was expressed more in the crude oil than the degummed oil. DTH tests are considered a recall response that is intrinsically different from the initial response to antigen by naïve T-cells.[19]

The humoural antibody titre (Figure 2) shows no significant increase ($p>0.05$) between group 6 (50 mg/kg b.w. of degummed oil) and group 7 (50 mg/kg b.w. of crude oil), probably the dosage did not achieve bioavailability to express any significant difference. It was observed that there was a reduction in the secondary titre values as shown in Figure 2 when compared with the primary titre values, except for groups 4, 4 and 5. The observed suppressive effect on the secondary anti-SRBC titres suggests that the oil exerted inhibition of immuno-regulatory functions as the treatment progressed. The secondary immune response mechanism is through the selection of B cells which has a high affinity for a given antigen; and this occurs in the germinal centres of secondary lymphoid follicles and requires enzyme activation-induced cytidine deaminase (AID).[20] The administration of the watermelon seed oil diminished retention period of the antigen (sheep red blood cell) at the germinal centre which is extremely important and might have affected the quality of secondary immune response observed in this study. The Ag-retaining reticulum of the germinal centers is comprised of specialised follicular dendritic cells (FDCs) that capture intact Ag in the form of Ag-Ab complexes by means of complement receptors or Fc receptors or both.[21]

After seven days of treatment, there was increase in the haemoglobin, haematocrit, and total white blood cell count, monocyte and platelet count. This result agreed with the work of Bushra *et al.*[22] The oil might have acted on hematopoietic cells and activated their differentiation into target cells. Pyrogallol which is a reducing agent might have accumulated in the bone marrow and kidney leading to alterations in the erythropoietic process as shown in group 2. Anaemia is a common phenomenon in immunosuppressive conditions.[23] The imbalance in redox reaction and inflammation brought by pyrogallol as well as *Candida albicans* created a hypoxic environment in the renal interstitium, (kidney being one of the target organs of oropharyngeal candidiasis) which resulted in impaired production of erythropoietin and subsequently result in anaemia. This however increases susceptibility to microbial infections, reactivation of 'inactive" (normal flora) pathogens. Madhavi *et al.*[11] observed that CLSO induced

lyses of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the oil may as well stabilise lysosomal membrane.[23]

Neutrophil activation is said to be compromised in disseminating candidiasis, through oral mucosa. It is possible that oil utilised a peculiar defense mechanism which controlled the evasiveness of candidiasis. Gradual increase in lymphocyte (at day-14) might be due to increased cellular response to *C. albicans* infection. Polymorphonuclear cells (PMNs), monocytes and macrophages are important for the main innate effector response. *Candida* exerts stimulatory effects on platelets through the release of antimicrobial peptide which activates thrombopoeitic activity to release inflammatory mediators. The aggregation of platelets releases microbicidal or microbiostatic substances that are active against *Candida spp*.[25]

CD4 T-cells represent a unique branch of the adaptive immune system that is vital in achieving a regulated effective immune response to pathogens, and their proper functioning is crucial to our survival. *Citrullus lanatus* seed oil might have triggered the proliferation/differentiation of CD4 T-cells against candidiasis. CD4 T-cell-mediated resistance to candidiasis is thought to take place via the production of T helper-1 cytokines (such as gamma interferon [IFN-g] and interleukin-2.) that enhance the candidacidal activity of phagocytic cells.[26, 27] AIDS patients, with reduced populations of CD4 T-cells, are susceptible to oropharyngeal candidiasis.[28]

Cytokines investigated in this study were that of pro-inflammatory such as tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) and anti-inflammatory such as interleukine-10 (IL-10) cytokine. It will make a physiologic sense to say that *C. albicans* ellicit a normal inflammatory response (TNF-α and INF-γ) expression which was down regulated by the administration of *Citrullus lanatus* seed oil (CLSO). This cytokines regulate the growth and differentiation of different lymphocyte subsets. The oil exhibited more anti-inflammatory potentials, since it inhibited the expression of TNF-α and IFN-γ at day 14 of the study. The anti-inflammatory activity of IL-10 dominated the inflammatory response to *C. albicans* resulting in decreases in pro-inflammatory cytokines investigated in this study. In addition, we observed that degummed oil [DO] (group 4 and 5) exerted more anti-inflammatory effect (day-14) probably due to enhanced nutritional values and removal of possible inhibitors of IL-10 in DO. IL-10 is a well-known anti-inflammatory cytokine and plays an important role in suppressing the inflammatory response in many *in vitro* or *in vivo* experimental models.[28, 29] It has been reported that IL-10 inhibits the release of pro-

inflammatory cytokines INF-γ and TNF-α by human monocyte/macrophage in response to polymethylmethacrylate (PMMA, spherical 1-10 μm) particle challenge *in vitro*.[30] TNF-α is essential for anti-*Candida* host defense through the recruitment of neutriphils and phagocytosis, and deficiency results in high mortality during experimental disseminated candidiasis.[29] INF-γ produced by CD4 T lymphocytes is also important for the stimulation of antifungal activity of PMNs. There is no report yet on cytokine analysis or immunoglobulin A (IgA) on both crude and degummed *Citrullus lanatus* oil. Sadeghi *et al*.[31] reported a decrease in TNF-α, IL-1β and IL-6 in response to dietary lipid (safflower oil, fish oil and coconut oil) in rats challenged with lipopolysaccharide (LPS).

Immunoglobulin A (IgA) is considered the isotype that predominantly mediates humoural immune defense at mucosal level.[32] The crude and degummed CLSO favoured immunoglobulin class switch to IgA after the rats were infected with *Candida albicans* SC5314 cells. The increase in IgA was sustained as the administration of the oil progressed up to day 14. Immunological protection of mucosal surfaces is mediated primarily by the secretory immune system, particularly immunoglobulin A (IgA) in secretions, which affords protection by inhibiting adherence and penetration of microorganisms and foreign proteins to mucosal tissues.[33] Mucosal IgA prevents attachment and penetration of microorganisms and molecular antigens, thereby blocking their potential effects on the host.[34] Group 3 showed a decrease in IgA up to day-14. This could be as a result of weakened immune system by pyrogallol, which was administered throughout the period of the study. This findings suggests that CLSO could enhance the immunoglobulin switching to IgA antibody to confer protection against *Candida albicans*. This was affirmed by the inability of group 3 to mount immunological protection against *Candida albicans* when compared with treated groups.

This study demonstrated that *Citrullus lanatus* seed oil has anti-inflammatory property which was expressed more in the crude than the degummed counterpart. However, the oil did not have favorable therapeutic effects on immune system.

REFERENCES

1. Harizi, H; Corcuff, J.B.; Gualde, N. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends Mol Med.* **2008**, 14(10), 461–910

2. Josef, E.; Gerhard, L.; Marion, E.; Margot, G.; Horst, R.; Gerd, S. Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. *PNAS* **2010**, 107 (17), 7817-7822.

3. Ecker, J.; Langmann, T.; Moehle, S.G. Isomer specific effects of conjugated linoleic acid on macrophage ABCG1 transcription by a SREBP-1c dependent mechanism. *Bioch and Biophy Res Comm.* **2007**, 352, 805–811.

4. Lisa, M.C.; Zena, W. Inflammatory Cells and Cancer. *J Exp Med.* **2001**, 193(6), F23-26.

5. McCarberg, B.H.; Cryer, B. Evolving therapeutic strategies to improve nonsteroidal anti-inflammatory drug safety. *Am J Ther.* **2014**, 22, e167-e178.

6. Koeberle, A.; Werz, O. Multi-target approach for natural products in inflammation. *Drug Discov Today* **2014**, 19(12), 1871-1882.

7. Atal, C.K.; Sharma, M.L.; Khariya, A. Immunomodulating agents of plant origin. *J Ethnopharm.* **1986**; 18, 133-141.

8. Gul, S.; Rashid, Z.; Sarwer, G. *Citrullus lanatus* (Watermelon) As Diuretic Agent: An *In vivo* Investigation on Mice. Citrullus Lanatus (Watermelon) As Diuretic Agent: An In vivo Investigation on MiceCitrullus Lanatus (Watermelon) As Diuretic Agent: An In vivo Investigation on Mice *Am J Drug Delivery and Therap.* **2014,** 1(4), 89-92.

9. Erukainure, O.L.; Oke, O.V.; Adenekan, S.O.; Ajiboye, J.A. Antioxidant activities, total phenolic and flavonoid levels of water melon rinds subjected to Saccharomyces solid media fermentation. *Fermentation Tech and Bioeng.* **2011**, 2, 11 – 16.

10. Jamuna, K.S.; Ramesh, C.K.; Srinivasa, T.R.; Raghu, K.L. *In vitro* antioxidant studies in some common fruits .*Intl J of Pharmacy and Pharma Sci.* **2011**, 3(1), 60 -63.

11. Madhavi, P.; Maruthi, R.; Kamala, V.; Habibur, R.; Chinna, E.M. Evaluation of Anti-inflammatory activity of Citrullus lanatus seed oil by In-vivo and *In-vitro* Models. *Intl Res J Pharmacy and Appl Sci.* **2012,** 2(4), 104-108.

12. Zufarov, O.; Stefan, S.; Stanislav, S. Degumming of rapseed and sunflower oils. *Acta Chimica Slovaca* **2008**, 1(1), 321-328.

13. Bafna, A.; Mishra, S. Antioxidant and immunomodulatory activity of the alkaloidal fraction of *cissampelos pareira* linn. *Sci. Pharm.***2010**, 78, 21–31.

14. Doherty, N.S. Selective effect of immunosuppressive agents against the delayed hypersensitive response and humoral response to sheep red blood cells in mice. *Agents Actions.* **1981**, 11, 237–242.

15. Puri, A.; Sexena, R.P.; Sexena, K.C.; Srinivasan, M.; Tandon, J.S. Immunostimulant activity of *Nyctanthes arbortristis* L. *J Ethnopharm.* **1994**, 42, 31-37.

16. Greve, B.; Cassens, U.; Westerberg, C.; Ghde, W.; Sibrowski, W.; Reichelt, D. A new no-lyse, no wash flow-cytometric method for the determination of CD4 T cells in blood samples. *Transfus. Med. Hemother.* **2003**, 30, 8-13.

17. Selvaraj, R.K.; Cherian, G. Dietary n-3 fatty acids reduce the delayed hypersensitivity reaction and antibody production more than n-6 fatty acids in broiler birds. *Euro J Lipid Sci and Tech.* **2004**, 106, 3-10.

18. Hall, J.A.; Jha, S.; Cherian, G. Dietary n-3 fatty acids decrease the leukotrienes B4 response

ex vivo and the bovine serum albumin-induced footpad swelling index in New Hampshire hens. *Canadian J Ani Sci.* **2007**, 87, 373-380.

19. Anderson, M.J.; Fritsche, K.L. Dietary polyunsaturated fatty acids modulate in vivo, antigen-driven CD4þT-cell proliferation in mice. *J Nutr*. **2004**, 134, 1978–1983.

20. Mwanaidi, Y.K.; Paushali, M.; Virander, S.C. Kinetics of Humoral and Memory B Cell Response Induced by the *Plasmodium falciparum* 19-Kilodalton Merozoite Surface Protein 1 in Mice. *Infection and Immunity* **2012**, 80(2), 633–642.

21. Halder, S.; Mehta, A.K.; Mediratta, P.K. Augmented humoral immune response and decreased cell mediated immunity by Aloe vera in rats, *Inflammopharmacol* **2012,** 20, 343–346.

22. Bushra, M.; Tahira, I.; Amer, J.; Faqir, M. Effect of β-Thalassemia on haematological and biochemical profiles of female patients. *Pak. J. Life and Soc. Sci.* **2013**, 11, 25-28.

23. Chinen, J.; Shearer, W. Secondary Immunodeficiencies, including HIV infection. *J. Allergy Clin. Immunol.* **2010**, 125(2), 195-203.

24. Chou, CT. The anti-inflammatory effect of Tripterygium wilfordii Hook F on adjuvant-induced paw edema in rats and inflammatory mediators release. *Phytotherapy Res.* **1997**, 11, 152-154.

25. Teresinha de Jesus, C.N.; Jair, O.; dos, S. Effect of Pathogenic Yeasts on Human Platelet Aggregation. *The Braz J Infectious Dis.* **2003**, 7(6), 370-374.

26. Strom, A.; Menart, B.; Simon, M.C; Pham, M.N; Kolb, H.; Roden, M.; Pozzilli, P. Cellular interferon gamma and interleukin-13 immune reactivity in type 1, type 2 and latent autoimmune diabetes: action LADA 6. *Cytokine*. **2012**, 58, 148–151.

27. Mayer, F.L. Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence* **2013**, 4(2), 119–128.

28. van de Veerdonk, F.L.; Mihai, G.N.; Leo, A.J.; van der Meer, J.W.M.; Bart, J.K. Novel strategies for the prevention and treatment of Candida infections: the potential of immunotherapy. *FEMS Microbiol Rev*. **2010**, 34, 1063–1075.

29. Netea, M.G.; Gow, N.A.; Munro, C.A.; Bate, S.; Collins, C.; Ferwerda, G.; Hobson, R.P.; Bertram, G.; Hughes, H.B.; Jansen, T.; Jacobs, L.; Buurman, *E.T.;* Gijzen, K.; Williams, D.L.; Torensma, R. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin Invest*. **2006**, 116, 1642–1650.

30. Trindade, M.C.D.; Lind, M.; Nakashima, Y.; Sun, D.; Goodman, S.B.; Schurman, D.J.; Smith, R.L. Interleukin-10 inhibits polymethylmethacrylate particle induced interleukin-6 and tumor necrosis factor-α release by human monocyte/macrophages in vitro. *Biomaterials.* **2001**, 22, 2067-2073.

31. Sadeghi, S.; Wallace, F.A.; Calder, P.C. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. *Immunology* **1999**, 96, 404-410.

32. Olivares, N.; Marquina, B.; Mata-Espinoza, D.; Zatarain-Barron, Z.L.; Pinzon, C.E.; Estrada, I.; Parada, C.; Collin, M.; Rook, G.; Hernandez-Pando, R. The protective effect of immunoglobulin in murine tuberculosis is dependent on IgG glycosylation. *Patho and Dis.* **2013**, 69, 176-183.

33. Kim, S.B.; Chang, B.Y.; Jo, Y.H.; Lee, S.H.; Han, S.B. Macrophage activating activity of pyrrole alkaloids from *Morus alba* fruits. *J Ethnopharm*. **2013,** 145, 393-396.

34. Kumar, S.K.; Singh, P.; Sinha, S. Naturally produced opsonizing antibodies restrict the survival of Mycobacterium tuberculosis in human macrophages by augmenting phagosome maturation. *Open Biol.* **2015**, 5, 150-171.

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Design, Development and *in vivo* Pharmacokinetic Evaluation of Zotepine Loaded Solid Lipid Nanoparticles for Enhanced Oral Bioavailability

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Running title: Zotepine lipid nanoparticles

ABSTRACT

The purpose of this work was to prepare and evaluate the zotepine (ZT) loaded solid lipid nanoparticles (SLNs) that might improve the oral bioavailability. ZT-SLNs were developed using homogenization method and characterized for optimal system based on physicochemical characteristics and in-vitro release. Optimized ZT-SLNs were evaluated for permeation, crystalline nature using DSC and XRD, surface morphology using SEM and physical stability. Further, pharmacokinetic (PK) studies of ZT-SLN were conducted in Wistar rats comparison with ZT-coarse suspension (ZT-CS). Optimized formulation showed Z-avg, PDI, ZP of 138.1±3.2, 0.23±0.02 and -26.4±1.5 mV, respectively. In-vitro release studies showed prolonged release, DSC and XRD studies revealed the conversion of ZT to amorphous form. SEM studies showed spherical shape. Permeability and PK studies showed 1.4-folds and 2.0-folds improvement in oral bioavailability, respectively in comparison with ZT-CS formulation. Therefore, the results concluded that SLNs could be considered as a new alternative delivery system for the enhancement of oral bioavailability of ZT.

Keywords: Zotepine, solid lipid nanoparticles, crystallinity, *in vitro* release, *ex vivo* permeation, oral bioavailability.

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INTRODUCTION

The amount of the drug reaching to the systemic circulation mainly depends on the rate of absorption, which in turn depends on the drug solubility[1]. Oral delivery of the drugs is the most conventional and widely used technique for the administration of majority of active moieties. However, several compounds are unsuccessful and failed owing to their low absorption and low bioavailability upon oral administration[2]. The drugs with poor oral bioavailability are unable to reach the minimum effective concentration to exhibit therapeutic action. Some of the reasons for poor bioavailability include: poor solubility; inappropriate partition coefficient as it influences the permeation of drug through lipid membrane; first-pass metabolism; P-glycoprotein (P-gp) mediated efflux; and degradation of drug in the gastrointestinal tract (GIT) due to pH of the stomach or enzymatic degradation or by chemical[3].

To overcome the solubility and oral bioavailability problems of Biopharmaceutical classification system (BCS) class II and IV drugs, the research in the present scenario is focused on bioavailability improvement by using various solubility enhancement techniques like liquisolid compacts[4,5], micronization using nanosuspensions[6], solid dispersions using complexation[7, 8], salt formation[9]. Majority of the drugs undergo first-pass effect after oral administration, there by the therapeutic concentration required in systemic circulation is lowered. However, by using the lipid based delivery systems both the solubility and first-pass effect problems are resolved and subsequently oral absorption was improved[3].

Lipid based drug delivery systems are one of the approaches, which favors the lymphatic transport of oral administered drugs by escaping the first-pass effect[10,11]. Colloidal lipid nanocarriers such as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are the promising approaches in the lipid based delivery systems. SLNs are mainly developed with the solid lipid carrier, used for incorporation of drug, and lipid dispersion is stabilized by the use of biocompatible surfactants[12]. SLNs offer remarkable advantages such as low toxicity, high bioavailability (pharmacokinetic and pharmacodynamic) of drugs, versatility of incorporation of hydrophilic and lipophilic drugs, and feasibility of large-scale production.SLN formulations have controlled drug release properties and provides enhanced chemical stability of drug molecules[13]. SLNs also used for drug targeting to various tissues such as liver[14] and ocular[15] for treatment of various diseases.

Zotepine (ZT) is an atypical anti-psychotic drug, which belongs to BCS class II category. The mechanism of action of ZT is mediated through combined dopa-

mine and serotonin antagonisms[16]. It has a poor oral bioavailability of about 7-13%, due to poor aqueous solubility (0.046 µg/L), highly lipophilic (log P 5.6), and also hepatic first-pass metabolism. The drug levels were also decreased in the presence of CYP enzyme[17,18]. In oral route, 30% of the drug metabolizes into Nor-zotepine (active metabolite) and remaining 70% of drug transforms to inactive metabolites such as 3-hydroxyzotepine, 2-hydroxyzotepine and zotepine-*S*-oxide[16]. Previously, Pailla et al.,[19] developed intranasal delivery of ZT for enhanced brain targeting, using nanosuspension formulation. The enhanced solubility and in vitro dissolution rate of ZT were observed and reported with self-emulsifying drug delivery systems[20]. But, till now there are no lipid nanocarrier systems were reported for enhanced oral delivery of ZT. Hence, in this attempt ZT loaded SLNs were developed could might be improve the oral bioavailability.

The objective of the present investigation was aimed to develop and characterize ZT loaded SLNs for enhanced oral delivery. Accordingly, ZT-SLNs were prepared using know methods and evaluated for an optimized formulation based on the physical and chemical parameters, in vitro release studies. Further, optimized formulation also characterized for solid state characterization for crystallinity, SEM for surface morphology and *ex vivo* permeation studies. *In vivo* performance of optimized ZT-SLN were observed by pharmacokinetic (PK) studies in male Wistar rats, comparison with ZT coarse suspension (ZT-CS) as control.

METHODOLOGY

Materials

Zotepine was a kind gifted sample from Symed labs, Hyderabad, India. Stearic acid (SA), glyceryl monostearate (GMS), Dynasan-118, Dyansan-116 and Dynasan-114 were purchased from Sigma-Aldrich, Hyderabad, India. Compritol 888 ATO was gift sample from Neuheit Pharma Technologies Private Ltd., Hyderabad. Soylecithin was a gifted sample from Lipoid, Ludwigshafen, Germany. Captex355, Captex200 were gift samples from Abitec corporation. All other chemicals were of analytical grade and solvents were of HPLC grade.

Methods

Solubility studies

ZT solubility in various solid lipids (stearic acid, glyceryl monostearate (GMS), Compritol ATO 888, Precirol ATO5, Dyansan-114, Dyansan-116 and Dynasan-118) was determined by using shaking method. Solid lipids were heated to above 5°C of their melting points. An excess amount of ZT was added and was

continuously stirred on gyrator shaker at 180 rpm for 48 h. The supernatant was collected and filtered through 0.45 μ membrane filter after centrifugation[21]. Necessary dilutions were made to the filtrate with methanol and estimated the drug concentration using UV-Visible spectrophotometer (SL-159, Elico, Hyderabad, India) at λ_{max} of 261 nm. Simultaneously, solubility of ZT in release media such as distilled water, 0.1N HCl (pH 1.2), phosphate buffer of pH 6.8 and p H 7.4.

Preparation of solid lipid nano particles (SLNs)

ZT-SLNs were prepared using homogenization-probe sonication method, based on film hydration method[22]. Required amounts of ZT, solid lipid (SA, GMS, Compritol ATO888), and soy lecithin were dissolved in 10 mL mixture of chloroform and methanol (1:1) in a round bottom flask. Organic solvents were removed using rota evaporator (Heidolph, Germany). Drug embedded lipid layer was molten by heating at 5°C above the melting point of the solid lipid. Aqueous phase was prepared by dissolving Poloxamer188 in double distilled water and heated to the same temperature of oil phase. Hot aqueous phase was added to oil phase. Homogenization was carried out at 12,000 rpm for 5mins to form pre-emulsion. The obtained emulsion was ultra-sonicated using Probe sonicator with 12T probe tip (Bandelin, Germany) for 20 mins. ZT-SLNs were obtained upon cooling to room temperature. The composition of the SLNs are given in Table 1.

Table 1: Composition of zotepine loaded solid lipid nanoparticles and control formulation

Ingredients (%w/v)	ZT-SLN1	ZT-SLN2	ZT-SLN3	ZT-SLN4	ZT-SLN5	ZT-SLN6	ZT-CS
Zotepine (mg)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glyceryl monostearate	1.0	-	-	2.0	-	-	-
Stearic acid	-	1.0	-	-	2.0	-	-
Compritol ATO 888	-	-	1.0	-	-	2.0	-
Soylecithin	1.0	1.0	1.0	1.0	1.0	1.0	-
Poloxamer 188	1.5	1.5	1.5	1.5	1.5	1.5	-
Na CMC*	-	-	-	-	-	-	0.05
Water (mL)	QS 10	QS 10	QS 10	QS 10	QS 10	QS 10	QS 10

*Na CMC – sodium carboxy methyl cellulose

Preparation of ZT coarse suspension (ZT-CS)

ZT-CS formulation was prepared by taking about 100 mg of sodium carboxy methyl cellulose (suspending agent) in a mortar and triturated for 15 mins. Then, 10 mg of ZT was added to it and together triturated. To it, 10 mL of double distilled water was added and again triturated for about 5 min to obtain ZT-CS (1 mg/mL). This was used as control formulation.

Characterization of ZT-SLNs

Measurement of particle size, PDI and zeta potential (ZP)

The mean size as Z-avg (nm), PDI and ZP of ZT-SLNs were measured by using photon correlation spectroscopy, using a Malvern Zetasizer (Nano ZS90, UK). About 100 µL of prepared SLN formulations were diluted to 5 mL with double distilled water to get optimum Kilo Counts Per Second (Kcps) of 50-200 for measurements in triplicate at 25°C[23].

Drug content and entrapment efficiency (EE)

About 0.1 mL of prepared ZT-SLN formulation was taken and diluted with chloroform: methanol (1:1) and drug content of the diluted samples was estimated by HPLC-method. The EE (%) in ZT-SLN formulation was calculated by estimating the concentration of the free ZT in the aqueous phase of an undiluted formulation, using an ultrafiltration method centrisort tubes (Sartorius, Germany). The aqueous medium was separated by ultra-filtration using centrisort tubes (Sartorius, Germany) which consisting a filter membrane (M.Wt.20,000 Daltons) at the base of the sample recovery chamber. Centrifugation was carried at 5000 rpm for 30 min[24]. The SLN along with the encapsulated drug remained in outer chamber and aqueous phase moved into sample recovery chamber through filter membrane. The amount of zotepine in aqueous phase was estimated by HPLC method. The EE was calculated by using the equation 1:

$$\% \text{ EE} = \left[\frac{\text{Di\_Df}}{\text{Di}} \right] \text{X } 100 \quad (1)$$

Where, D_i is the total ZT content and D_f is the free ZT present in the aqueous phase.

In vitro drug release studies

The *in vitro* drug release study of ZT-SLN and ZT-CS formulation was performed using dialysis method. Dialysis membrane (Hi media, Hyderabad, India) having average pore size 2.4 nm and molecular weight cut-off between12,000-14,000 Daltons were used for the release studies[25]. Dialysis membrane was soaked overnight in double distilled water prior to the study. Release studies were carried out for first 2 h in 0.1N HCl, followed by pH6.8 phosphate buffer for 48 h by open tube method. Dialysis membrane was tied to open tube which acts as a donor compartment. Temperature of receptor compartment was maintained at 37±0.5°C. Two mL sample was withdrawn at regular time points of 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 36, and 48 h from receiver compartment and replenished with same volume of fresh buffer. The collected samples were suitably diluted and analyzed by UV-Visible spectrophotometer.

Ex vivo studies by normal sac method

Ex vivo studies were performed for optimized ZT-SLN formulation by using evert-sac method[26]. The studies were conducted with prior approval of Institutional Animal Ethical Committee (IAEC/01/UCPSC/KU/2018). Evert-sac studies were carried out in order to investigate the permeation behavior of the control formulation and optimized ZT-SLN formulation (1 mL) across the small intestine. In this study, male Wistar rats of 200 ± 30 g (n=3) were taken and subjected to overnight fasting. The rats were sacrificed by cervical dislocation technique and jejunum of 4 cm were isolated, flushed with saline solution and transferred into oxygenated Krebs's ringer solution. The one end of the sac was tied with thread and filled with the optimized ZT-SLN or ZT-CS as control (1 mL) formulations and the other end was tied. The segment was immersed in 100 mL in Krebs's ringer solution and the medium was oxygenated using aerator. At regular time intervals (0, 15, 30, 45, 60, 90 and 120 min) samples were withdrawn from beaker and analyzed for drug content[27]. The permeability coefficient of the SLN and control formulation was calculated from the slope of linear portion, by plotting the percentage drug permeated vs time[28].

Lyophilization of ZT-SLNs

The optimized ZT-SLN formulation was subjected to lyophilization. The SLN formulation was prepared, using 10% w/w of trehalose dihydrate as cryoprotectant. Prepared ZT-SLN formulation was kept in -80°C freezer for overnight and subjected to lyophilization (Lyodel, Chennai, India) with applied vacuum[29, 30]. Lyophilized formulation was subjected to particle size analysis, drug content, EE and *in vitro* release before and after lyophilization.

Solid-state characterization

Differential Scanning Calorimetry (DSC)

DSC experiments were through using Perkin Elmer DSC 4000 (USA) model to find out the presence of any interaction between drug and the excipients and also to check the alterations in crystallinity of the drug. About 8 mg of the pure drug, physical mixture and optimized lyophilized ZT-SLN formulation samples were taken in aluminium pans, using dry nitrogen as purging gas. The heating rate was 10°C/min and the obtained thermograms were observed for any type of interaction[31].

X-ray diffraction studies

Crystalline nature of Powder X-ray diffractometer (XRD-6000, Shimazdu, Japan) was used for diffraction studies (Arun et al., 2017). Powder XRD studies were performed on the samples by exposing them to nickel filtered CuKα radiation (40 kV, 30 mA) and scanned from 2° to 70°, 2θ at a step size of 0.045° and step time of 0.5 s. Samples used for PXRD analysis were pure ZT, pure lipid and lyophilized ZT loaded solid lipid nanoparticles[32].

Surface morphology by scanning electron microscopy (SEM)

The morphology of pure ZT and freeze-dried ZT-SLN formulation were investigated by scanning electron microscope (SEM S-3700, Hitachi, Japan). The samples were fixed on a brass stub and were coated by thin layer of gold to make electrically conductive[25]. SEM images were recorded at various magnification levels.

Bioavailability study

Study design and sampling schedule

Healthy male Wistar rats (weighing 210±30 g) were used for the PK study. The animals were fasted overnight and had free access to water. The studies were conducted with prior approval of Institutional Animal Ethical Committee (IAEC/01/UCPSC /KU/2018). The animals were divided into two groups (each with n=6) and were orally administered with ZT-CS and optimized ZT-SLN formulation at a dose of 2.2 mg/kg body weight. Both the formulations were administered orally with the help of sterilized rat oral feeding tube. At predetermined time intervals, 0.5 mL of blood samples were collected at predetermined (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h) time intervals by retro-orbital venous plexus puncture. The blood samples were allowed to clot and centrifuged for 15 mins at 3000 rpm. The serum was separated and transferred into clean micro centrifuge tubes and stored at - 20 °C until analysis.

HPLC method

HPLC analysis was conducted with C_{18} column (250 mm X 4.6 mm; 5 µm). Mobile phase consisting of 45: 55 v/v ratio of acetonitrile and phosphate buffer adjusted to pH 4.7 at a flow rate of 1 mL/min at 261 nm with UV detector was used for detection of peaks.

Extraction procedure from rat serum sample

To 100 µL of serum, 100 µL of internal standard (Ramipril, 2 µg/mL), 300 µL of methanol was added. After vortex mixing for 5 min at room temperature, the samples were centrifuged at 5000 rpm for 15 min. After centrifugation the whole organic layer was separated and transferred into another eppendorf tube. Finally, a volume of 20 µL was injected into HPLC system.

Estimation of Pharmacokinetic parameters and statistical significance

Kinetica 2000 software (version 5.0, USA) was used for the estimation of PK parameters like Cmax, Tmax, AUC, $t_{1/2}$ and MRT and the values were expressed in mean ± SD. One-way ANOVA was performed using Graph pad prism software (version 5.03, San Diego, CA, USA) for statistical comparison of data at p value of less than 0.05.

Physical stability studies

An optimized ZT-SLN formulation was stored at room temperature and refrigerated temperature conditions over two months to assess the physical and chemical stability. Z-avg, PDI, ZP, assay and EE of the samples were observed at predetermined time intervals and checked for statistically significance.

RESULTS AND DISCUSSION

Solubility studies

Solubility of ZT was studied in various solid lipids. SA, GMS, Compritol ATO 888 were selected as solid lipids for SLN formulations development. In order to determine the effect of mono and mixed glycerides on the SLN formulation SA, GMS and Compritol ATO 888 was selected. Order of solubility of ZT in solid lipids were GMS, SA, Dynasan-118, Dynasan-114 (showed nearly same solubility)>Compritol ATO888 > Dynasan-112 >Precirol ATO5. The solubility of ZT was decreased with increased pH of dissolution media and indicates pH dependent solubility. The order of solubility in release media as follows: water>0.1N HCl>pH 6.8 phosphate buffer>pH 7.4 phosphate buffer.

Characterization of ZT-SLNs

ZT-SLNs formulations were prepared using three different lipids such as GMS, SA and Compritol ATO 888, each at 1 and 2% w/v, respectively. Homogenization followed by probe sonication method used for the preparation of ZT-SLNs. The homogenization time and probe sonication time used at 12000 rpm for 5 mins and 20 mins at 40% amplitude, respectively. The conditions were optimized based on the earlier reported methods[23].

Measurement of particle size, PDI and ZP

Prepared ZT-SLNs were characterized for particle size, PDI and ZP, using Zetasizer and reported in Table 2. Particle size, PDI and ZP of the ZT-SLNs formulations (ZT-SLN1 to ZT-SLN6) were ranged from 137.3±3.5 to 340.4±3.0 nm, 0.22±0.01 to 0.54±0.08, -10.6±0.3 to -27.4±2.5 mV, respectively. From the results, as the concentration of lipids increased from 1% w/v to 2% w/v, increased in particle size and PDI, decreased in ZP were observed. This might be due to the changes in the orientation of the drug and presence of the void spaces of lipid and drug. PDI of the formulations (SLN1-SLN5) were below 0.5, except for formulations prepared with Compritol ATO® 888. The PDI value of up to 0.5 were considered to be homogeneous dispersion and was evidenced from the earlier reports. [15] ZP of the ZT-SLNs were crucial factor the stability assessment. In this case, the combination of surfactants imparts the electrostatic repulsion and steric stabilization as well. The presence of Poloxamer 188 in the development of SLNs could be favored for the maintenance of this ZP[33]. In general, ZP with ±30 mV could be considered as stable dispersion system. Formulations prepared with GMS showed above -25 mV and indicates stability. But, formulations prepared with SA had below -20 mV of ZP.

Table 2: Physicochemical characteristics - size, PDI, Zeta potential, entrapment efficiency and assay of zotepine loaded solid lipid nanoparticle formulations (mean ± SD, n=3)

Formulation	Size (nm)	PDI	ZP (mV)	Assay (mg)	EE (%)
ZT-SLN1	137.3±3.5	0.22±0.01	-26.7±1.7	9.9±0.4	95.1±2.1
ZT-SLN2	154.5±4.7	0.26±0.06	-27.4±2.5	9.2±0.4	90.4±3.2
ZT-SLN3	187.3±2.8	0.46±0.05	-10.6±0.3	9.1±0.5	85.7±2.6
ZT-SLN4	253.7±5.8	0.34±0.03	-18.4±2.4	8.7±0.4	88.9±2.5
ZT-SLN5	340.4±3.0	0.29±0.07	-29.4±2.1	8.9±0.1	92.6±1.6
ZT-SLN6	303.3±2.1	0.54±0.08	-25.6 ± 1.0	8.8±0.4	94.8±3.5

Drug content and entrapment efficiency

All the formulations were analyzed for EE and total drug content by HPLC (Table 2). EE of the all the formulations (ZT-SLN1 to ZT-SLN6) were ranged from 85.7 ± 2.6 to 95.1 ± 2.1%. As the concentration of lipid increases, EE of the ZT-SLNs were decreased, but not significant difference. This is due to the presence of less orientation of drug molecule in the lipid matrix. Among all the developed formulations ZT-SLN1 formulation is having the high EE. Drug content of all the formulations were in the range of 8.7±0.4 to 9.9±0.4 mg.

In vitro dissolution studies

All the prepared SLNs were subjected to *in vitro* release studies, using dialysis method. 0.1N HCl pH 1.2 followed by pH 6.8 phosphate buffer for the release studies. *In vitro* release of ZT from SLN formulations were observed to 69 ± 2.1 to 82.9 ± 3.6% range, over a 48 h (Figure 1). However, formulations prepared with 1% and 2% w/v lipid concentrations were not significantly influence the drug release behavior from the SLN formulations**.** This could be due to the high EE (more than 90%) and also free drug availability in the outer lipid phase was very less. Formulations prepared with GMS, SA and Compritol ATO888 showed 83.3 ± 2.4 and 75.7 ±3.2 % (from SLN1 and SLN2), 76.8 ± 1.9 and 73.6± 2.3% (from SLN3 and SLN4), 71.5 ± 1.2% and 68.5 ± 1.8% (from SLN5 and SLN6), respectively. The crystal lattice of the lipid matrix might be the reason for difference in the drug release from ZT-SLNs. Monoglyceride formulations (GMS and SA) showed slightly better release then mixed glyceride (Compritol ATO®888) formulations. The drug release from the ZT-CS formulation showed 50.2 ± 2.2% in 120 min (data not showed). In all the prepared formulations, ZT-SLN1 formulation prepared with GMS was having less particle size and PDI, stable ZP, with high EE, acceptable drug content and also showed maximum drug release in 48 h, comparison with other formulations. Therefore, ZT-SLN1 formulation was considered as an optimized formulation and selected for further studies.

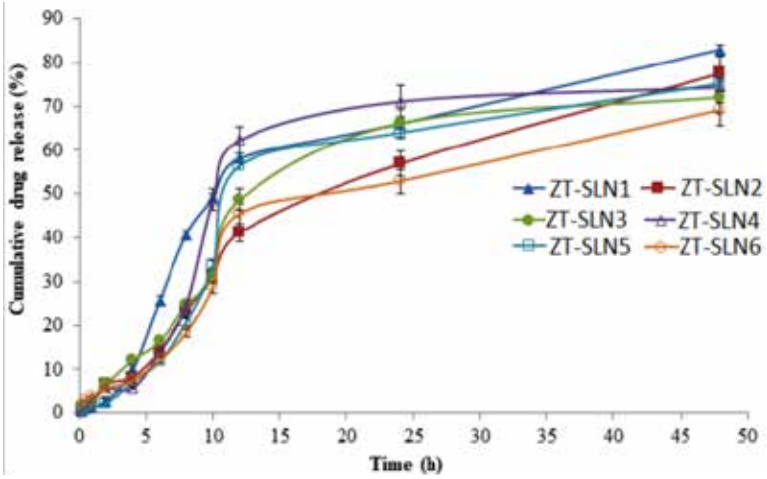

Figure 1: In vitro release profiles of zotepine from ZT-SLNs (mean ± SD, n=6)

Ex vivo permeation studies

Ex vivo permeation studies were performed for optimized ZT-SLN1 formulation in comparison with ZT-CS formulation through rat intestine using everted sac perfusion method. The duration of the study was 120 min and represented in Figure 2. From the results, the % of ZT permeation from ZT-SLN1 and ZT-CS formulation was 18.8 ± 1.6% and 28.3 ± 2.2%, respectively. Statistically significance difference ($p<0.05$) was observed from the ZT-SLN1 compared with ZT-CS formulation. The prolonged release of the ZT from the ZT-SLN formulation was observed from the *in vitro* release and *ex vivo* permeation studies. Furthermore, the permeability rate coefficient of the ZT-SLN1 formulation was calculated from the slope of linear portion by plotting percentage drug permeation against time. From the results, permeability rate of the ZT-SLN1 and ZT-CS formulation was 0.19 and 0.13 ml/min. cm^2, respectively. About 1.4-folds enhancement in the permeation of ZT-SLN1 was observed compared with ZT-CS formulation.

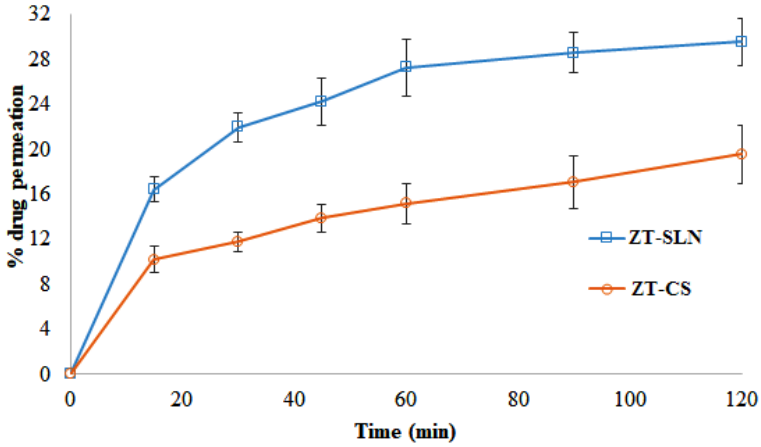

Figure 2: *Ex vivo* permeation profiles of zotepine from ZT-CS and ZT-SLN1 formulations (mean ± SD, n=3)

Lyophilization of ZT-SLN

The crystalline nature, surface morphology and conversion of lipid nanoparticles into solid dosage forms by incorporating in capsules or compression to tablets were easy by the conversion of dispersion to solid form of the nanoparticles. This is generally achieved with either spray drying or freeze drying techniques. Optimized ZT-SLN formulation was subject to lyophilization, using 10 %w/w of trehalose as cryoprotectant. The cryoprotectant and its concentration were selected based on the previously reported methods[24]. The lyophilized formulation was characterized for before and after lyophilization and presented in Table 3. From the results, about two to three-fold increase in the particle size and PDI of the formulation was observed after lyophilization. This could be due to the aggregation of the particles during the process of freeze-drying. But, there were no significant changes noticed in the other parameters.

Table 3: Physicochemical characteristics of optimized ZT-SLN formulation before and after lyophilization (mean ± SD, n=3)

Condition	Size (nm)	PDI	ZP (mV)	Assay (mg)	EE (%)
Pre-lyo	145.2±2.7	0.23±0.05	-27.6±1.4	9.8±0.3	94.4±2.8
Post-lyo	288.9±5.2	0.38±0.06	-29.1±3.5	9.9±0.6	93.6±1.6

Solid state characterization

Differential Scanning Calorimetry

DSC studies were used to determine the compatibility status of the solid lipids and other excipients used in the SLN formulation, crystalline nature of the drug in the nanoformulations and was based on the fact that different lipids possessed different melting points and enthalpies. DSC thermograms of pure ZT, pure GMS, physical mixture of ZT and GMS in 1:1 ratio and lyophilized ZT-SLN1 formulation are shown in Figure 3.

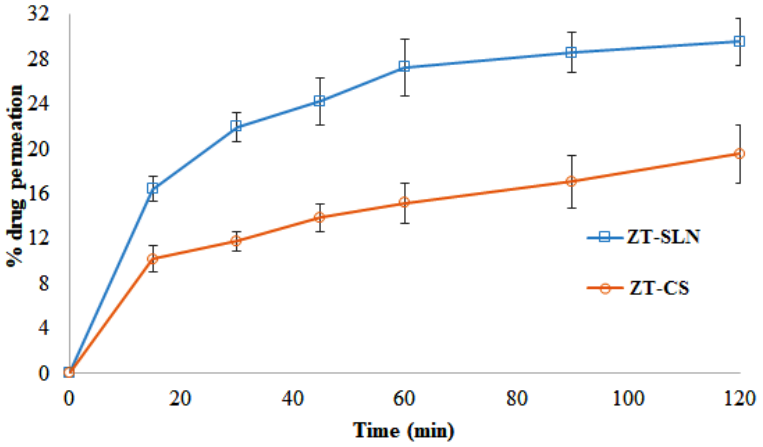

Figure 3: DSC thermograms of pure ZT (A), pure Dynasan-118 (B), physical mixture of ZT + lipid (1:1) (C) and lyophilized optimized ZT-SLN1 formulation (D)

The DSC thermogram of pure ZT showed a sharp endothermic peak at 97.76 °C and it is corresponding to reported melting point. Pure GMS showed a sharp endothermic peak at 72.06 °C. Physical mixture of ZT with GMS showed drug peaks at 98.82 °C, however with less enthalpy compared with pure ZT enthalpy. But, melting endotherm of drug was well preserved with slight changes in terms of shifting in the temperature of the melt. It is known that the quantity of material used, especially in drug-excipient mixtures, could influence the peak shape and enthalpy. Thus, these minor changes in the melting endotherm of drug could be due to the mixing of drug and excipient, which lowered the purity of each component in the mixture and this, might not necessarily indicate potential incompatibility. The absence of endotherm peak of drug in lyophilized ZT-SLN1 formulation unravels the conversion of native crystalline state of the drug to amorphous state.

XRD studies

XRD studies of pure ZT, pure lipid and lyophilized ZT-SLN1 were showed in Figure 4. From the results, powder-XRD patterns of ZT showed sharp peaks at 2θ scattered angles of 10.3, 11.4, 13.5, 17.1, 18.3, 19.8, 20.3, 23.2 and 24.1 degrees, these were indicating the crystalline nature of drug. These characteristic peaks of ZT peaks were absent in the lyophilized ZT-SLN1 sample. This specified that the ZT was not in crystalline form and converted to amorphous nature, after lyophilization of optimized ZT-SLN1. This reduction in crystallinity or conversion to amorphous state of ZT from ZT-SLN was noticed in DSC analysis as well[34].

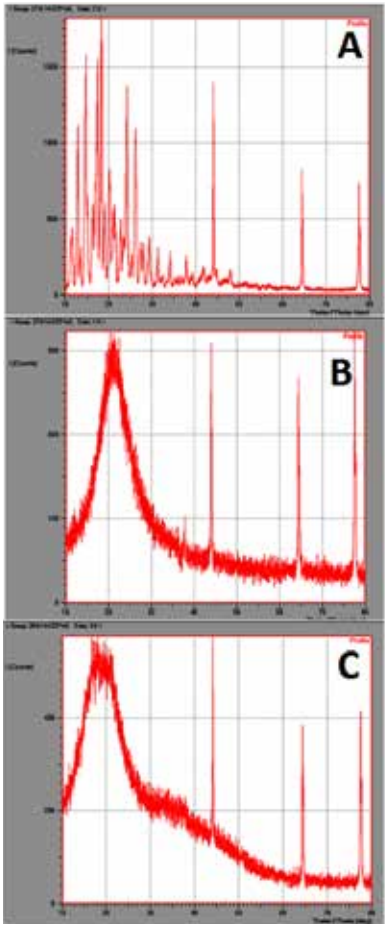

Figure 4: XRD spectra of pure ZT (A), pure Dynasan-118 (B), p and lyophilized optimized ZT-SLN1 formulation (C)

Scanning electron microscopy

The shape of the ZT before and after loading onto SLN formulation was observed using SEM method. For this purpose, surface morphology study was conducted for pure drug and lyophilized ZT-SLN formulation. The results are showed in Figure 5. SEM picture revealed that pure drug of ZT particles are possessed different shaped (cubic and nearly spherical) but highly aggregated (Figure 5A). Optimized formulation of ZT-SLN1 particles are nearly spherical shaped and also aggregated[35]. The results of the SEM studies also correlated with particle size of after lyophilized sample.

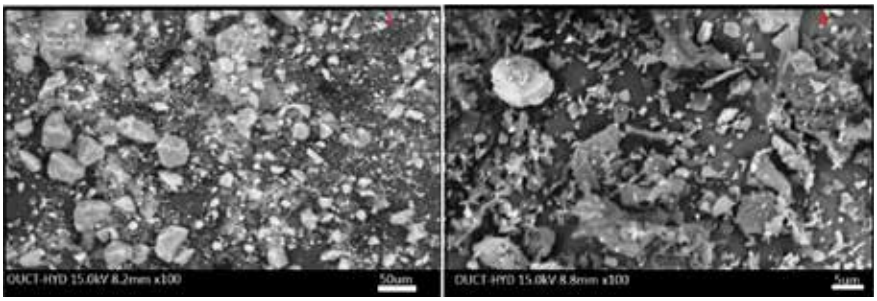

Figure 5: SEM images of pure ZT (A) and optimized ZT-SLN formulation (B)

Stability study of optimized ZT-SLN

The stability of the optimized ZT-SLN was monitored by storage at room temperature (25 ºC) and refrigerated temp (4ºC) for 60 days. The formulations were analyzed on 1st day, 30thday, and 60th day respectively for size, PDI, ZP, EE and drug content. The results indicated that no significant variations were noticed in all the measured parameters, and found to be stable up to two months (Table 4).

Table 4: Stability studies of optimized formulation of ZT-SLN (F1) at both room temperature (25°C) and refrigerated (4°C) for a period two of months (mean ± SD, n=3)

Time (day)	At room temperature (25ºC)					At refrigerated temperature (4ºC)				
	Size (nm)	PDI	ZP	EE	Assay	Size (nm)	PDI	potential	EE	Assay
1	138.1±3.2	0.23±0.02	-26.4±1.5	9.9±0.3	94.7±3.1	138.1±3.2	0.23±0.02	-26.4±1.5	9.9±0.3	94.7±3.1
30	140.5±2.7	0.23±0.04	-27.1±1.0	9.8±0.4	92.3±2.4	145.5±4.2	0.24±0.06	-25.4±1.9	9.8±0.5	91.4±2.8
60	144.3±2.7	0.24±0.05	-6.2±2.3	9.8±0.6	91.7±2.5	148.3±5.3	0.25±0.03	-24.3±1.4	9.7±0.4	90.2±2.4

PK study

Oral bioavailability study for ZT-CS and optimized ZT-SLN1 formulation was performed in male Wistar rats with n=6 in each group. The collected serum samples were analyzed for drug content, using HPLC method. Ramipril is used as internal standard. Retention times of both ZT and IS were 7.8 and 4.5 min, respectively. The linearity of the ZT was observed in the concentration range of 0.25-12 μg/mL. The limit of detection and limit of quantification of the ZT were 0.1 and 0.2 μg/mL, respectively. About 98.5% of extraction efficiency was observed from recovery studies.

The PK parameters of ZT from testing formulations were calculated using Kinetica 2000 software with non-compartmental model. Mean serum concentration vs time profiles of ZT-CS and ZT-SLN formulation after oral administration of single dose are shown in Figure 6. PK parameters like AUC_{total}, C_{max}, T_{max}, MRT and $t_{1/2}$ of the formulations were calculated and shown in Table 5. A significant difference observed in C_{max} and MRT of the F1 formulation, indicates the prolonged release of the ZT. AUC is the major indicator of the bioavailability of the formulations. From the results, AUC values of ZT-CS and ZT-SLN1 formulations were found to be 18.2±1.3 and 37.1±2.2 μg/mL.h, respectively and were statistically significant ($p < 0.05$). From the results, about 2.0-folds improvement in the bioavailability observed from ZT-SLN formulation in comparison with ZT-CS formulation. The improved oral bioavailability of SLNs of ZT could be due to the contribution of individual and/or combined effects of nanosized particles of the SLNs. The effective surface would influence the adhesion to GIT. Consequently, there is increased contact time of the SLN particles[36-42]. In addition, the soylecithin and poloxamer could alter the permeability characters of the GI membrane. The fatty acid chains present in the lipids of SLNs improve the uptake by lymphatic transport. This lymphatic transport minimizes the first-pass effect of the drug[43-47].

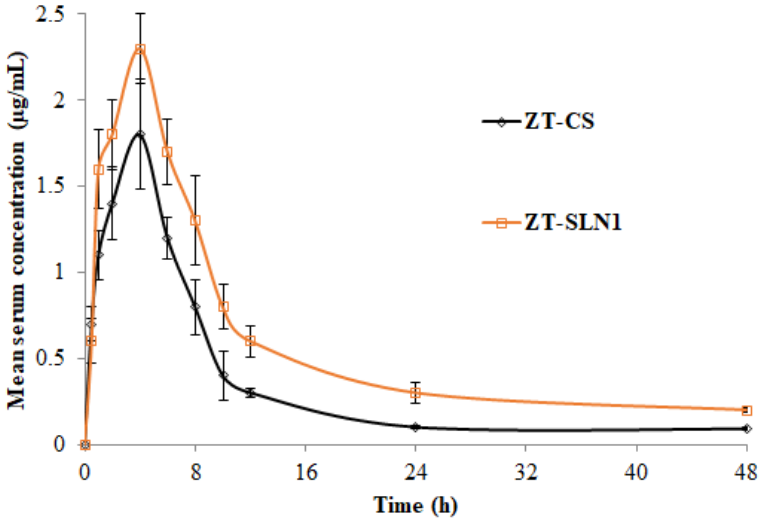

Figure 6: Mean serum concentration – time profiles of zotepine from ZT-CS and optimized ZT-SLN1 formulation after oral administration in rats (mean ± SD, n=6)

Table 5: Pharmacokinetic parameters of zotepine from zotepine coarse suspension (ZT-CS) and zotepine solid lipid nanoparticle (ZT-SLN1) formulations after oral administration in rats (mean ± SD, n=6)

Parameter	ZT-CS	ZT-SLN1
C_{max} (µg/mL)	1.8±0.3	2.3±0.2*
T_{max} (h)	4	4
AUC_{total} (µg/mL).h	18.2±1.3	37.1±2.2*
$t_{1/2}$ (h)	18.6±1.2	18.5±2.1
MRT (h)	17.7±2.6	23.8±1.8*

*indicates statistically significant at $p<0.05$ in comparison with ZT-CS.

ZT loaded solid lipid nanoparticles were successfully developed for the improved oral delivery. The ZT-SLNs were prepared with well-known reported method and characterized for an optimal system. DSC and XRD studies revealed the conversion to amorphous form of ZT in SLN formulation. *In vitro* and *ex vivo* permeation studies confirms the sustained release of the ZT from SLN formulation comparison with coarse suspension as control. ZT-SLN formulation stable for two months at storage conditions. Pharmacokinetic study in male Wistar rats revealed that there was 2.0-fold improvement for SLN when compared to coarse suspension. The overall results indicated that the lipid based delivery systems considerably improved the bioavailability of ZT.

Disclosure statement

The authors declare no conflict of interest in this study.

REFERENCES

1. Louis L, Harvey W. Predicting oral drug absorption: Mini Review on physiologically- based pharmacokinetic models. Pharmaceutics. **2017**; 9(4): 41.

2. Narendar D, Kishan V. Candesartan cilexetil nanoparticles for improved oral bioavailability. Ther Deli. **2017**; 8(2): 79-88.

3. Zhang L, Wang S, Zhang M, Sun J. Nanocarriers for oral drug delivery. J Drug Target. **2013**; 21: 515–527.

4. Arun B, Narendar D. Enhancement of solubility and dissolution rate of trandolapril sustained release matrix tablets by liquisolid compact approach. Asian J Pharma. **2015**; 9 (4): 290-297.

5. Mei L, Haonan X, Jingzheng J, et al. Liquisolid technique and its applications in pharmaceutics. Asian J Pharm Sci. **2017**; 12: 115-123.

6. Nagaraj K, Narendar D, Kishan V. Development of olmesartan medoxomil optimized nanosuspension using Box-Behnken design to improve oral bioavailability. Drug Dev Ind Pharm. **2017**; 43(7): 1186-1196.

7. Yanbin H, Wei-Guo D. Fundamental aspects of solid dispersion technology for poorly soluble drugs. Acta Pharm Sin B. **2014**; 4(1): 18-25.

8. Palem CR, Narendar D, Satyanarayana G, Varsha BP. Development and optimization of Atorvastatin calcium-cyclodextrin inclusion complexed oral disintegrating tablets for enhancement of solubility, dissolution, pharmacokinetic and pharmacodynamic activity by central composite design. Int J Pharm Sci Nanotech. **2016**; 9(2): 1-11.

9. Serajuddin AT. Salt formation to improve drug solubility. Adv Drug Deliv. Rev. **2007**; 59(7): 603-616.

10. William NC, Christopher JHP. Lipophilic prodrugs designed for intestinal lymphatic transport. Adv Drug Deliv Rev. **1996**;19: 149-169.

11. Natalie LT, William NC, Christopher JHP. Lipid- based delivery systems and intestinal lymphatic drug transport: A mechanistic update. Adv Drug Deliv Rev. **2008**; 60: 702-716.

12. Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. Eur J Pharm Biopharm. **2000**; 50(1): 161–77.

13. Mehnert W, Mäder K. Solid lipid nanoparticles. production, characterization and applications. Adv Drug Deliv Rev. **2012**; (64): 83-101.

14. Wenyu W, Xiuli Z, Haiyang H, Dawei C, Jianchun G, Yihui D, Jin S. Galactosylated solid lipid nanoparticles with cucurbitacin B improves the liver targetability. Drug Deliv **2010**; 17(3): 114–122.

15. Tatke A, Dudhipala N, Janga KY, Balguri SP, Avula B, Monica MJ, Majumdar S. In situ gel of triamcinolone acetonide-loaded solid lipid nanoparticles for improved topical ocular delivery: tear kinetics and ocular disposition studies. Nanomaterials (Basel). **2018**; 27: 9(1).

16. Ben G. Zotepine: a clinical review. Expert Opin Drug Metab Toxicol. **2009**; 5(2): 81-86.

17. Tanaka O, Kondo T, Otani K, Yasui N, Tokinaga N, Kaneko S. Single oral dose kinetics of zotepine and its relationship to prolactin response and side effects. Ther Drug Moni **1998**; 20(1): 117-119.

18. Tanaka O. Pharmacokinetics of zotepine and various factors affecting that of zotepine. Nihon Shinkei Seishin Yakurigaku Zasshi. **1996**; 16(2): 49-52.

19. Pailla SR, Talluri S, Rangaraj N, Ramavath R, Challa VS, Doijad N, Sampathi S. Intranasal zotepine nanosuspension: intended for improved brain distribution in rats. Daru. **2019**, 29. doi: 10.1007/s40199-019-00281-4.

20. Dalvadi H, Patel N, Parmar K. Systematic development of design of experiments (DoE) optimised self-microemulsifying drug delivery system of zotepine. J Microencapsul. **2017**; 34: 308-18.

21. Mangesh B, Prashant U, Ashwini M. Formulation and characterization of solid lipid nanoparticles for an anti-retroviral drug darunavir. App Nanosci. **2017**; 7(1-2): 47–57.

22. Narendar D, Kishan V. Improved anti-hyperlipidemic activity of Rosuvastatin Calcium via lipid nanoparticles: pharmacokinetic and pharmacodynamic evaluation. Euro J Pharm Biopharm. **2017**;110 (1): 47-57.

23. Narendar D, Kishan V. Candesartan cilexetil loaded solid lipid nanoparticles for oral delivery: characterization, pharmacokinetic and pharmacodynamic evaluation. Drug Deliv. **2014**;23(2): 395–404.

24. Narendar D, Kishan V. Pharmacokinetic and pharmacodynamic studies of nisoldipine-loaded solid lipid nanoparticles developed by central composite design. Drug Dev Ind Pharm. **2015**; 41(12): 1968-77.

25. Arun B, Narendar D, Kishan V. Development of olmesartan medoxomil lipid-based nanoparticles and nanosuspension: preparation, characterization and comparative pharmacokinetic evaluation. Artif cells. Nanomed. Biotechnol. **2017**, 46(1), 126-137.

26. Mai MG, Sana MM, Marwa AS. Hexagonal liquid crystalline nanodispersions proven superiority for enhanced oral delivery of rosuvastatin: in vitro characterization and in vivo pharmacokinetic study. J Pharma Sci. **2017**; 106: 3103-3112.

27. Mohammad M, Hadi V, Parvin ZM. Bortezomib-loaded solid lipid nanoparticles: preparation, characterization, and intestinal permeability investigation. Drug Dev Ind Pharm. **2018**; 44(10): 1598-1605.

28. Narendar D, Riyaz PMD, Ahmed AY, Nagaraj B. Effect of lipid and edge activator concentration on development of Aceclofenac loaded transfersomes gel for transdermal application: in vitro and ex vivo skin permeation. Drug Dev Ind Pharm. **2020**; 46(8):1334-1344.

29. Butreddy A, Dudhipala N, Janga KY, Rajendra PG. Lyophilization of small-molecule injectables: an industry perspective on formulation development, process optimization, scale-up challenges, and drug product quality attributes. AAPS PharmSciTech. **2020**; 21: 252.

30. Thirupathi G, Swetha E, Narendar D. Role of isradipine loaded solid lipid nanoparticles in the pharmacodynamic effect of isradipine in rats. Drug Res. **2017**; 67(03): 163-169.

31. Narendar D, Karthik YJ. Lipid nanoparticles of zaleplon for improved oral delivery by Box-Behnken design: Optimization, in vitro and in vivo evaluation. Drug Dev Ind Pharm. **2017**; 43(7):1205-1214.

32. Narendar D, Karthik YJ, Thirupathi G. Comparative study of nisoldipine-loaded nanostructured lipid carriers and solid lipid nanoparticles for oral delivery: preparation, characterization, permeation and pharmacokinetic evaluation. Artificial cells. Nanomed Biotech. **2018**; 46(S2): 616-625.

33. Shi L, Li Z, Yu L, Jia H, Zheng L. Effects of surfactants and lipids on the preparation of solid lipid nanoparticles using double emulsion method. J Disp Sci Techno. **2011**; 32(2): 254-259.

34. Xin, W. L.; Xiao, H. L.; Li, Q. Z.; ; Hong, Z.M. Preparation, characterization, and in vitro release of chloramphenicol loaded solid lipid nanoparticles. J Disp Sci Techno. **2008**, 29(9), 1214-1221.

35. Pitta S, Dudhipala N, Narala A, Veerabrahma K. Development and evaluation of zolmitriptan transfersomes by Box-Behnken design for improved bioavailability by nasal delivery. Drug Dev Ind Pharm. **2018**; 44(3):484-492.

36. Arik D, Amnan H. The effect of different lipid based formulations on the oral absorption of lipophilic drugs: The ability of in vitro lipolysis and consecutive ex vivo intestinal permeability data to predict in vivo bioavailability in rats. Euro J Pharm Biopharm. **2007**; 67: 96-105.

37. Narendar D. A comprehensive review on solid lipid nanoparticles as delivery vehicle for enhanced pharmacokinetic and pharmacodynamic activity of poorly soluble drugs. Int J Pharm Sci Nanotech. **2019**; 12(2): 4421-4440.

38. Dudhipala N, Puchchakayala G. Capecitabine lipid nanoparticles for anti-colon cancer activity in 1, 2-dimethylhydrazine-induced colon cancer: Preparation, cytotoxic, pharmacokinetic, and pathological evaluation. Drug Dev Ind Pharm. **2018**; 44(10): 1572-1582.

39. Narendar D, Thirupathi G. Neuroprotective effect of ropinirole lipid nanoparticles enriched hydrogel for Parkinson's disease: in vitro, ex vivo, pharmacokinetic and pharmacodynamic evaluation. **2020**. Pharmaceutics: 12(5), E448. doi: 10.3390/pharmaceutics12050448

40. Tirumalesh C, Suram D, Dudhipala N, Banala N. Enhanced Pharmacokinetic Activity of Zotepine via Nanostructured Lipid Carrier System in Wistar Rats for Oral Application. Pharm Nanotechnol. **2020**; 8(2):148-160.

41. Nagaraj B, Tirumalesh C, Dinesh S, Narendar D. Zotepine loaded lipid nanoparticles for oral delivery: development, characterization, and in vivo pharmacokinetic studies. Fut J Pharm Sci. **2020**; 6: 1-11.

42. Ahmed AAY, Narendar D, Mujumdar S. Ciprofloxacin loaded nanostructured lipid carriers incorporated into in-situ gels to improve management of bacterial endophthalmitis. Pharmaceutics. **2020**; 12(6):572.

43. Krishna MV, Kumar BV, Dudhipala N. In-situ intestinal absorption and pharmacokinetic investigations of carvedilol loaded supersaturated self-emulsifying drug system. Pharm nanotech. **2020**; 8(3):207-224.

44. Dudhipala N, Ay AA. Amelioration of ketoconazole in lipid nanoparticles for enhanced antifungal activity and bioavailability through oral administration for management of fungal infections. Chem Phy Lipids. **2020**; 104953.

45. Suvarna G, Narender D, Kishan V. Preparation, characterization and in vivo evaluation of rosuvastatin calcium loaded solid lipid nanoparticles. Int J Pharm Sci Nanotech. **2015**; 8(1): 2779-2785.

46. Sandeep V, Narendar D, Arjun N, Kishan V. Lacidipine loaded solid lipid nanoparticles for oral delivery: preparation, characterization and in vivo evaluation. Int J Pharm Sci. Nanotech. **2016**; 9(6):3524-30.

47. Gondrala UK, Dudhipala N, Kishan V. Preparation, characterization and in vivo evaluation of felodipine solid-lipid nanoparticles for improved oral bioavailability. Int J Pharm Sci Nanotech. **2015**; 8 (4): 2995-3002.

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A Stability Indicating UV-HPLC Method for the Determination of Potential Impurities in Nandrolone Phenylpropionate Active Pharmaceutical Ingredient

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ABSTRACT

A reverse phase stability indicating HPLC method has been developed for the identification and quantification of potential impurities in Nandrolone phenylpropionate active pharmaceutical ingredient. The chromatographic separation of potential impurities was achieved in Inertsil ODS-3Vcolumn (250 mm x 4.6 mm, 5 µm) using gradient elution method. Mobile phase-A was prepared using 0.1% orthophosphoric acid in water and methanol mixture in a ratio of (90:10, v/v) and mobile phase-B was only acetonitrile. The developed method was validated as per the ICH guidelines for specificity, linearity, precision and accuracy. Specificity of the method was confirmed by peak purity analysis using photodiode array (PDA) detector. The value of correlation coefficient was greater than 0.999 for Nandrolone phenylpropionate and its six impurities. Accuracy of the method was established between 93.3% to 109.0% for all impurities. Nandrolone was found to be the major degradation impurity. The proposed method is suitable for routine as well as stability studies.

Keywords: Nandrolone phenylpropionate, nandrolone, anabolic, steroid.

INTRODUCTION

Nandrolone phenylpropionate (NPP) is known as 19-noretestorone β-phenyl propionate [1]. It is an anabolic-androgenic steroid. It is a fast-acting ester derivative form of Nandrolone and has strong anabolic effect with weak androgenic

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effect. NPP is injected intramuscularly every once in a week for 12 weeks and the dose may vary from 25 mg/mL to 50 mg/mL of steroid dissolves in oily formulation[2]. Nandrolone Esters have been used in the treatment of osteoporosis, anemia, increase in muscle mass, to induce protein synthesis in skeletal muscles and improve respiratory muscle functions in COPD (Chronic Obstructive Pulmonary Disease)[3-7]. The effect of NPP in rats has been reported by Hale (1972)[8].

The related substances method by TLC and assay method by UV spectrophotometer have been reported in Indian Pharmacopoeia and United States Pharmacopoeia respectively[9,10]. The work on the development and validation of multi-residue method for the detection of wide range of hormonal anabolic compounds in hair using gas-chromatography-tandem mass spectrometry has been carried out [11]. The effect of Nandrolone esters in the growth and histology of urine and hair has been studied by Groot et al[12]. The validated GC-MS and UHPLC-MS method have been reported for the quantitative determination of anabolic steroids in formulation products and dietary/nutritional supplements[13-16]. Findings on the effect of side chain, the injection site and the injection volume on the pharmacokinetics and pharmacodynamics of Nandrolone esters in an oil vehicle in men is also available[17]. Various research articles have also reported for the development and analysis of Nandrolone esters[18-21].

However, reverse phase HPLC method has not been reported yet for the determination of potential impurities in NPP (including major pharmacopoeias such as USP, Ph.Eur, BP, JP, and IP). Therefore, it is important to have a stability indicating method for the quantification of potential impurities in NPP which is specific and accurate. The quality, efficacy and safety are significantly affected by the impurities present in the drug product[22]. As per the current ICH Guideline of stability testing of drug substances, forced degradation must be carried out to establish that the method is stability indicating and the analytical methods need to be validated[23-26]. The current work involves method development, forced degradation and method validation for NPP.

METHODOLOGY

Materials and chemicals

NPP sample (99.7% purity, 10 g, Batch number: NPP/009/005), Imp-2 (98.3% purity, 1 g, Batch number: ND/031/016), Imp-3 (98.7% purity, 500 mg, Batch number: BD/035/009), Imp-4 (98.5% purity, 500 mg, Batch number: NPP/009/018), Imp-5 (98.9% purity, 500 mg, Batch number: NPP/009/023), Imp-6 (98.1% purity, 500 mg, Batch number: NPP/009/029) were obtained from chemical research division, Ipca laboratories Ltd. (Vadodara, India). Imp-1

(99.0% purity, 100 g, Lot number: STBD4764V) was obtained from Sigma-Aldrich (Steinheim, Germany). HPLC grade water for the analysis purpose was obtained from Milli-Q plus water purification system (Millipore, Bedford, MA, USA). HPLC grade acetonitrile and methanol was purchased from Merck India (Mumbai, India).

Instrumentation and HPLC conditions

The instruments used in the study include: HPLC separation module (2695) with UV detector (2487) and photodiode array detector (2996) (Waters Corporation, Milford, MA, USA); A photostability chamber model NEC-104RTS (Newtronic, Mumbai, India).

Inertsil ODS-3V column (250 mm x 4.6 mm, 5 µm) HPLC column was used in the analysis (GL Sciences). For the gradient elution method, mobile phase-A and mobile phase-B were used. Mobile phase-A was prepared using 0.1% orthophosphoric acid in water and methanol mixture in a ratio of (90:10, v/v) and mobile phase-B was only acetonitrile. The column temperature was kept at 40°C for the analysis; flow rate of 1.0 mL/min; injection volume was 20 µL; detector wavelength was fixed at 240 nm and 210 nm; sample temperature was 25°C. Diluent was a mixture of water and acetonitrile in a ratio of (20:80, v/v). Gradient program is tabulated in Table 1.

Table 1. Linear gradient program.

Time/minutes	Mobile phase-A, %	Mobile phase-B, %
0	45	55
25	20	50
35	10	90
40	45	55
50	45	55

Preparation of sample and stock solution for validation

NPP test sample was prepared by transferring about 20 mg of sample into 50 mL volumetric flask, added10 mL of acetonitrile to it and sonicated for 1.0 min and made uptothe volume with diluent (400 µg/mL).

Individual stock solution of about 800 µg/mL was prepared by transferring about 20 mg each of NPP, imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 into separate 25 mL volumetric flasks, added 10 mL of acetonitrile to it and sonicated for 1.0 min and made upto the volume with diluent.From the above individual stock solution transfer 0.75 mlinto separate 20 mL volumetric flasks, made upto the volume with diluent and shake well. This solution was labeled as standard stock solution (30.0µg/mL). Further from this standard stock solution, the desired solutions of different concentrations were prepared for validation.

Preparation of forced degradation samples

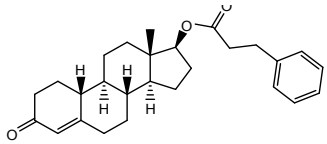
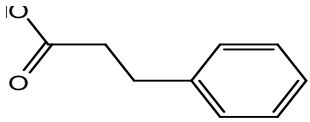
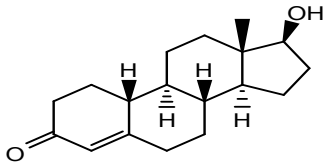
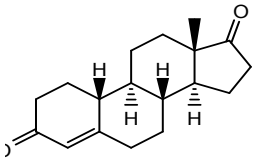
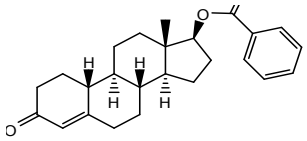
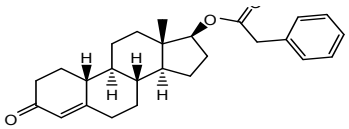
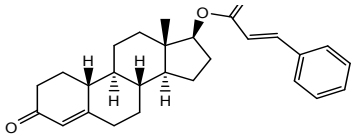
About 20 mg each of sample was weighed and transferred into 3 separate 50 mL volumetric flasks and labeled as 1, 2 and 3. 10 mL of acetonitrile was added into each volumetric and the sample was dissolved by sonicated for 1.0 min. 2 mL of 0.5 N hydrochloric acid solution, 5.0 % hydrogen peroxide solution and 0.5 N sodium hydroxide was also added into each volumetric flask. The flasks were kept in a water bath at 60°C for 2 h (acid hydrolysis), 60°C for 6 h (oxidative degradation) and at room temperature for 1 h (base hydrolysis) respectively. The excess of acid or base in volumetric flask 1 and 3 were neutralized and made upto the mark with diluents. Corresponding blank solutions were prepared. Thermal degradation was performed on solid NPP sample at 70°C for 48h. Photolytic degradation was performed by spreading the sample on petri dish and kept in a photostability chamber model NEC-104RTS (Newtronic) to get the light intensity of 1.2 million Lux hours for white light and 200 Wh/m^2 for ultraviolet region.

RESULTS and DISCUSSION

HPLC method development

The literature search was done to check the method availability but little information was available regarding the quantification of related impurities of NPP. Hence it was decided to initiate the reverse phase HPLC method development. The aim of the proposed method is to achieve the baseline separation between all related impurities and NPP. Structure and details of NPP and its impurities are listed in Table 2. The λ_{max} of NPP and its potential impurities were showed in Figure 1.

Table 2. Structure and details of NPP and its impurities.

S.No	Name	Structure	Code	Source
1	Nandrolone phenylpropionate		NPP	Drug
2	3-Phenylpropionic acid		Imp-1	Degradation Impurity
3	Nandrolone		Imp-2	Raw material and degradation Impurity
4	Bolandione		Imp-3	Process impurity
5	Nandrolone benzoate		Imp-4	Process impurity
6	Nandrolone phenyl acetate		Imp-5	Process impurity
7	Nandrolone phenylcinnamoate		Imp-6	Process impurity

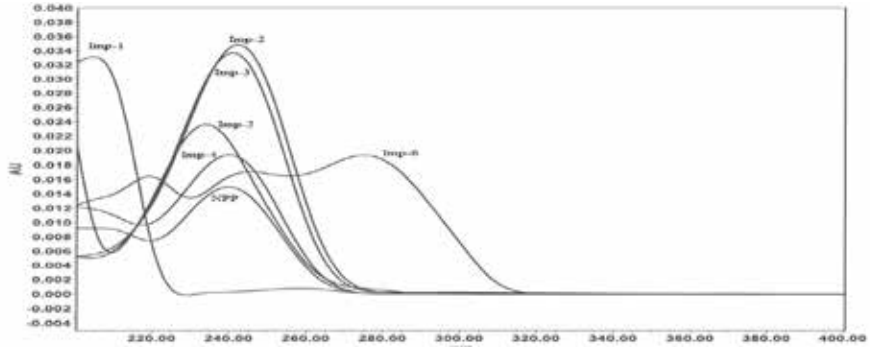


Figure 1. The λ_{max} of NPP and its potential impurities.

The method development was initiated using water and acetonitrile as mobile phase-A and Mobile phase-B in gradient elution method, using C-8 column with dimension (150 mm x 4.6 mm, 5 µm).The peak of NPP and imp-6 co-elutes and the peak shape of imp-1 was found distorted. Finally after conducting several experiments the method was finally developed using linear gradient program. For the gradient elution method, mobile phase-A and mobile phase-B were used. Mobile phase-A was prepared using 0.1% orthophosphoric acid in water and methanol mixture in a ratio of (90:10, v/v) and mobile phase-B was only acetonitrile. Inertsil ODS-3Vcolumn (250 mm x 4.6 mm, 5 µm) was used. The column temperature was kept at 40°C and detector wavelength was selected as 240 nm and 210 nm (210 nm for imp-1) throughout the analysis. The NPP peak eluted at about 26 min with the base line separation of all impurities (Figure 2 and 3).

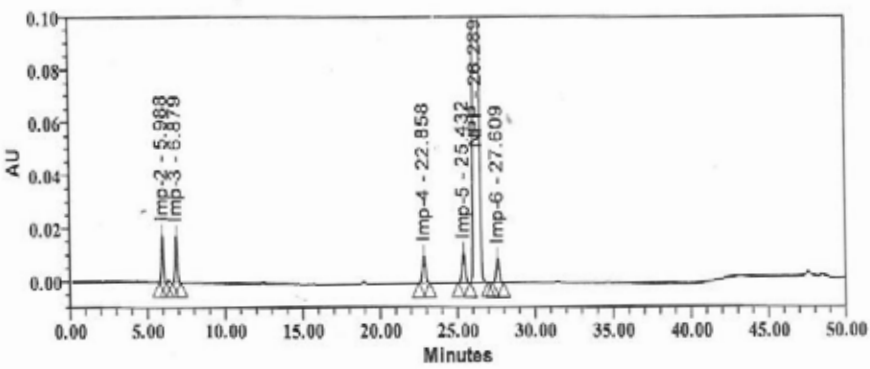


Figure 2. Chromatogram of spiked impurities in NPP at wavelength 254 nm.

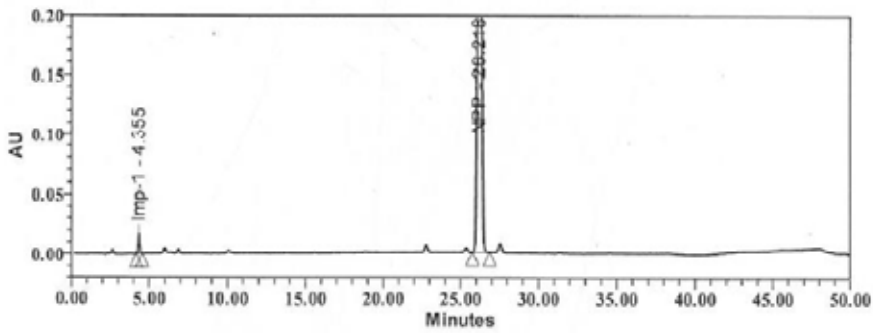


Figure 3. Chromatogram of spiked impurities in NPP at wavelength 210 nm.

Forced degradation study

The forced degradation of NPP sample was conducted in acidic, basic, thermal, oxidative and photolytic conditions. The significant degradation of NPP was observed in acidic and basic conditions. In both the conditions, the impurity formed was identified as imp-2 at 240 nm and imp-1 in 210 nm. There was no impact of oxidative, thermal and photolytic conditions on NPP. The peak purity analysis of the analyte peak obtained from PDA detector in all stress samples confirmed the spectral purity of NPP peak demonstrates the stability indicating capability of the method. The chromatograms are shown in Figure 4 and 5 and data is tabulated in Table 3.

Table 3. Percentage of degradation under different conditions using NPP sample at 400 µg/mL.

S No.	Condition	Degradation %
1	Acidic (0.5N HCl, 60°C, 2 h)	3.5
2	Basic (0.5N NaOH, room temperatute, 1 h)	6.7
3	Oxidation (5% H2O2, 60°C, 6 h)	-
4	Thermal (70°C, 48 h)	-
5	Photolytic degradation	-

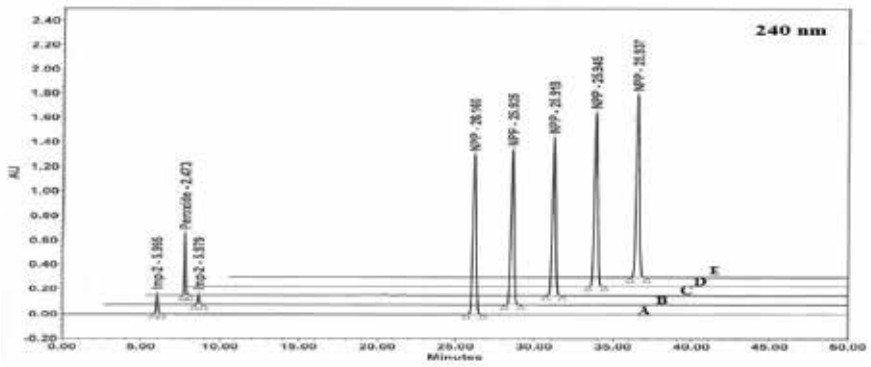


Figure 4. Chromatogram of forced degradation studies of NPP at 240 nm: (A) base hydrolysis, (B) acid degradation (C) oxidative degradation, (D) thermal degradation, (E) photolytic degradation.

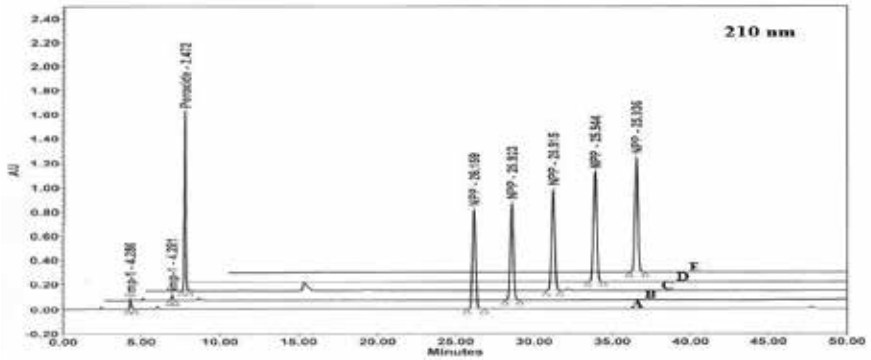


Figure 5. Chromatogram of forced degradation studies of NPP at 210 nm: (A) base hydrolysis, (B) acid degradation (C) oxidative degradation, (D) thermal degradation, (E) photolytic degradation.

Method validation

As per ICH guidelines the method must be validated to establish specificity, solution stability, sensitivity, linearity, precision, accuracy and robustness. The method validation was carried out for imp-1, imp-2, imp-3, imp-4, imp-5, imp-6 and NPP. A summarized result of method validation is tabulated in Table 4.

Table 4. Method Validation summary data

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	NPP
Retention Time (RT)	4.36	5.99	6.88	22.86	25.43	27.61	26.29
Relative RT	0.17	0.23	0.26	0.87	0.97	1.05	1.00
Resolution	76.00	3.85	53.85	6.97	2.27	3.50	-
Symmetry factor	1.04	1.03	1.02	1.00	1.00	1.01	1.03
Response factor	0.56	0.71	0.69	0.77	0.68	0.88	1.00
Linearity	0.9998	0.9991	0.9997	0.9997	0.9993	0.9997	0.9996
Detection Limit (µg/mL)	0.0721	0.0362	0.0364	0.0360	0.0358	0.0359	0.0361
Quantitation Limit (µg/mL)	0.1503	0.1205	0.1212	0.1199	0.1193	0.1195	0.1202
Intra-day precision (n=6, % RSD)	1.95	0.47	0.57	1.14	0.60	0.83	--
Inter-day precision (n=6, % RSD)	0.92	0.66	1.32	1.57	1.48	1.70	-
Accuracy at LOQ (n=3, %)	94.6	106.7	103.3	104.4	108.0	93.3	--
Accuracy at 100 (n=3, %)	102.7	108.1	107.8	105.9	105.9	105.9	--
Accuracy at 150 (n=3, %)	103.9	106.3	109.0	106.0	105.4	104.5	--

Specificity

Specificity is the ability of chromatographic method to separate the analyte peak from blank interference, impurities and degradants. Specificity was done by injecting blank, NPP sample and NPP sample Co-spiked with impurities. The NPP peak was found well resolved from all six impurities and no blank interference was observed. Forced degradation study was also performed to demonstrate method specificity as well as stability indicating capability of the proposed method.

Solution stability

Stability of solutions was established by injecting spiked and unspiked sample solution kept in HPLC vial at 25°C in auto sampler. Area of each impurity was checked after 24 hrs against initial (0 h) and the % difference was found less than 10%, hence sample solution are stable for 24 h at 25°C.

Limit of detection and quantitation (Sensitivity)

The detection Limit (LOD) and Quantitation limit (LOQ) determines the sensitivity of the proposed method. The LOD for each impurity (imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) and NPP were calculated and found to be in the range of 0.0358 µg/mL to 0.0721 µg/mL. The LOQ for each impurity (imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) and NPP were calculated and found to be in the range of 0.1193 µg/mL to 0.1503 µg/mL.

Linearity and range

Linearity determines the ability of the analytical method to get the test results that are directly proportional to the analyte concentration within the given range. The solutions were prepared at six different concentration levels for all specified impurities and sample from LOQ level to 150% of impurity limit [0.15% of the drug concentration (400 µg/mL). The result of correlation coefficient obtained for all analyte were found to be greater than 0.999, confirms positive correlation between peak area and concentration of impurities and NPP peak (Figure 6).

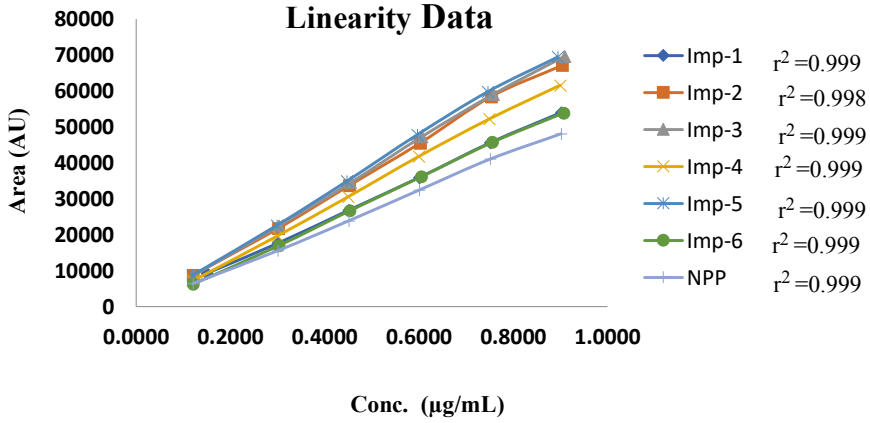


Figure 6. Linearity data of all 6-impurities and NPP.

Accuracy

The accuracy of the method was expressed in the term of percentage recovery. The accuracy of the present method was performed at LOQ level, 100% level (0.15% of the drug substances) and 150% level (0.225% of the drug substances). The experiment was performed in triplicate. The percentage recovery of all impurities ranged between 93.3% to 109.0% indicating the accuracy of the method.

Precision

The precision (Intra-day precision) of the method was evaluated by preparing six individual preparations of NPP sample and spiked with 0.15 %(0.6 µg/mL) of each impurity with respect to sample concentration(400 µg/mL). Peak area of each impurity was checked and percentage relative standard deviation was calculated and found less than 2.0 % for each impurity.

The precision (inter-day precision) was also determined by performing the same procedure in same lab by different analyst on different instrument and on different dates. The percentage RSD for each impurity was found below 2.0% in both the precision indicated the good precision of the proposed method.

Robustness

The robustness of the analytical method was checked by evaluating the influence of small deliberate modifications in the HPLC method parameter. The studies showed that there was no impact on the method even by deliberately changing the chromatographic parameters (i.e. column temperature, flow rate and mobile phase composition). It was observed that the resolution among all the impurities and NPP peak was higher than 2.0. This showed the reliability of the method during routine usage of the method.

The proposed method was found to be better and advantageous over previous reported methods for quantification of potential impurities as compared to the TLC and UV spectrophotometer method describes in Indian pharmacopoeia and United States Pharmacopoeia respectively. The proposed method has been validated as per the current ICH guidelines and easy to use for routine analysis as well as stability studies.

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REFERENCES

1. O'Neil MJ. The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biological, 15th ed. Merck& Co.,Inc.,Whitehouse station: New Jersey; **2013**. 1702 p.

2. Llewellyn W. Anabolics.10th ed. Molecular Nutrition: Jupiter, Florida; **2011**. 460-467p.

3. Wijnhoven H, Ennen L, Rodenburg R, Dekhuijzen PNR. Mitochondrial Function in Diaphragm of Emphysematous Hamsters after treatment with nandrolone. Int J Chron Obstruct Pulmon Dis. **2006**; 1(1): 83-89.

4. Haupt HA, Rovere GD. Anabolic steroids: A Review of a Literature. Am J Sports Med. **1984**; 12:469-484.

5. Choo JJ, Emery PW, Rothwell NJ. Dose-Dependent Effect of an Anabolic Steroid, Nandrolonephenylpropionate (Durabolin), on Body Composition and Muscle Protein Metabolism in Female Rats. Ann Nutr Metab. **1991**; 35: 141-147.

6. Snow DH, Munro CD, Nimmo MA. Effect of Nandrolone Phenylpropionate in the Horse: (1) Resting Animal. Equine Vet J. **1982**; 14:219-223.

7.Bagchus WM, Smeets JMW, Verheul HAM, DeJager-Van DerVeen SM, Port A, Geurts TBP. Pharmacokinetic Evaluation of Three Different Intramuscular Dose of Nandrolone Decanoate: Analysis of Serum and Urine Samples in Healthy Men. J Clin Endocrinol Metab. **2005**; 90: 2624-2630.

8. Hale DH. The Effect of Orchidectomy and Administration of Testosterone Propionate or Nandrolone Phenylpropionate to Orchidectomised Rats on their growth and carcass composition. *S Afr J Anim Sci*. **1973**; 1: 39–44.

9. Indian pharmacopoeia, Government of India, Ministry of Health and Family Welfare. 8th ed.; **2018**. Indian Pharmacopoeia Commission: Ghaziabad; pp 2692.

10. The United States Pharmacopoeia (USP) and the National Formulary (NF). USP 40- NF 35. Rockville, Md. **2017**; pp 5278-5279.

11. Rambaud L, Monteau F, Deceuninck Y, Bichon E, Andre F, Bizec BL. Development and Validation of a Multi-Residue Method for the Detection of a Wide Range of Hormonal Anabolic Compounds in Hair Using Gas Chromatography-Tandem Mass Spectrometry. Anal Chim Acta. **2007**; 586: 93-104.

12. Groot MJ, Lasaroms JJP, Vanbennekom EO, Meijer T, Vinyeta E, Vanderklis JD, Nielen MWF. Illegal Treatment of Barrows with Nandrolone Ester: Effect on Growth, Histology and Residue Levels in Urine and Hair. Food Addit Contam: Part A. **2012**;29: 727-735.

13. Neves DBDJ, Caldas ED. GC–MS Quantitative Analysis of Black-Market Pharmaceutical Products Containing Anabolic Androgenic Steroids Seized by the Brazilian Federal Police. *Forensic Sci Int*. **2017**; *275*: 272-281.

14. Tricova B, Bosakova Z, Kozlik P. Development of an Ultra High-Performance Liquid Chromatography–Tandem Mass Spectrometry Method for the Determination of Anabolic Steroids Currently Available on the Black Market in the Czech Republic and Slovakia. Drug Test Anal. **2019**; 11: 355-360.

15. Thuyne WV, Delbeke FT. Validation of a GC-MS Screening Method for Anabolizing Agents in Solid Nutritional Supplements. Biomed Chromatogr. **2004**; 18: 155-159.

16. Odoardi S, Castrignano E, Martello S, Chiarotti M, Strano-Rossi S. Determination of Anabolic Agents in Dietary Supplements by Liquid Chromatography-High-Resolution Mass Spectrometry. Food Addit Contam: Part A .**2015**;32: 635-647.

17. Minto CF, Howe C, Wishart S, Conway AJ, Handelsman DJ. Pharmacokinetics and Pharmacodynamics of Nandrolone Esters in Oil Vehicle: Effect of Ester, Injection Site and Injection Volume. J pharmacol Exp Ther. **1997**; 281: 93-102.

18. Cavrini V, DiPietra AM, Raggi MA, Sarti R. Analysis of Nandrolone Esters in Oily Injections by Reversed-Phase High Performance Liquid Chromatography. *J Pharm Biomed Anal*. **1987**;*5*: 21-32.

19.Mukherjee J, Das A, Chakrabarty US, Sahoo BK, Dey G, Choudhry H, Pal TK. Development and Validation of RP-HPLC Method to Determine Nandrolone Phenylpropionate in Different Pharmaceutical Formulations. Acta Pol Pharm. **2011**;68: 155-160.

20. Li Y, Yu P, Wang Q. Determination of Anabolic Hormones in Porcine Hair by High Performance Liquid Chromatography-Mass Spectrometry. Anal Lett. **2016**; 49: 2895-2906.

21.He L, Huang XH, Fang B, Huang S, Cao Y, Chen J, Zeng Z, Chen Z. Determination of Eleven Steroid Hormones in Animal Muscle Tissues and Eggs using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry. Chin J Chromatgr. **2008**; *26*: 714-719.

22. ICH Harmonised Tripartite. Impurities in New Drug Substances.*Guideline*.**2006**, Q3A(R2).

23. ICH Harmonised Tripartite. Stability Testing of New Drug Substances and Products. *Guideline*. **2003**, Q1A (R2).

24. ICH Harmonised Tripartite. Photostability Testing of New Drug substances and Products. *Guideline*.**1996**, Q1B.

25. ICH Harmonised Tripartite. Validation of Analytical procedure: Text and Methodology. *Guideline*.**2005**, Q2(R1).

26. Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of Forced Degradation and Stability Indicating Studies of Drug-A Review. J Pharm Anal. **2014**;4: 159-165.

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Stability Indicating Method Development and Validation of Ranolazine Extended Release Tablets

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ABSTRACT

The objective of this study was to develop and validate the stability-indicating method for newly developed Extended-Release tablet formulation of Ranolazine. First, new Ranolazine tablet formulation was developed. These tablets were analyzed by using a High-Performance Liquid Chromatography system with a UV detector at 220 nm wavelength and by using C8-3 column (150 mm x 4.6 mm i.d; 5 µm particle size). The injection volume of the system was 10 µl. The validation parameters; Selectivity, linearity, accuracy, robustness, precision and limit of quantification and detection parameters were proved good results. A highly sensitive and simple HPLC-UV analytical method of the Ranolazine tablet formulation was developed in accordance with ICH Guideline Q2 and Q3.

Keywords: Ranolazine, HPLC, forced degradation, stability indicating method, validation

INTRODUCTION

Ranolazine, (RNZ, N-(2,6-dimethylphenyl)-2-{4-[2-hydroxy-3-(2-methoxyphenoxy) propyl] piperazine-1-yl}acethamide) is a piperazine derivative drug substance, which has anti-ischemic and antianginal effects[1-3]. The chemical structure of RNZ[4] is given in Figure 1. RNZ is a stereo isomer molecule. The pharmacological activity of enantiomers shows statistically insignificant differences[5, 6].

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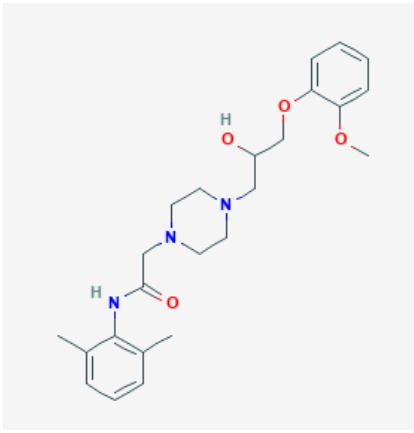

Figure 1. The chemical structure of RNZ

In the past decades, RNZ is the only anti arrhythmic medication developed and marketed for the treatment of chronic angina[7]. RNZ inhibits selectively fast and late sodium current. RNZ is a well-tolerated drug without showing any bradycardia and/or hypotension effect in contrast to β-blockers, Ca^{++} blockers, and nitrates[8, 9]. Although there is no convincing proof, RNZ has been shown to improve glycometabolic homeostasis in rats by enhancing insulin function [10, 11].

Many patients sustain persistent angina, despite the using combined drug therapies[12]. In some cases, the use of additional antianginal agents with a novel mechanism of action, such as RNZ, is unavoidable. RNZ extended release (ER) tablets are developed and marketed due to the short half-life of RNZ immediate release tablets. Because of the increasing consumption of RNZ, separate chromatographic methods for its analysis were reported in the literature. There is no official monograph for RNZ in the pharmacopoeias, in this context developing and validating stability indicating chromatographic method could be useful.

The aim of developing RNZ ER tablets is to reduce the severity and frequency of angina pectoris symptoms and improving the life standard of patient[13]. Common granulation techniques such as wet and dry granulation were used to prepare RNZ ER tablets, indicated in the literature. The effect of different pH-dependent[14-16] and/or pH-independent polymers on the release of RNZ ER tablets were observed[17-19] in these studies.

The objective of this study was developing and validating stability indicating method for prepared ER tablet formulation of RNZ. The developed method was validated RNZ and its impurities. The proposed chromatographic method was applied to the assay of commercial Ranexa ER tablet and prepared RNZ ER tablet.

METHODOLOGY

Chemical, reagents and materials

Ranolazine, Ranolazine Working Standard (WS), Zen-I Impurity WS, Zen-II Impurity WS, and Zen-III Impurity WS were purchased from Aarti Healthcare (Mumbai-India). Acetonitrile, methanol, hydrochloric acid, perchloric acid, phosphoric acid, ammonium dihydrogen phosphate, sodium hydroxide, ethanol, and triethylamine were purchased from J.T.Baker (AP-USA). Fumaric acid was a kind gift from Merck Millipore (Darmstadt-Germany). Povidon (Kollidon® K30) was purchased from BASF (Florham Park-USA). Xanthan gum was purchased from CP-Kelco (USA). Hypromellose (Benecel™ K15M) was a gift from Ashland (Covington-USA). Magnesium stearate was purchased from Faci (Carasco GE Italy).

Preparation of RNZ ER tablets

RNZ 500 mg ER tablets were prepared by wet granulation method. The high shear mixer was used for granulation. The granulation process designed in two stages. At the first stage, fumaric acid and Ranolazin were granulated with granulation solvent consist of purified water and PVP. The wet mass sieved and dried in the oven. Afterward, dried bulk was sieved and this premix blended with hypromellose and xanthan gum. The second granulation was carried out with ethanol. The wet mass sieved and dried in the oven. The dried mass eluted and mixed with magnesium stearate to obtain the final blend. The unit formula and the function of the raw materials of RNZ ER tablets are shown at the Table 1. The final blend was compressed by using rotary tablet press machine (Manesty XSpress).

Table 1. The unit formula and the function of the raw materials of RNZ ER tablet

Raw materials	Function	% w/w
Ranolazine	Active ingredient	63,69
Fumaric acid	Diluent	25,48
Povidon	Binder	2,42
Hypromellose	Controlled release agent	2,04
Xanthan gum	Controlled release agent	5,10
Magnesium stearate	Lubricant	1,27
Purified water*	Granulation solvent	-
Ethanol*	Granulation solvent	-

*purified water and ethanol were used as granulation solvent and do not exist in the finished product.

Instrumentation and analytical conditions

Analysis method of RNZ and its impurities were chromatographic. A Waters *High Performance Liquid Chromatography* (HPLC) system (New Castle-USA) with UV detector was used. The method validation was made in accordance with ICH Guideline Q2 and Q3[20, 21].

Chromatographic condition for quantization of RNZ

Inertsil C8-3 column (150 mm x 4.6 mm i.d; 5 µm particle size) from GL Sciences (Japan), was performed for chromatographic separation of RNZ. The temperature of column was set and maintained at 30°C. The UV detection was fixed to 220 nm wavelength and the injection volume was 10 µl. The mobile phase was consisting of acetonitrile (A) and buffer solution (B) (30:70). Buffer solution was prepared with triethylamine: purified water; 4.2:1000 (v/v) and the pH of solution was adjusted to 3.00 ± 0.05 with phosphoric acid. The flow rate was set to 1.0 ml/min. The injection time was 9 minutes.

Chromatographic condition for quantization of impurities

Waters XTerra RP18 column (250 mm x 4.6 mm i.d; 5 µm particle size) from Waters (New Castle-USA), was performed for chromatographic separation of Zen-I (2-((2-methoxyphenoxy) methyl) oxirane), Zen-II (2-chloro-N-(2,6-dimethylphenyl) acetamide), and Zen-III, RNZ impurities. The UV detection was fixed to 210 nm wavelength and the injection volume was 20 µl. The mobile phase X was consisting of ammonium dihydrogen phosphate solution (C) and methanol (D) (80:20). The mobile phase Y was consisting of ammonium dihydrogen phosphate solution (C) and methanol (D) (20:80). Ammonium dihydrogen phosphate solution was prepared with ammonium dihydrogen phosphate: purified water; 5.75:1000 (w/v) and filtered (0.45 µm PET filter). The injection time was 40 minutes. The following gradient, at a fixed flow rate of 1.0 ml/min was used: from 0 to 5 min the composition was X: Y, 90:10. From 5 to 15 min the composition was X: Y, 80:20. From 15 to 27 min the composition was X: Y, 40:60. From 27 to 32 min the composition was X: Y, 20:80. From 32 to 40 min the composition was X: Y, 90:10.

Preparation of standard and sample solutions

Preparation of standard and sample solutions for validation of RNZ

Preparation of standard solution: 50 mg RNZ WS was accurately weighed to a 20 ml volumetric flask and then 10 ml 0.1 N HCl solution was added to the flask, mixed 1 min by vortex and then kept in an ultrasonic bath during 15 min to obtain a clear solution. The volumetric flask was complemented to designated

volume with 0.1 N HCl solution. 5.0 ml of this solution precisely was transferred to a 50 ml volumetric flask and complemented to designated volume with the mobile phase. The obtained solution was filtered 0.45 µm PET filter and transferred to HPLC vials (the concentration of solution 0.25 mg/ml).

Preparation of sample solution: 20 RNZ ER tablets were weighed and pulverized by grinding with the help of mortar and pestle. The obtained powder, equivalent to 250 mg RNZ was accurately weighed and transferred to a 100 ml volumetric flask. The volumetric flask was complemented to designated volume with the mobile phase. The obtained solution was filtered 0.45 µm PET filter and transferred to HPLC vials (the concentration of solution 0.25 mg/ml).

Preparation of RNZ standard stock solution: 125 mg RNZ WS was accurately weighed to a 50 ml volumetric flask and, then 25 ml 0.1 N HCl solution was added to the flask, mixed 1 min by vortex and then kept in an ultrasonic bath during 15 min to obtain a clear solution. The volumetric flask was complemented to designated volume with 0.1 N HCl solution (the concentration of solution 2.5 mg/ml).

Preparation of standard and sample solution for validation of impurities

Preparation of RNZ standard solution: 20 mg RNZ WS was accurately weighed to a 50 ml volumetric flask and then 30 ml mobile phase X solution was added to the flask, mixed 1 min by vortex. 5.0 ml of this solution precisely was transferred to a 50 ml volumetric flask and complemented to designated volume with mobile phase X. 5.0 ml of this solution precisely was transferred to a 50 ml volumetric flask and complemented to designated volume with the mobile phase X. The obtained solution was filtered 0.45 µm PET filter and transferred to HPLC vials (the concentration of solution 0.002 mg/ml).

*Preparation of placebo solution:*124 mg RNZ ER tablet placebo was weighed to a 100 ml volumetric flask and, then 70 ml mobile phase X solution was added to the flask, and kept in an ultrasonic bath during 10 min. The volumetric flask was complemented to designated volume with the mobile phase X solution and kept in an ultrasonic bath during 10 min. The obtained solution was filtered 0.45 µm PET filter and transferred to HPLC vials.

Preparation of sample solution: The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was accurately weighed and transferred to a 100 ml volumetric flask and, then 70 ml mobile phase X solution was added to the flask and kept in an ultrasonic bath during 10 min. The volumetric flask was complemented to designated volume with mobile phase X solution and kept in an ultrasonic bath

for 10 min. The obtained solution was filtered 0.45 μm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml).

Preparation of Zen-I, Zen-II, and Zen-III standard stock solution: 7.5 mg Zen-I WS was accurately weighed to a 25 ml volumetric flask and then 10 ml mobile phase X solution was added to the flask, and kept in an ultrasonic bath during 15 min. The volumetric flask was complemented to designated volume with mobile phase X solution and mixed by a vortex (the concentration of solution 0.3 mg/ml). The same procedure was applied to obtain Zen-II and Zen-III standard stock solutions.

Preparation of 100 % preparative sample solution: The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was accurately weighed and transferred to a 100 ml volumetric flask and then 70 ml mobile phase X solution was added to the flask and kept in an ultrasonic bath during 10 min. From each standard stock solutions respectively Zen-I, Zen-II, and Zen-III, 1.0 ml solution were added to the flask and complemented to designated volume with the mobile phase X solution and kept in an ultrasonic bath during 10 min. The obtained solution was filtered 0.45 μm PET filter, and transferred to HPLC vials (C_{RNZ}:2.0 mg/ml, C_{ZEN-I}:0.003 mg/ml, C_{ZEN-II}: 0.003 mg/ml, $C_{ZEN-III}$:0.003 mg/ml).

Preparation of 200 % preparative sample solution: The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was accurately weighed and transferred to a 100 ml volumetric flask and then 70 ml mobile phase X solution was added to the flask and kept in an ultrasonic bath during 10 min. From each standard stock solution respectively Zen-I, Zen-II, and Zen-III, 2.0 ml solution were added to the flask and complemented to designated volume with mobile phase X solution and kept in a ultrasonic bath during 10 min. The obtained solution was filtered 0.45 μm PET filter, and transferred to HPLC vials (C_{RNZ}:2.0 mg/ml, C_{ZEN-I}:0.006 mg/ml, C_{ZEN-II}: 0.006 mg/ml, $C_{ZEN-III}$:0.006 mg/ml).

Method validation protocol

Analytical method validation is done to demonstrate that the obtained results are precise and valid and to show the applicability of the method. The stability-indicating method was validated according to ICH Guidelines Q2 and Q3. In this context linearity, selectivity, accuracy, robustness, precision, the limit of quantification and detection were evaluated.

Forced degradation studies

Forced degradation studies were performed to prove the stability indicating capability of the method. Prepared samples and placebos were exposed to:

Acidic condition (1N HCl / room temperature for 24 hours)

Basic condition (1 N NaOH / room temperature for 24 hours)

Oxidative condition (H_2O_2 30% / room temperature for 24 hours)

Thermal condition (60 ºC ± 2 ºC / for 1 week)

Light (1.2 million lux hours)

Acidic condition

The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was weighed to a 100 ml volumetric flask and then 20 ml ammonium dihydrogen phosphate solution was added to the flask and kept in an ultrasonic bath during 15 min. Then 10 ml methanol was added to the flask and kept in ultrasonic bath for 1 min. Afterward 15.0 ml 1 N HCl was added. The prepared solution-maintained room temperature for 24 hours. At the end of time the solution was neutralized with 15.0 ml 1 N NaOH and the volumetric flask was complemented with ammonium dihydrogen phosphate solution to designated volume. The flask was kept in an ultrasonic bath for 15 min. The obtained solution was filtered 0.45 µm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml). The same procedure was applied to the placebo.

Basic condition

The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was weighed to a 100 ml volumetric flask and, then 20 ml ammonium dihydrogen phosphate solution was added to the flask and kept in an ultrasonic bath for 15 min. Then 10 ml methanol was added to flask and kept in ultrasonic bath for 1 min. Afterward 15.0 ml 1 N NaOH was added. The prepared solution-maintained room temperature for 24 hours. At the end of time the solution was neutralized with 15.0 ml 1 N HCl and the volumetric flask was complemented with ammonium dihydrogen phosphate solution to designated volume. The flask was kept in an ultrasonic bath for 15 min. The obtained solution was filtered through 0.45 µm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml). The same procedure was applied to the placebo.

Oxidative condition

The pulverized RNZ ER tablets, equivalent to 200 mg RNZ was weighed to a 100 ml volumetric flask and then 20 ml ammonium dihydrogen phosphate solution was added to the flask, and kept in an ultrasonic bath for 15 min. Then 10 ml methanol was added to the flask and kept in ultrasonic bath for 1 min. Afterward 15.0 ml H_2O_2 30% was added. The prepared solution-maintained room

temperature for 24 hours. At the end of time the volumetric flask was complemented with ammonium dihydrogen phosphate solution to designated volume. The flask was kept in an ultrasonic bath for 15 min. The obtained solution was filtered 0.45 μm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml). The same procedure was applied to the placebo.

Thermal condition

RNZ ER tablets and placebo were kept at 60 °C ± 2 °C in the oven for a week. At the end of the time the pulverized RNZ ER tablets, equivalent to 200 mg RNZ was weighed to a 100 ml volumetric flask, and then 70 ml ammonium dihydrogen phosphate solution was added to the flask and kept in an ultrasonic bath for 10 min. The solution was kept at room temperature for 5 min and complemented with ammonium dihydrogen phosphate solution to designated volume. The flask was kept in an ultrasonic bath for 10 min. The obtained solution was filtered 0.45 μm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml). The same procedure was applied to the placebo.

Light (1.2 million lux hours)

RNZ ER tablets and placebo were exposed to light for 24 hours (1.2 million lux hours). The procedure applied according to ICH guideline Q1B. At the end of the time the pulverized RNZ ER tablets, equivalent to 200 mg RNZ was weighed to a 100 ml volumetric flask and then 70 ml ammonium dihydrogen phosphate solution was added to the flask and kept in an ultrasonic bath for 10 min. The solution was kept at room temperature for 5 min and complemented with ammonium dihydrogen phosphate solution to designated volume. The flask was kept in an ultrasonic bath for 10 min. The obtained solution was filtered 0.45 μm PET filter and transferred to HPLC vials (the concentration of solution 2.0 mg/ml). The same procedure was applied to the placebo.

RESULTS and DISCUSSION

Method optimization

In order to optimize the assay method, several mobile phase solvents were tested. In this context 0.1 N HCl, pH 6.8 phosphate buffer, acetonitrile: buffer (ammonium dihydrogen phosphate) (80:20), methanol: water (80:20), acetonitrile: water (80:20), acetonitrile: pH 6.8 phosphate buffer (80:20), acetonitrile: 0.1 N HCl (80:20) were tried and the condition that enabled a suitable resolution and shape of peaks was acetonitrile: buffer (80:20). Also different sample and standard preparation solvents: acetonitrile + 0.1 N HCl (1:10); water + 0.1 N HCl (1:5); mobile phase and different sample preparation processes (mixing with vor-

tex, keeping in an ultrasonic bath) were tried. The results of the analysis of these changes showed that for sample preparation RNZ ER tablet using mobile phase and for standard preparation using 0.1 N HCl as described at *2.4. Preparation of standard and sample solutions* were the best way of solving RNZ.

To optimize and validate the analytical method of RNZ impurities for RNZ ER tablets, data obtained from *AARTI Healthcare Related Substances Analytical Method* was modified and used.

The obtained features provided the chromatogram reported in Figure.2. a good peak shape and peak resolution were obtained within acceptable analysis time (20 min).

Method validation

Selectivity

To assess the selectivity of the method, placebo solution, mobile phases, sample solution of RNZ ER tablet, RNZ WS, Zen-I, Zen-II, and Zen-III impurity solutions were injected in duplicate and ability of chromatographic separation of each sample was evaluated. The chromatogram shown Figure. 2 was obtained. Five peaks were obtained from the chromatogram. Between the peaks of RNZ, solvent, mobile phases, placebo and impurities did not observe any interference. All peaks completely separated from another one.

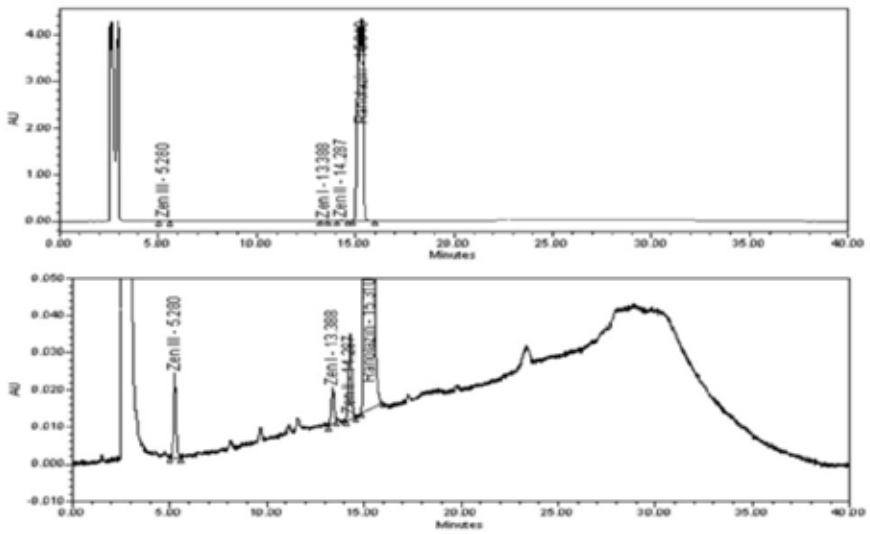

Figure 2. HPLC chromatogram (λ=220 nm) of a solution containing RNZ ER tablet, impurities Zen-I, Zen-II and Zen-III.

Linearity

The linearity of the method was evaluated for five concentration levels by diluting the standard stock solution corresponding to 20, 50, 80, 100, and 120 %. The prepared solutions were filtered and injected in triplicate. For impurities, response and correction factor were calculated using the formula shown below:

$$RRF = \frac{m_i}{m_s} \qquad CF = \frac{1}{RRF}$$

RRF: Response factor

CF: Correction factor

m_i: the slope value obtained from the calibration curve of the impurity standard solutions

m_s: the slope value obtained from the calibration curve of the active substance standard solutions

The obtained data showed that in the expected concentration RNZ and its impurities are linear. The summary of the results is reported in Table 2. The determination coefficient of all compounds was found greater than 0,9999, these results show the precision of the proposed methods[22].

Table 2. The summary of five levels calibration graphs for RNZ, Zen-I, Zen-II and Zen-III (y=ax+b), three replicates for each level (n=15).

	RNZ	Zen-I	Zen-II	Zen-III
Range (µg/ml)	50 – 300	0.09 – 5.76	0.07 – 5.97	0.07 – 6.01
Slope (a)	20801294.3595	40503993.06	83381036.74	63496097.58
Intercept (b)	5977.0612	2263.45	1162.55	879.75
r^2	0.9998	1.00	1.00	1.00
RRF	-	0.70	1.44	1.09
CF	-	1.43	0.70	0.91
StDev*	229	303	401	179
95% CI for µ*	(114617; 115135)	(111394; 112081)	(247206; 248114)	(190099; 190505)
SE Mean*	132	175	232	103

* 100% concentration data

Accuracy

To assess the accuracy of the method, three parallel samples are prepared by spiking the placebo with known amounts of RNZ WS 80, 100, 120 %. For impurities with the known amounts Zen-I WS, Zen-II WS, and Zen-III WS, LOQ, 100, and 200 % solutions were prepared. Three injections from each sample were run on HPLC. The recovery values were calculated according to the standard solution. The obtained results of accuracy studies were shown in Table 3.

Table 3. The summary of accuracy studies for RNZ and its impurities Zen-I, Zen-II and Zen-III

RNZ Level %	**Concentration (mg/ml)**	**Recovery %**	**Mean %**	**Zen-I Level %**	**Concentration (µg/ml)**	**Recovery %**	**Mean %**
80	0.20	100.5 100.1 100.8	100.5	LOQ	0.096	98.6 98.0 97.1	97.9
100	0.25	99.9 99.9 100.2	100.0	100	3.000	93.3 92.9 90.1	92.1
120	0.30	100.2 99.9 98.8	99.6	200	6.000	84.6 90.3 90.5	88.47
Zen-II Level %	Concentration (µg/ml)	Recovery %	Mean %	**Zen-III** Level %	Concentration (µg/ml)	Recovery %	Mean %
LOQ	0.096	85.6 98.0 84.6	89.4	LOQ	0.096	102.6 103.6 103.2	103.1
100	3.000	100.9 102.2 98.7	100.6	100	3.000	96.4 96.6 95.2	96.1
200	6.000	92.6 99.2 100.0	97.3	200	6.000	91.3 99.1 99.5	96.6

Robustness

To determine the robustness of the method, the effect of different flow rates (0.8 – 1.2 ml/min), column temperature (28 – 32 °C), and filter (Nylon – PET – RC) were evaluated. The consistency between the results without changing the analysis conditions and the results found after the change was evaluated. For the method of RNZ impurities the effect of different wavelength (208 nm-212 nm) and filter (RC – PET) were evaluated. The obtained data of robustness studies summarized in Table 4 and 5.

Table 4. The summary of robustness studies for RNZ

Factor	Flow rate	Column Temperature ºC	Filter	RRT (min)	*Conformity %
Flow Rate	1.0 mg/ml	30	PET	4.204	-
	0.8 mg/ml			5.159	99.0
	1.2 mg/ml			3.464	99.3
Column Temperature	1.0 mg/ml	30	PET	4.204	-
		28		4.164	99.0
		32		4.151	99.3
Filter	1.0 mg/ml	30	PET	4.204	-
			Nylon		100.9
			RC		100.5

* conformity is a description of how the change rate fits with the routine of the study

Table 5. The summary of robustness studies for Zen-I, Zen-II and Zen-III

Factor	Flow rate (mg/ml)	Column Tempera-ture (ºC)	Filter	λ (nm)	Conformity Zen-I %*	Conformity Zen-II %*	Conformity Zen-III %*
Wavelength	1.0	30	PET	210	-	-	-
				208	100.8	98.6	102.0
				212	102.2	99.5	98.0
Filter	1.0	30	PET	210	-	-	-
			Nylon		100.1	101.9	101.8
			RC		100.0	100.0	100.0

* conformity is a description of how the change rate fits with the routine of the study

Precision

The precision of the method was evaluated at three levels: repeatability, intermediate precision and, reproducibility. To assess the reproducibility six separate sample solutions were run on HPLC. Two injections were made for each sample. To assess the repeatability of the method six separate standard solutions were run on HPLC. Field values were evaluated as a result of sequential injections. To evaluate the intermediate precision of the method, the samples identified in the method accuracy were prepared by a different analyst. The samples were injected into a different HPLC system. Quantity values were calculated for each sample. The RSD values obtained from twelve samples were evaluated. The low RSD values of the precision studies indicate that the method is precise[23]. As a System Suitability Parameters, the RSD value of the peak areas

obtained from six measurements of the standard solution should be a maximum of 5.0%. The results are given in the Table 6-8.

Table 6. Results of reproducibility studies for RNZ and its impurities Zen-I, Zen-II and Zen-III

Sample No	RNZ	Zen-I	Zen-II	Zen-III
	Result (%)	Recovery (%)	Recovery (%)	Recovery (%)
1	96.7	96.1	101.4	101.8
2	97.4	96.5	102.7	102.9
3	97.5	94.2	98.6	98.7
4	98.6	94.7	99.5	99.9
5	97.5	100.4	104.6	106.0
6	97.9	99.7	104.3	104.1
Mean	97.6	96.9	101.9	102.2
RSD %	0.7	2.7	2.4	2.6

Table 7. Results of repeatability studies for RNZ and its impurity standard

Sample No	RNZ	RNZ Impurity Standard
	Peak Area	Peak Area
1	5123875	115881
2	5096835	115681
3	5109472	115363
4	5092544	114367
5	5094318	115862
6	5109169	115198

Table 8. Results of intermediate precision studies for RNZ and its impurities Zen-I, Zen-II and Zen-III

Sample No	RNZ Result (%)		Zen-I Recovery (%)		Zen-II Recovery (%)		Zen-III Recovery (%)	
	1st analyst	2nd analyst	1st analyst	2nd analyst	1st analyst	2nd analyst	1st analyst	2nd analyst
	Instrument A	Instrument B	Instrument A	Instrument B	Instrument A	Instrument B	Instrument A	Instrument B
1	96.7	96.8	96.1	94.5	101.4	100.9	101.8	99.4
2	97.4	95.6	96.5	94.8	102.7	103.5	102.9	100.4
3	97.5	96.9	94.2	94.3	98.6	100.3	98.7	99.1
4	98.6	95.8	94.7	94.6	99.5	98.8	99.9	100.9
5	97.5	95.1	100.4	94.4	104.6	99.4	106.0	99.1
6	97.9	96.6	99.7	94.3	104.3	99.6	104.1	101.9
Mean	97.6	96.1	96.9	94.5	101.9	100.4	102.2	100.1
RSD %	0.7	1.0	2.7	0.2	2.4	1.7	2.6	1.1

Limit of quantification and detection

The LOD and LOQ concentrations were obtained from the linearity studies. It is found by calculating the signal to noise ratio (LOD: signal to noise ratio=3; LOQ: signal to noise ratio=10). Data obtained of LOD and LOQ for RNZ and its impurities Zen-I, Zen-II, and Zen-III were showed in Table 9.

Table 9. Results of LOD and LOQ studies for RNZ and its impurities Zen-I, Zen-II and Zen-III

Sample	LOD %	LOQ Mean Peak Area / RSD %	LOQ (mg/mL)	LOD (mg/mL)
RNZ	0.002	2357 / 5.6	0,0000399	0,000012
Zen-I	0.005	3607 / 2.9	0,0000960	0,000029
Zen-II	0.004	4753 / 3.1	0,0000717	0,000022
Zen-III	0.004	4619 / 1.8	0,0000722	0,000022

Forced degradation

RNZ ER tablets were exposed to heat, light, acid, base, and oxidation. Impurity analyses were performed before and after exposure. The analysis results were given in Table 10.

A comprehensive stability study was performed including forced degradation studies. A highly sensitive, simple HPLC-UV method developed and validated for the determination of ranolazine and its impurities. The method validation was performed in accordance with ICH guidelines. The highest degradation occurred in the H_2O_2 environment, indicating that Ranolazine is highly sensitive to the oxygen[24].

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REFERENCES

1. Dobesh, PP, Trujillo TC. Ranolazine: a new option in the management of chronic stable angina. Pharmacotherapy, **2007**; 27(12)**:** 1659-1676. doi: 10.1592/phco.27.12.1659.

2. Ranolazine, Medscape, 2016; Availiable from: https://reference.medscape.com/. Accessed on: 01.05.**2017**

3. Dobrev D, Nattel S. New antiarrhythmic drugs for treatment of atrial fibrillation, new drug class. Lancet. **2010**; 375: 1212- 1223.

4. Pubchem, Ranolazine chemical structure, 2020; Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Ranolazine. Accessed on: 22.09.2020

5. Moen AR, Karstad R, Anthonsen T. Chemo-Enzymatic synthesis of both enantiomers of the anti-anginal drug ranolazine. Biocatal Biotransfor, **2005**; 23(1): 45-51. https://doi.org/10.1080/10242420500067357

6. Jerling M. Clinical pharmacokinetics of ranolazine. Clin Pharmacokinet, **2006**; 45(5): 469-491. https://doi: 10.2165/00003088-200645050-00003.

7. Thireau J, Pasquie J, Martel E, Guennec J, Richard S. New drugs vs. old concepts: a fresh look at antiarrhythmics. Pharmacol Therapeut, **2011**; 132: 125-145. https://doi: 10.1016/j.pharmthera.2011.03.003.

8. Szél T, Koncz I, Jost N, Baczkó I, Husti Z, Virág L et al. Class I/B antiarrhythmic property of ranolazine, a novel antianginal agent, in dog and human cardiac preparations. Eur J Pharmacol, **2011**; 662 (1-3): 31-39. https://doi: 10.1016/j.ejphar.2011.04.042.

9. Ranexa™ Extended Release Tablets, FDA, Accessdata, Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/021526s029lbl.pdf . Accessed on: 05.05.2018.

10. Cattaneo M, Poretta AP, Gallino A. Ranolazine: Drug overview and possible role in primary microvascular angina management. Int J Cardiol, **2015**; 181: 376-381. https://doi.org/10.1016/j.ijcard.2014.12.055

11. Kosiborod M, Arnold SV, Spertus JA, McGuire DK, Li Y, Yue P et al. Evaluation of ranolazine in patients with type 2 diabetes mellitus and chronic stable angina. J Am Coll Cardiol, **2013**; 61: 2038 – 2045. https://doi: 10.1016/j.jacc.2013.02.011

12. Keating GM. Ranolazine: A Review of its use as add-on therapy in patients with chronic stable angina pectoris. Adis Drug Evaluation, **2013**; 73: 55-73. https://doi: 10.1007/s40265-012-0005-z.

13. Truffa AM, Newby LK, Melloni C. Extended-release ranolazine: critical evaluation of its use in stable angina. Vasc Health Risk Manag. **2011**; 7: 535-539. https://doi: 10.2147/VHRM.S15560

14. Tegk M, Mukkala BVP, Babu S. Formulation and evaluation of ranolazine extended release tablets: influence of polymers. Asian J Pharm Sci, **2011**; 5: 162-166.

15. Deepika D. Formulation and evaluation of film coated ranolazine sustained release matrix tablets. Int J Appl, **2015**; 2(5): 38-53.

16. Kumari MV, Reddy PV, Sudhakar M. Formulation and evaluation of sustained release matrix tablets of ranolazine. Int J Pharm, **2012**; 2(1): 224-229.

17. Bidada J, Gonjari I, Bhusari A, Raut C, Dhule A. Development of extended release matrix tablets of ranolazine containing polyacrylic and ethylcellulose polymers. Der Pharmacia Lettre. **2011**; 3(4): 215-226.

18. Asaduzzaman Rahman R, Khan SR, Islam A. Development of sustained release matrix tablet of ranolazine based on methocel K4M CR, in vitro drug release and kinetic approach. J. Appl Pharm Sci, **2011**; 01(08): 131-136.

19. Rahman M, Jha MK, Ahsan Q, Begum T. Effect of various grades of hydroxypropylmethylcellulose matrix systems as oral sustained release drug delivery systems for ranolazine. Journal of Pharmaceutics and Cosmetology, **2011**; 1(2): 82-92.

20. ICH Q2, Validation of Analytical Procedures: Text And Methodology, **2005**, Available from: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf. Accessed on: 01.06.2017.

21. ICH Q3B, Impurities in New Drug Products Q3B, 2006, Available from: https://database.ich.org/sites/default/files/Q3B%28R2%29%20Guideline.pdf. Accessed on: 01.07.2017.

22. Esim Ö, Gümüştaş M, Hasçiçek C, Özkan SA. A novel stability-indicating analytical method development for simultaneous determination of carboplatin and decitabine from nanoparticles. J Sep Sci, **2020**; 43(17): 3491-3498. https://doi.org/10.1002/jssc.202000320

23. Dinesh ND, Vishukumar BK, Nagaraja P, Made Gowda NM, Rangappa RK. Stability indicating RP-LC determination of sildenafil citrate (Viagra) in pure form and in pharmaceutical samples. J Pharm Biomed Anal, **2020**; 29(4): 743-748. https://doi: 10.1016/s0731-7085(02)00123-1

24. Kurmi M, Kumar S, Singh B, Singh S. Implementation of design of experiments for optimization of forced degradation conditions and development of a stability-indicating method for furosemide. J Pharm Biomed Anal, **2014**; 96: 135-143. https://doi: 10.1016/j.jpba.2014.03.035

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Effect of Seed Oil and Methanol Leaf Extract of *Dialium Guineense* Steud on Wound and Inflammation

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ABSTRACT

Dialium guineense native to the Sub-Saharan Africa serves as a good source of vitamins particularly Vitamin C for children, it is used in folklore medicine as treatment for cough, fever, stomachache, ulcer, wounds and in improving lactation in women. This study aims at investigating the anti-inflammatory and wound healing effects of the crude methanol extract, seed oil, ointments of the extract and seed oil. The phytochemical analysis of the leaf and seed of *Dialium guineense* were assayed for secondary metabolites. The egg-albumin method was adopted for the anti-inflammatory study while the excision wound model was used to determine the wound healing effect in rats. The presence of Anthraquinones, flavonoids, saponins and sterols were present in the leaf and seed of *Dialium guineense*. The leaf extract and seed oil of *Dialium guineense* exhibited anti-inflammatory effect which were not significantly different at the various doses tested (200 mg/kg, 100 mg/kg, 50 mg/kg). The ointment of the leaf extract as well as the seed oil exerted pronounced wound healing effect of 77.8% and 85.7% wound contraction respectively on day 19.The seed oil of *Dialium guineense* showed a faster wound healing effect than the leaf by creating occlusive effect on the wounds against moisture. Furthermore, the presence of phenolic compounds in the leaf and seed oil exerted a synergistic anti-inflammatory and wound healing effect.

Keywords: *Dialium guineense*, wound healing, anti-inflammatory, seed oil, leaf.

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INTRODUCTION

Dialium species of the family Fabaceae are common to the Sub-Saharan Africa, with about twenty-four species, five of these species are indigenous to the West African countries[1] of which *Dialium guineense* is one of them, it is commonly known as the velvet tamarind, black velvet or tumble tree, Awin (Yoruba), Icheku (Igbo), Tsamiyar Kurmi (Hausa)[2,3]. It grows to a height of about 30m and 0.8m in diameter[4].

In Nigeria, the fruits of *D. guineense* are abundant between the months of January and May [5]. These edible fruits are good source of protein, minerals, and Vitamin C [6].

In traditional medicine, various parts of *D. guineense* are used for treating diseases such as cough, fever, stomachache. The fruits are taken to improve lactation in women [2], treat malaria, jaundice, stomachache, ulcer, malnutrition, as well as in treating wounds, hemorrhoid [7] and in the prevention of cancer.

Several studies have reported the biological activities such as antidiarrhoeal , antimicrobial [8], antiulcerogenic , antibacterial [3,9,10,11] and anticancer activities [12] of *D. guineense*. In addition, some secondary metabolites have been identified in the leaf and stembark of *D. guineense,* these include tannins, alkaloids, flavonoids, saponins, sterolds and cardiac glycosides [13,14,15].

This study aims at investigating the anti-inflammatory and wound healing activities of the methanol leaf extract and seed oil of *Dialium guineense*.

METHODOLOGY

Materials

The leaf and seed of *Dialium guineense* were collected from growing tree in Ibadan and Sagamu, Oyo and Ogun States, respectively. The plant was authenticated at the Herbarium Department of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria where a voucher specimen was deposited.

Drying

The leaves were dried under shade while the seeds were removed from the pulp and air dried. The dried leaves and seeds were milled to powder and stored in containers for further use.

Extraction

300g of powdered leaf sample was extracted by cold maceration in 70% methanol for 72 hrs while 300g of the powdered seeds were macerated in Hexane for 72hrs. These were filtered separately, and the filtrates concentrated to dryness under reduced pressure in a Rotary evaporator.

Phytochemical Screening

The powdered leaf and seed of *D. guineense* were analysed for secondary metabolites [16,17].

Anti-inflammatory Activity

The anti-inflammatory activity of the leaf extract and seed oil were carried out in wistar rats using the egg albumin model [18]. Healthy Wister rats obtained from the Animal House of Babcock University Ilishan, Ogun State, Nigeria were used for the study. They were allowed to acclimatize and had access to water *ad libitum* and rats' pellets. The weights of the Wistar rats ranged from 160 g to 200 g.

The hind right paws of the animals were induced with 0.2 mL of fresh hen egg albumin and the diameter of the paws were taken and recorded as values for 0 min (T_o).

Table 1: Animal Grouping

Group A	Rats received 200 mg/kg of the methanol leaf extract
Group B	Rats received 100mg/kg of the methanol leaf extract
Group C	Rats received 50 mg/kg of the methanol leaf extract
Group D	Rats received I mL of seed oil
Group E	Rats received 0.5mL of seed oil
Group F	Rats received 0.25mL of seed oil
Group G	Rats received 5 mg/kg of Diclofenac sodium (Reference drug)
Group H	Rats received I mL of water only (Untreated Group).

Thirty minutes after administering extract, seed oil, Diclofenac and water to respective groups, orally, 0.2 mL of fresh egg albumin was injected into the right hind paw of each rats in the different groups. The linear circumference of the injected paws was measured and recorded immediately after injecting with egg albumin (0 hr); the diameter of the paw was then taken at an interval of 30 minutes for five hours.

The percentage inhibition of oedema was measured using the following formula

$$\% \text{ Inhibition of oedema} = \frac{Io - I_1}{Io} \times 100$$

Io – Initial paw circumference

I_1 – Change in paw circumference at time interval

Wound Healing

The wound healing effect of the methanol leaf extract and seed oil of *Dialium guineense* was carried out by the Excision wound model [19,20].

Excision Wound Model

The excision wound model was used to evaluate the wound healing activity of *Dialium guineense* leaf extract and seed oil. The wound excision was made through full thickness of the skin with sterile blade. The length and Circumference of the wounds were measured using a tracing paper on the wound and measuring the circumference with a meter ruler [21]. The length and circumference of the wound were taken on day 1, 3, 5, 9, 12, 15, 19.

The doses of the leaf extract and seed oil ointments were 2% w/w, 5% w/w, 10% w/w and 100% seed oil. Povidone iodine was used as standard drug, and these were applied topically on the animal wounds every day for nineteen days.

Simple Ointment

Simple ointment based on the British Pharmacopoeia (BP) was prepared using white soft paraffin, wool fat, cetostearyl alcohol, Hard paraffin and the leaf extract and seed oil of *D. guineense* [22,23].

Statistical Analysis

Data of the study are presented as Percentage, mean ± Standard error of mean (SEM) of sample size (n = 5). Mean values were compared statistically by one-way analysis of variance (ANOVA) followed by post hoc Turkey's test multiple comparison using Statistical package for Social Sciences (SPSS version 20). $P<0.05$

RESULTS and DISCUSSION

The skin serves as a medium between the internal and external environment, any disruption of the anatomy of the epidermis by physical, biological, and thermal stimuli leads to wound [24]. Inflammation is one of the processes of wound healing by protection against tissue damage through the elimination of pathogens and cell debris [25], however when the inflammation becomes severe, it leads to production of excess oxidative stress which acts as etiologic factor for chronic diseases[19]

Wound healing occurs through diverse phases which involves hemostasis, inflammation, proliferation, and remodeling of tissues. During these processes of healing, a wound matrix is involved followed by a breakdown and cleanup of tissues and pathogen debris.

The leaf extract ointment of *D. guineense* at 2%, 5% and 10% all produced healing effect on the wounds of the animals with percentage contraction of 77.8%, 60.8% and 66.1% respectively. Though there was no dose dependent effect observed, wound contractions became obvious from day 5. In addition, the healing effect of the different doses of the ointments incorporated with the leaf extract were not significantly different from that exhibited by the standard drug which had 66.7% contraction. (Table 2).

Table 2: Percentage wound contraction of *Dialium guineense* leaf and seed oil

Percentage wound contraction (%)							
Group (Treatment)	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 12**	**Day 15**	**Day 19**
2% leaf extract	9.3± 6.8	25.4 ±8.7	33.2 ±5.3	61.9 ±3.1	69.9 ±4.3	73.9± 3.9	77.8 ±3.4
5% leaf extract	15.0 ±4.9	25.3 ±7.6	35.6 ±7.6	45.6 ±5.5	54.6 ±3.4	57.4 ±2.9	60.8 ±3.0
10% leaf extract	5.9 ±2.9	16.5 ±7.9	28.8 ±5.0	48.0 ±3.1	55.5 ±2.0	58.5 ±2.0	66.1 ±2.4
2% oil extract	13.1 ±4.2	20.8 ±2.6	39.6 ±4.2	57.3 ±2.1	64.0 ±1.2	69.6 ±2.2	80.4 ±1.4
5% oil extract	9.6 ±3.9	18.4 ±6.5	29.0 ±2.3	48.3 ±2.3	58.6 ±1.5	62.4 ±2.3	70.7 ±0.5
10% oil extract	12.2 ±6.1	26.1 ±12.1	27.9 ±11.9	59.6 ±8.5	64.1 ±6.2	67.5 ±5.4	85.7 ±1.1
100% oil	5.5 ±1.4	18.8 ±3.8	25.7 ±1.5	46.9 ±5.3	56.9 ±4.5	60.8 ±5.1	74.5 ±5.6
Standard drug	5.6 ±1.2	8.7 ±2.0	19.6 ±1.8	32.3 ±0.4	46.2 ±1.8	51.8 ±2.2	66.7 ±0.0

$$\% \text{ wound contraction} = \frac{\text{Day 1 wound circumference - Day n wound circumference}}{\text{Day 1 wound circumference}} X100$$

The seed oil ointment at 2% and 10% exerted more pronounced healing effect of 80.4% and 85.7% contraction than those exhibited by the leaf extract (77.8% and 66.1% contraction) on Day 19. Though the seed oil alone healed the wound by 74.5% contraction, the effect was not as prominent as those of the seed oil ointments of 2% and 10%.

The ability of plant oils to act as a protective barrier to the skin when applied on the skin surface thereby making available the active ingredients present in the oil for the skin have been well documented. In addition, ointments incorporated

with plant oils are used in the treatment of wounds because of their ability to create an occlusive effect against moisture, hence the use of simple ointment incorporated with the leaf extract and seed oil of *Dialium guineense* in this study. Furthermore, the choice of use of ointment in this study is also because of the ability to sustain drug release at application site and as barrier for moisture [23].

The presence of tannins, terpenoids and flavonoids have been previously reported to play vital roles in wound healing [26,27] by affecting one or more phases of wound healing process [28]. The presence of these secondary metabolites in the leaf extract and seed of *Dialium guineense* are probably responsible for their ability to heal the wounds by increasing the wound contraction through the activation of fibroblasts, stimulation of collagen deposit, a process assisted by phenolic constituents such as those detected in the leaf and seed of *D. guineense*. These phenolic compounds also aid wound healing by collagen formation, wound closure, and epithelialization due to the anti-inflammatory, antibacterial and antioxidant properties which they possess [24]

The phytochemical analysis of *Dialium guineense* in this study revealed the presence of free and combined Anthraquinones, saponins, Tannins in high quantities, Alkaloids and sterols in moderate quantities, trace of flavonoids and absence of cardiac glycosides.

Table 3: Phytochemical Analysis of Leaf and Seed of *Dialium guineense*

Morphological part	Anthraquinone		Flavonoids	Saponins	Tannins	Alkaloids	Sterols	Cardiac glycosides
	Free	Combined						
Leaf	+++	+++	+	+++	+++	++	++	-
Seed	+	+	+	++	-	-	+	+

+++ = Highly Present; ++ = moderately present; += Present; - = Absent.

In previous studies, the presence of tannins, alkaloids, flavonoids, saponins, steroids and cardiac glycosides in the leaf and stembark of *D. guineense* were reported [13,14]. Furthermore, the fruit coat extract has been shown to possess alkaloids and saponins in high concentration, flavonoids and steroids in trace quantities[29] while, the presence of anthraquinone, alkaloids, flavonoids, tannins and saponins [30] were moderately present.

The variations in the secondary metabolites present in the different morphological parts of *D. guineense* could be due to diverse factors relating to the environment, time of the day and season of collection. In addition, this could also be responsible for the different biological activities reported in the morphological parts of *Dialium guineense*.

In this study, the egg albumin induced assay was used to induce inflammation in the animals. The egg albumin causes inflammation by the release of histamine and serotonin during the second phase of inflammation thereby causing vasodilation and increased permeability.

The leaf extract of *D. guineense* exhibited mild anti-inflammatory effect while, the seed oil of *D. guineense* exerted time -dependent anti-inflammatory activities which was more pronounced than the effect of the standard drug. At a dose of 1 mL and 0.5 mL the effect shown were not significantly different from each other over the test period. Figure 1 and 2.

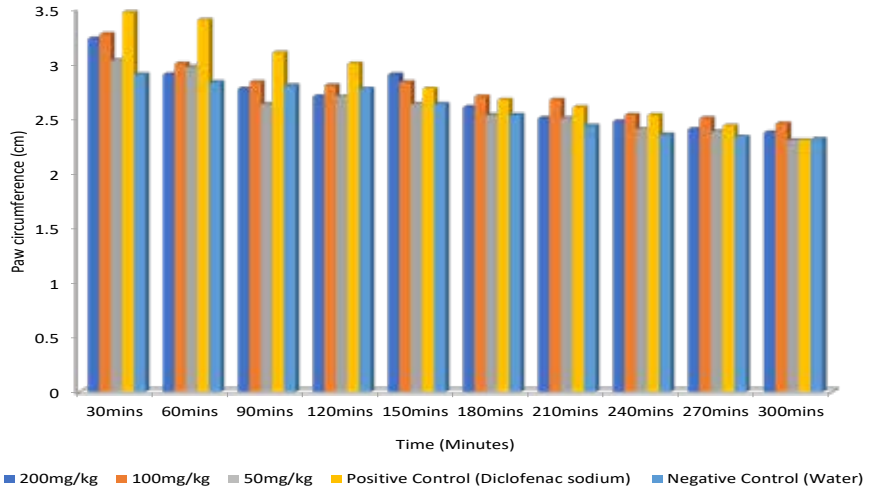

Figure 1: Anti-Inflammatory Effects of *Dialium guineense* Methanol Leaf Extract.

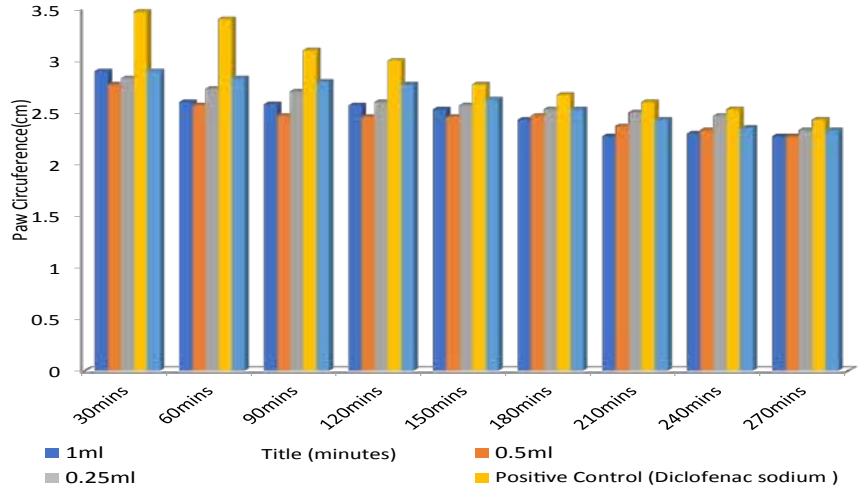

Figure 2: Anti-Inflammatory effects of *Dialium guineense* Seed Oil.

The presence of anthraquinones, flavonoids and sterols in the leaf extract and seed oil of *D. guineense* had a culminative effect which helped in wound healing and anti-inflammatory activity in the test animals. Tannins present in high concentration in the leaf could act by detoxifying as well as inhibit microbial growth [26]. The sterols present both in the leaf extract and seed oil have astringent properties while flavonoids have potency in acting as free scavengers as well as prevent prostaglandin synthesis a major factor responsible for the second phase of inflammation.

Furthermore, these phenolic compounds in the leaf and seed oil of *D. guineense* are also able to exert wound healing and anti-inflammatory effect by synergy thereby aiding cell migration, the proliferation of cells, fibroblasts, keratocytes, tissue repairs, thereby promoting blood vessel contraction, collage deposit, modulating the production of reactive oxidative stress, chelating free radicals as well as inhibiting the production of nitric oxide at wound site.

In previous study, the wound healing effect of the Dichloromethane fraction of the fruit coat of *Dialium guineense* have been reported [28]. This study further confirms and shows the ability of the phytoconstitutents in *D. guineense* to heal wounds as well as reduce inflammation through possible diverse mechanism of actions. One of which is possible by suppressing or inhibiting the release of histamine, serotonin and prostalglandin. This study therefore has been able to justify the use of *Dialium guineense* in folklore medicine as an agent of wound healing.

STATEMENT OF ETHICS

Approval was obtained from the Animal Ethical Committee (OOU/PCG/AEC/2020001) and animals were handled according to the National Institutes of Health guide for the care and use of Laboratory Animals

CONFLICT OF INTEREST

The Authors declare that there is no Conflict of Interest.

The Authors declare they have no competing Interests.

FUNDING SOURCE

This study did not receive financial support towards this study.

AUTHORS CONTRIBUTION

Adediwura Fred-Jaiyesimi conceived the study, concept, and design, Oluwakemi Ogunleye and Mary Adesina conducted most of the Laboratory experiments, Peter Segun, Modupe Adebowale and Katherine Olufolabo contributed to the proposal. Adediwura Fred-Jaiyesimi and Modupe Adebowale contributed to the supervision of the study. All authors read and approved the final manuscript.

REFERENCES

1. Besong EE, Balogun ME, Djobissie SFA, Obu DC, Obimma JN. Medicinal and Economic value of Dialium guineense. Afr J Biomed Res. **2016**; 19: 163 – 170.

2. Nwosu MO. Plant resources used by traditional woman as herbal cosmetics in South West Nigeria. Arzte fur natur Fahr. **2000**; 41: 760 - 767.

3. Akinpelu, AD, Awoterebo TO, Agunbiade OM, Aiyegoro AO, Okoh IA. Antivibrio and preliminary phytochemical characteristics of crude methanolic extract of the leaves of Dialum guineense (Wild). J Med Plt Res. **2011**; 511: 2398 – 2404.

4. Hutchinson J, Dalziel MJ. Flora of West Tropical Africa. 2nd edition. Milbank, London: Crown Agents for Overseas Governments and Administration. Millbank; **1958**. 567-569 p.

5. Keay RWJ. Trees of Nigeria. London: Oxford Clarendon Press; **1989**, pp476.

6. Arogba SS, Ajiboro A, Odukwe I. A physico-chemical study of Nigeria velvet tamarind (Dialium guineense L) fruit. J Sci Food and Agric. **2006**; 66: 533 – 534.

7. Bero J, Ganfen H, Jo ville MC, Frederich M, Gbaguidi F, De MP. In vitro antiplasmodial activity of plants used in traditional medicine to treat malaria. J Ethnopharmacol. **2009**; 122: 439 – 444.

8. Odukoya OA, Houghton PJ, Adelusi A, Omogbai EKI, Sanderson L, Whitfield PJ. Molluscicidal triterpenoid glycosides of Dialium guineense. J Nat Prod. **1996**; 59: 632 – 634.

9. Oyegoke RA, Oladiji AT. Antiulcerogenic Activity of Dialium guineense Fruit pulp meal-based diet in Aspirin-induced ulcerogenic rats. Nig J Biochem Mol Bio. **2014**; 29: 77 – 93.

10. Ezeja MI, Omeh YS, Ezeigbo A, Ikechukwu A. Evaluation of the analgesic activity of the methanolic extract of Dialium guineense (Wild). Ann Med Health Sci Res. **2011**; 1: 55 – 62

11. Orji JO, Alo MN, Anyim C, Okonkwo EC. Antibacterial activities of crude leaf and bark extracts of "icheku" Dialium guineense on bacterial isolates from bronchitis patients. IOSR J Pharm Biol Sci. **2012**; 1: 21 – 25.

12. Balogun ME, Oji JO, Besong EE, Umahi GO. Evaluation of the anti-ulcer properties of aqueous leaf extract of Dialium guineense (Velvet tamarind) on experimentally induced ulcer models rats. Int J Dev Res. **2013**; 3: 106-110.

13. David AA, Olaniyi AT, Mayowa AO, Olayinka AA, Okoh IA. Anti-vibrio and preliminary phytochemical characteristics of crude methanolic extracts of the leaves of Dialium guineense (Wild). J Med Plants Rev. **2011**; 5: 2398 – 2404.

14. Ogu GI, Amiebenomo AR. Phytochemical analysis and in vivo antidiarrheal potential of Dialium guineense (Wild) stem bark extract. J Intercult Ethnopharmacol. **2012**; 1: 105 – 110.

15. Ajiboye AE, Ameen MT, Adedayo MR. Antimicrobial activity and phytochemical screening of the fruit pulp of Dialium guineense (Velvet Tamarind) on some microbial isolates. J Microbiol Antimicrob. **2015**; 7: 33 – 41.

16. Harborne JA. Phytochemical Methods A guide to modern Techniques of plant Analysis. 3rd ed. London: Chapman and Hall; **1998**. 15-36.

17. Evans WC. Trease and Evans' Pharmacognosy. 15th ed. London: J and A Churchill Ltd; **1996**. 234-492 p.

18. Sindhu RK, Sood N, Puri V, Arora S. Various Animal Models for Preclinical Testing of Antiinflammatory Agents. Inter J Pharm Sci Res. **2017**, 8: 1550 – 1557.

19. Kumar N, Gupta AK. Wound-healing activity of Onosma hispidum (Ratanjot) in normal

and diabetic rats. J Herbs Spices Med Plt. **2010**; 15: 342-351.

20. Demilew W, Adinew GM, Asrade S. Evaluation of the wound healing activity of the crude extract of leaves of Acanthus polystachyus Delile (Aanthanceae). Evidence Based Complementary and Alternative Medicine. **2018**; 9.

21. Kokane DD, More RY, Kale MB, Nehese MN, Mehendale PC, Gadgoli G. Evaluation of wound healing activity of root of Mimosa Pudica J Ethnopharmacol. **2009**; 124: 311 – 315.

22. British Pharmacopoeia (BP). Department of health and social security Scottish home and health Department office of the British Pharmacopoeia Commission, UK. **1988**; 2: 713.

23. Ansel H, Popovich N. Preparation of Topical Dosage forms. Introduction to Pharmaceuical Dosage forms. 4th edition. Philadelphia, USA: Lea & Febiger; **1985**.

24. Yadav E, Singh D, Yadav P, Verma A. Antioxidant and anti-inflammatory properties of Prosporis cineria based phenolic rich ointment in wound healing. Biomed Pharmacother. **2018**; 108: 1572 – 1583.

25. Yadav E, Singh D, Yadav P, Verma A. Ameliorative effect of biofabricated ZnO nanoparticles of Trianthema portulacastrum Linn. on dermal wounds via removal of oxidative stress and inflammation. RSC Adv. **2018**; 8: 21621 – 21635.

26. Kipngeno CD, Mshimba SM, Gilbert C. Antimicrobial activity and phytochemical investigation of crude extracts of the fruits of Solanum incarnum (Solanaceae) and Dovgalis abbysimica (Flacourtiaceae). Sci J Microbiol. **2014**; 1 – 4.

27. Agra LC, Ferro JNS, Barbosa FT, Barreto E. Triterpenes with healing activity. A systematic Review. J Dermatol Treat. **2015**; 26: 465 – 470.

28. Premarathnaa AD, Ranahewaa TH, Wijesekerab SK, Wijesundaraa RRMKK, Jayasooriyac AP, Wijewardanaa V, et al. Wound healing properties of aqueous extracts of Sargassum illicifolium: An in vitro assay. Wound Med. **2019**; 24: 1-7

29. Okeke NC, Udeani TKC, Onyebuchi UL. Wound – healing and Antimicrobial properties of dichloromethane fraction of Dialium guineense (Wild) fruit coat. Res Pharm Sci. **2016**; 11: 219 – 226

30. Olajubu FA, Akpan I, Ojo DA, Oluwalana SA. Antimicrobial potential of Dialium guineense (Wild) stem bark on some clinical isolates in Nigeria. Int J Appl Basic Med Res. **2012**; 2: 58 – 62.

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Effect of Ethanol Extract of *Irvingia Gabonensis* (Aubrey Lecomte Ex O. Rorke) Baill. (Irvingiaceae) Seeds on Diet Induced Obesity in Wistar Rats

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ABSTRACT

Obesity is a chronic condition associated with high morbidity and mortality rates. Its comorbidities include cancer, diabetes mellitus, and cardiovascular diseases. Traditional medicines have found increasing use due to cost effectiveness and minimal side effects. This study evaluated the Anti-obesity effect of ethanolic extract of *Irvingia gabonensis* seeds.

Wistar rats were divided into control and treatment groups. Untreated group I on normal diet (negative control), untreated group II on high fat diet (HFD) and 10 mL/Kg Tween 80 (positive control), extract treated groups III-V (50, 100 and 200 mg/Kg) on HFD. Orlistat treated group VI (200 mg/Kg) on HFD (standard). Body mass index (BMI), weight of abdominal fat, cholesterol levels and hepatic enzymes were evaluated after 12 days of treatment.

Body mass index (BMI) and abdominal fat of (treatment groups) reduced. However, the seed extract and orlistat did not significantly increase liver enzymes. Conclusion: *Irvingia gabonensis* seed extract showed anti-obesity properties.

Keywords: Obesity, Body mass index, lipid profile, *Irvingia gabonensis* ethanolic extract, High fat diet.

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INTRODUCTION

Obesity is a pathological condition in which excess body fat is accumulated and resulting in adverse effects on health and life expectancy. It is a chronic disorder involving a complex interaction between genetic and environmental factors [1]. Obesity normally occurs when energy intake exceeds energy expenditure. It is a complex health issue resulting from a combination of causes and individual factors such as behavior and genetics. Behaviors can include physical activity, inactivity, dietary patterns, medication use, and other exposures. Additional contributing factors include the food and physical activity environment, education and skills, and food marketing and promotion.[2]

Medicinal plant preparations containing naturally active ingredients have been used either to enhance satiety or boost metabolism of high energy dense food to speed up weight loss [3]. Anti-obesity mechanisms for herbal plants included reduction in lipid absorption, reduced energy intake, increased energy expenditure, decreased pre-adipocyte differentiation and proliferation, or decreased lipogenesis and increased lipolysis[4]. Adipose tissue as an endocrine organ contributes in the mechanisms of obesity by secreting cytokines like interleukin 6 (IL-6) and Tumor necrosis factor alpha (TNFα): which therefore could regard obesity as a chronic inflammatory disease[5]. The physiological mechanism of high-fat diet induced obesity is related to the energy derived from fat which has a greater effect on body weight gain than the energy from non-fat diet [6]. Another mechanism involved in obesity is in hyperleptinemia and leptin resistance on high fat diet which involved the hypothalamic leptin receptors and signaling pathways[7]. Another contributory factor to obesity is stress; long term stress is associated with increase food intake and thus promotes fat and weight gain in human subjects [8].

Currently, 80% of the world population use plant-derived drugs as the first-line in treatment of obesity because of their efficacy and minimally tolerable side effects[9]. *Allium cepa* (Onion; Family: Amaryllidaceae), *Trigonella foenum-graecum* (Fenugreek; Family: Fabaceae) *Cyamopsis tetragonoloba* (Guar; Family: Leguminoceae) and *Hibiscus cannabinus* (Kenaf; Family: Malvaceae) are examples of plants with proven antiobesity activity[10]. Some families of plants with anti-obesity effects include: Apocynaceae, Rutaceae, Leguminosae, Malvaceae, Moraceae, Compositae, Rubiaceae and Zingiberaceae [11].

The Irvingiaceae family is a small woody family that consists of 10 species in three genera. The genera include *Desbordesia* Pierre ex Tiegh(one species), *Klainedoxa* Pierre ex Engl. (two species) and *Irvingia* Hook. f. (seven species) which are distributed in central and west Africa except *Irvingia malayana* Oliv., which is found in southeast Asia. The family was formerly placed in the

Simaroubaceae family or as a distinct family in the Rutales. Later on, the Angiosperm Phylogeny group placed it in the Malpighiales on the basis of molecular evidence[8]. To gain insight into family relationships, 63 embryological characters of two previously unstudied African species, *Irvingia gabonensis* and *I. smithii*, were investigated by Tobe and Raven in 2011 and were compared to other Malpighiales and the sister group Oxalidales. Embryologically, *Irvingia* was found to be characterized by the absence of an integumentary tapetum and by having a non-multiplicative inner integument, a multiplicative testa, many discrete fascicles of vascular bundles running in the testa from the raphe to antiraphe, and a fibrous exotegmen. Comparisons showed that *Irvingia* did not resemble any of the Linaceae, Caryocaraceae, Erythroxylaceae, Rhizophoraceae, or any of the other malpighialean families. The genus rather resembled Huaceae and Connaraceae (Oxalidales) in seed coat structure. It has been reported to have a number of beneficial medicinal effects such on gastrointestinal discomfort and diabetes [12]. It is also known to have analgesic and antioxidant properties[13].

Irvingia gabonensis (gabonensis) (Aubrey Lecomte ex O. Rorke) Baill. is a sweet variety which is a large tree that grows up to 15-40 meters and a diameter of about 120 cm. Its trunk is slightly buttressed; it has a dense, compact crown, branchlets ending in a narrow, curved, stipular sheath covering the leaf bud. Leaves are elliptic or slightly obovate, acute to acuminate and cuneate. They are 5-15x2.5-6 cm in size and usually have 5- 10 pairs of irregular lateral veins. The leaves are dark green in color and leathery and glossy. Flowers are yellow or greenish-white in color. They are slender; they have racemes which are slender or small panicles above the leaves or on the branches. Their flower stalk is slender and about 6mm long. Fruits are yellow in color when ripe, broadly ellipsoid and are variable in size. They are 5-7.5 cm in size with a yellow, fibrous pulp surrounding its large seed[14]. The seed has medicinal, nutritional and industrial uses and very rich in fats, proteins and in lipids than other oil seeds and legumes such as groundnut, melon, soybeans and cotton seed. The seeds are a source of human food and constitute important part of the diet in most parts of Nigeria. The seeds are ground and used as thickening agents in soups. Other parts of the tree, like the bark and the leaves have been used in gastrointestinal conditions as laxatives and its oil processed into soap or pharmaceuticals [15].

The nutritional analysis of *I. gabonensis* (gabonensis) shows that its seeds contain 3.36% m moisture, 7.70% crude protein, 65.46% crude fat, 2.26% mineral ash, 10.23% crude fiber and 10.93% carbohydrate. The pulp contains 80% moisture, 1.09% crude protein, 1.06% crude fat, 0.8% mineral ash, 0.4% crude fiber and 10.7% carbohydrate. The physicochemical evaluation of the pulp contains 0.21% water soluble ash, 459.7 mg/100 mL reducing sugars, 49.1% non-

reducing sugars, 0.112 cm^3 titratable acidity, 10.0 (Brix) soluble solids, 10.0% total solids, 1.012 specific gravity and 1.3355 refractive index [16]. The phytochemical analysis of the seeds of *I. gabonensis* (gabonensis) revealed the presence of alkaloids, flavonoids, tannin, volatile oils, saponins, terpenoids, carbohydrate and cardiac glycosides but no resins were found [17].

Ethnopharmacology uses of *I. gabonensis* (gabonensis) include as antifertility, antimicrobial and antidiabetic agents. This *in vivo* study sought to compare the effect of *Irvingia gabonensis* seed and Orlistat (Reference standard) on diet induced obesity as can be adaptable in humans.

METHODOLOGY

Experimental animals

Male Wistar rats weighing (60 - 70 g) were used in the study. They were purchased from the Central Animal House, University of Ibadan and housed in plastic cages at room temperature and had free access to rodent pellet diet and water *ad libitum*. The animals were allowed to acclimatize for one week before the experiment. All rats were handled in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and OECD guidelines for testing of chemicals (NIH publication #85-23, 1985).

The experimental protocol was in conformity with the Ethics Committee Guidelines of the University of Ibadan with ethical approval number (UI-ACUREC/17/0105) as well as the US guidelines of internationally accepted principles for laboratory animal use and care.

Plant materials

Fresh seeds of *Irvingia gabonensis* (gabonensis) were obtained from the Bodija market, Ibadan, Oyo State, Nigeria. The seeds were authenticated by Mr. Adeyemo of the Forestry Research Institute of Nigeria, Ibadan with the voucher specimen number, FHI: 111042.

Extraction

The seeds of *I. gabonensis* (gabonensis) were broken into smaller pieces. They were then placed in an oven at a temperature of 36°C and 40°C to dry. After a few hours in the oven, a blender was used to grind the seed pieces to powder which was then poured into a macerating jar. The ethanol extract of *I. gabonensis* (gabonensis) was obtained by first defatting it in distilled *n*-hexane twice. The marc from the seeds was filtered and air- dried and was macerated in 70% distilled ethanol at room temperature for 72 h. The extract was filtered and concentrated using a rotary evaporator at 40°C.

Experimental Design and Drug Treatment

Thirty rats were randomly divided into six groups of five animals each (n=5). Group I consisted of normal diet control rats (ND), group II consisted of the untreated high fat diet (HFD) fed rats treated with 10 mL/Kg vehicle (Tween 80), groups III-V consisted of rats fed with high fat diet but treated with 50, 100 and 200 mg/kg ethanol extract of *I. gabonensis* (gabonensis) seed (EIGS). Group VI also consist of rats fed with high fat diet but treated with 200 mg/kg orlistat. The rats were fed till their BMI was above 310, for twelve days before treatment. Body mass index was calculated using the Lee's index formula:

$$BMI = \frac{\sqrt[3]{\text{weight}}(g)}{Length\ (cm)} \times 1000$$

The length was measured from the nose to the anus (naso-anal) for each animal after every 3 days during treatment. Animals were sacrificed 24 h after the last treatment. Abdominal fats and blood (through cac puncture) were collected for lipid profile and biochemical analysis.

Biochemical parameters and lipid profile

Blood samples were collected in heparinized tubes and the blood plasma was obtained by centrifugation at 1000 rotations per minute (rpm) for 15 minutes. The lipid profiles, Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were evaluated using the Randox® kits.

Statistical Analysis

Data were analysed by using GraphPad Prism software version 7 and were presented as mean ± standard error of mean (SEM). Statistical analysis of data was carried out using one-way and two-way Analysis of Variance (ANOVA), followed by Dunnet's test for comparison between groups. p values less than 0.05 ($p< 0.05$) were regarded as statistically significant.

RESULT and DISCUSSION

Obesity is a chronic disorder characterized by increased concentration of various forms of lipids and increased fat deposition. This is mostly due to an imbalance consumption and expenditure of energy resulting in an increase in the number of adipocytes [18].

A number of health issues particular reno-cardiovascular among others have been associated with obesity [19]. In recent past, a number of drugs have been designed to correct the imbalance in energy intake and expenditure, but most

of these drugs despite their efficacy have limited clinical use because of the severity of their side effects and exorbitant cost [20]. Thus, this study investigated the anti-obesity activity and probable mechanism of *Irvingia gabonensis* seed extract by evaluating its effect on lipid profile and biochemical parameters.

The decreased deposition of fat in the visceral region i.e. perianal and perirenal indicates antiobesity activity of compounds [21]. *I. gabonensis* (50-200 mg/kg) decreased the body mass index and abdominal fat deposition in high fat diet-induced obese rats.

High fat diet induces obesity by significantly increasing the intake of energy and causing a magnified imbalance in energy expenditure. One of pharmacological approach to treatment of obesity is by blocking fat uptake, and newer approaches have focused on control of energy balance[22]. High fat diet induce obesity by increasing level of total cholesterol, triglyceride, and LDL in serum, demonstrating development of hyperlipidemia.

Irvingia gabonensis seed fiber might bind to bile acids in the gut and transport them out of the body through faeces. This might induce the body to convert more cholesterol into bile acids hence reducing cholesterol levels [23].

Irvingia gabonensis seed extract significantly reduced cholesterol (at 50 and 100 mg/kg; 69.6, 73.2 vs. 77.2), triglyceride (at 50 mg/kg; 54.6 vs. 58.8), and LDL (at 50, 100 and 200 mg/kg; 21.0, 20.8, 21.0 vs. 25.2) compared to HFD group. The result suggests that *I. gabonensis* might possess compounds or active principles which might be useful in preventing the adverse drug effect associated hyperlipidemia. Orlistat, the standard anti-obesity drug used in the study is a pancreatic lipase inhibitor which suppress food intake (hyperphagia) via inhibiting re-uptake of 5-Hydroxytryptamine (5HT) at hypothalamic site in the central nervous which regulates appetite [24]. Although the interaction of *I. gabonensis* with monoaminergic transmitters and its endocrine effect particularly on ghrelin were not established in the study, further works still remain to be done in this regard.

The BMI of the rats fed with high fat diets range between 311.6±2.2 and 329.8±2.3 calculated as Lee's index. The administration of IBG (50, 100, 200 mg/kg) and orlistat (200 mg/kg) significantly [$F(5, 24) = 19.73$; $p < 0.0001$] reduced the BMI of treated rats (287.6±4.4, 297.6±2.4, 300.2±3.5 and 288.7±3.6 vs 324.4±5.4) after twelve days of treatment when compared with HFD (Figure 1).

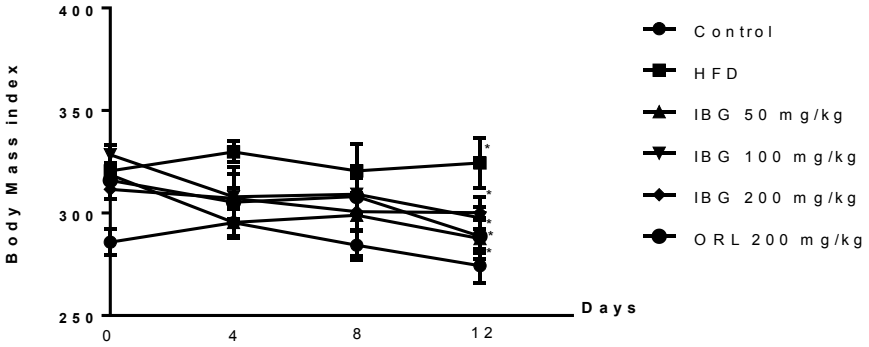

Figure 1: Effect of Ethanol Extract *Irvingia gabonensis* (IBG) on Body Mass Index (BMI)

All values are expressed mean ± SEM. Data were analyzed using two way ANOVA for multiple comparison and Dunnett's post-hoc test. *= significant at $p<0.05$ in comparison with the group of rats fed with HFD.

Body mass index (BMI) of the rats decreased over time during treatment with *I. gabonensis*, thereby establishing that the plant is beneficial in management of obesity. This was observed from the decreasing Lee's index values from the result as BMI is directly proportional to Lee's index [25]. The measurement of Lee's index is to determine obesity level in the rodent models. This agrees with a similar study carried out which revealed that *I. gabonensis* plant possess hypolipidemic effect [26].

The anti-obesity activities of *I. gabonensis* extract might be due to synergistic actions of secondary metabolites present. The presence of alkaloids, flavonoids, saponins, tannins and phenols has been reported to contribute to anti-obesity effects [27]. Flavonoids act by activating β-adrenergic receptors involved in the burning of fats by exhibiting PPAR-γ ligand binding activity, similar to PPAR-γ agonists [28]. Similarly, flavonoids and phenols have also been reported to function as antioxidants, thus preventing obesity by modulating oxidative stresses in the body [29]. In many other studies, alkaloids present in plant extracts significantly reduce the expression levels of several adipocyte marker genes including enhancer binding proteins and proliferator activated receptor hence inhibiting adipogenesis [30].

The administration of IBG (200 mg/kg) and orlistat (200 mg/kg) significantly [$F(5, 24) = 39.57$; $p < 0.0001$] reduced the abdominal fat of the animals (5.8±0.4, 3.4±0.1 vs 6.8±0.2) when compared with the group fed with HFD. At 50 and 100 mg/kg, IBG did not significantly reduce the abdominal fat.

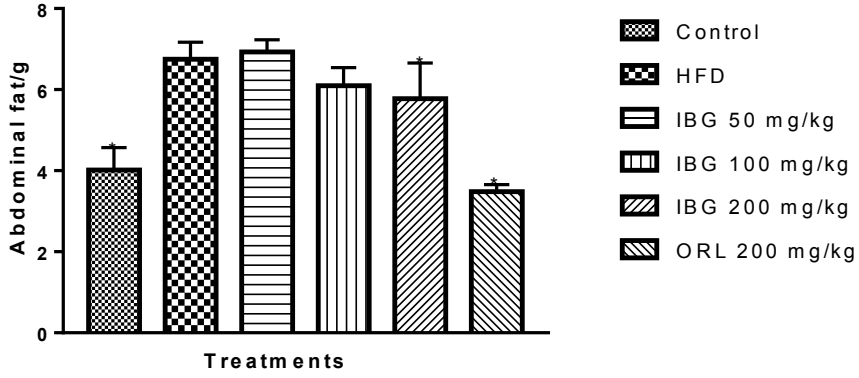

Figure 2: Effect of IBG on abdominal fat

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test. *= significant at $p<0.05$ in comparison with the group of rats fed with HFD.

The doses of IBG used in the study did not significantly reduce the level of cholesterol in the serum. The orlistat (200 mg/kg) did not significantly reduce cholesterol in serum (Figure 3).

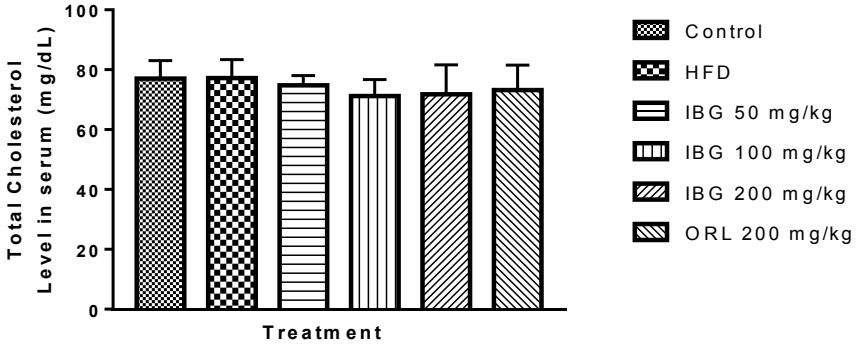

Figure 3: Effect of IBG on total cholesterol in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

*= significant at $p<0.05$ in comparison with the group of rats fed with HFD.

The doses of IBG used in the study did not significantly reduce the level of triglyceride in the serum. The orlistat (200 mg/kg) did not significantly reduce triglyceride in serum (Figure 4).

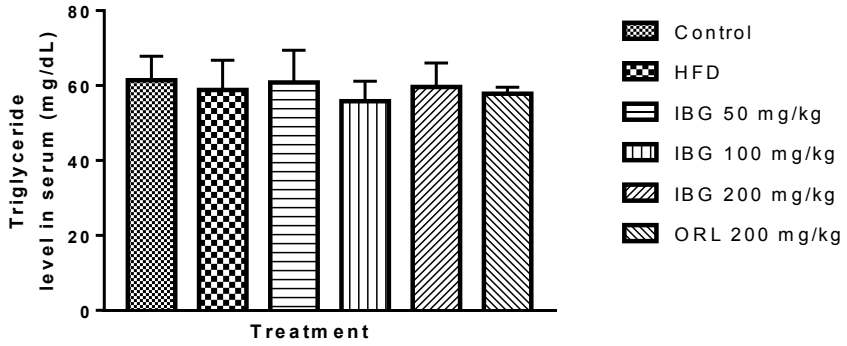

Figure 4: Effect of IBG on triglyceride in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

*= significant at $p<0.05$ in comparison with the group of rats fed with HFD.

The doses of IBG used in the study did not significantly reduce the level of HDL in the serum. Orlistat (200 mg/kg) did not also significantly reduce HDL in serum (Figure 5).

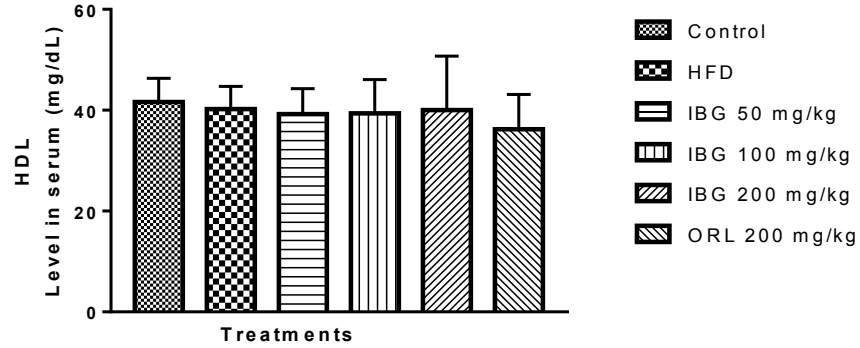

Figure 5: Effect of IBG on High Density Lipoproteins (HDL) in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

*= significant at $p<0.05$ in comparison with the group of rats fed with HFD.

The administration of IBG (200 mg/kg) significantly reduced the level of low density lipoproteins (LDL) in the serum. Orlistat (200 mg/kg) did not significantly reduce LDL in serum (Figure 6).

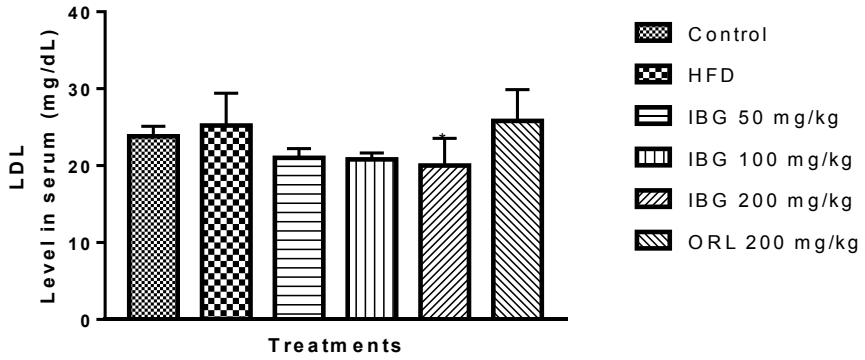

Figure 6: Effect of IBG on Low density lipoprotein (LDL) in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.*$p<0.05$ in comparison with the group of rats fed with HFD.

The administration of IBG did not significantly reduce the level of Very low density lipoproteins (VLDL) in the serum. Orlistat (200 mg/kg) did not significantly reduce VLDL in serum (Figure 7).

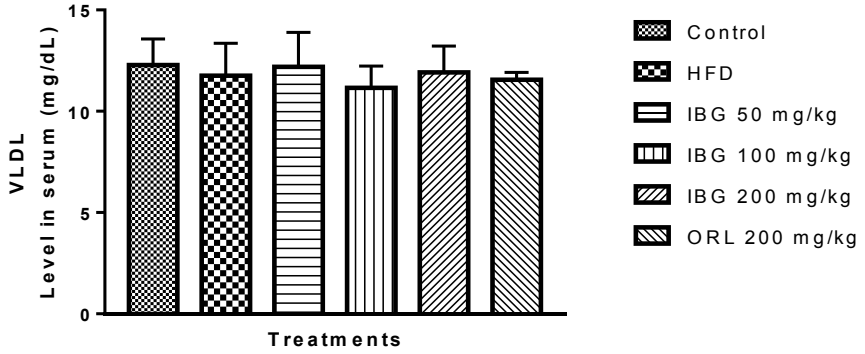

Figure 7: Effect of IBG on VLDL in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

It is known that plasma AST and ALT levels are the most reliable laboratory indicators of hepatotoxic effects [31]. The normal ALT level for rats is between 10 to 40 units per liter (U/L) and that of AST for rats is 50 to150 U/L [32]. The ALT, AST, and creatinine levels in serum of animals treated with *I. gabonensis,*

revealed no detectable adverse toxic effects as they were comparable to levels in reference Orlistat treated animals. The ALT values for the rats treated with IBG 50 – 200 mg/kg ranged from 26 - 28 U/L and the ALT value of the rats treated with orlistat 200 mg/kg was 27 U/L. The ALT value for the HFD control group was 26 U/L. Also, the AST values of the rats treated with IBG 50 - 200 mg/kg ranged from 38-39 U/L and the AST value of the rats treated with Orlistat was 37 U/L. The AST value for the HFD control group is 38 U/L. This shows that the ALT values were normal for all the treated rats and the AST values were below the normal range. Also, there was no increase in AST and ALT levels of EIGS treated rats compared to the HFD control group. Hence, the usage of EIGS is shown to be safe as it has no hepatotoxic effect with short term use. Creatinine level is used to check the kidney function. Normal creatinine levels in rats range from 0.5 to 2.2 mg/dL. The creatinine values for animals treated with EIGS ranged from 0.52-0.56 mg/dL vs 0.56 mg/dL for the HFD control group. Hence, EIGS is shown to have no toxic effect on the kidney.

The doses of IBG used in the study did not significantly increase the level of glucose in the serum. Also the orlistat (200 mg/kg) did not significantly increase glucose level in serum (Figure 8).

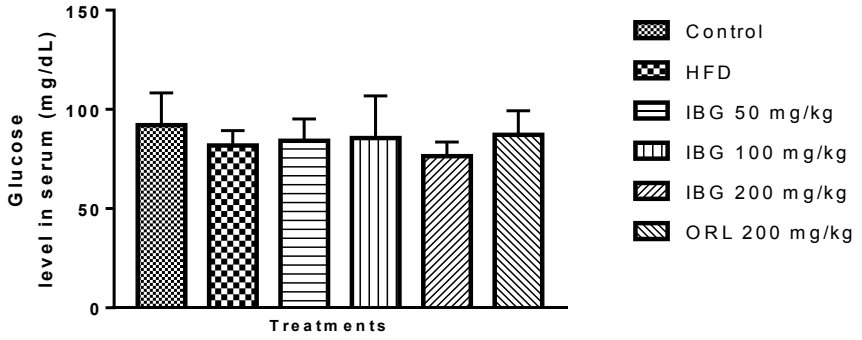

Figure 8: Effect of IBG on glucose in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

Also, *Irvingia gabonensis* seeds might delay stomach emptying which leading to a more gradual absorption of dietary sugar and this effect reduces the raise in blood glucose that is typical after a meal [23]. EIGS at 50, 100 and 200 mg/kg revealed no significant change in the glucose level of the rats compared to the HFD group.

The doses of IBG used in the study did not significantly increase the level of ALT and AST in the serum. Also the orlistat (200 mg/kg) did not significantly increase ALT and AST in serum (Figure 9).

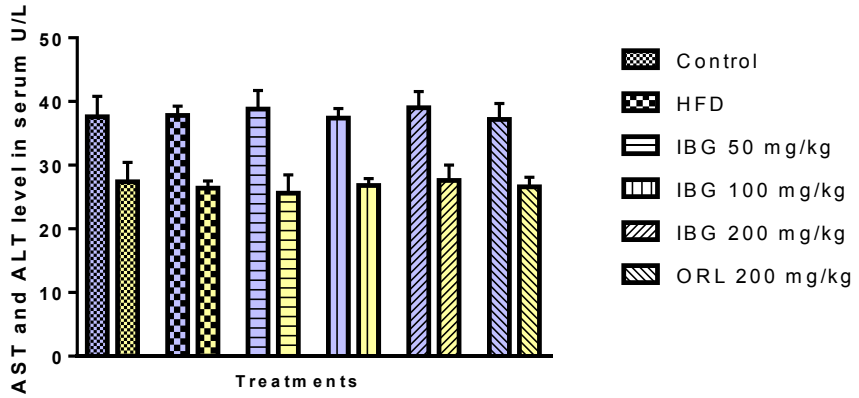

Figure 9: Effect of IBG on AST and ALT in serum

All values are expressed mean ± SEM. Data were analyzed using one way ANOVA for multiple comparison and Dunnett's post-hoc test.

AST = Aspartate aminotransferase (blue bar)

ALT = Alanine aminotransferase (yellow bar)

The doses of IBG used in the study did not significantly increase the level of creatinine in the serum. Also the orlistat (200 mg/kg) did not significantly increase creatinine level in serum (Figure 10).

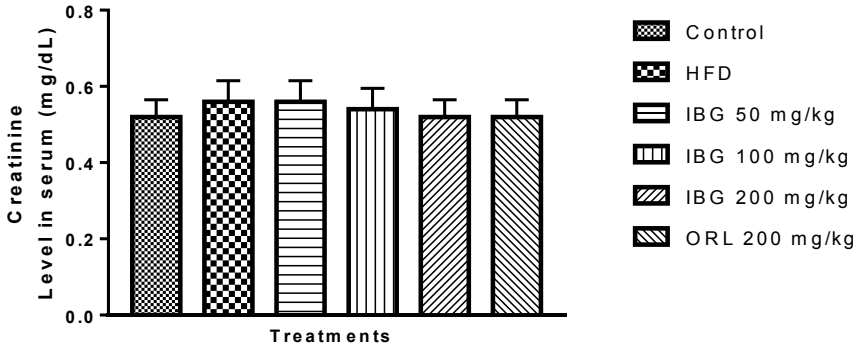

Figure 10: Effect of IBG on creatinine in serum

All values are expressed mean ± SEM. Data were analyzed using one-way ANOVA for multiple comparison and Dunnett's post-hoc test.

In the present study, there was no toxicity outcome in both the Orlistat and IBG treated rats. No significant difference ($p>0.05$) was observed in the serum levels of HDL, Triglyceride, total cholesterol and LDL in both the Orlistat and IBG extract treated groups. However, there was a significant decrease in IBG treated rats at 200 mg/kg in the LDL serum level when compared to the orlistat treated rats. There was also a significant reduction in abnormal fat (g) in groups treated with Orlistat at 200 mg/kg and IBG treated animals (200 mg/kg) which could be as a result of dose dependent pattern respectively. In the serum glucose levels of all the experimental animals, there were no significant changes among the Orlistat and IBG treated animals respectively as observed in the present study. There was also no significant changes in the Serum levels of liver enzymes and creatinine of Orlistat treated rats in comparison with the IBG treated groups as obtained. This reveals that the ethanol extract of *Irvingia gabonensis* seeds might possess comparable anti-obesity effects by decreasing the BMI and weight of abdominal fat via reduction of the plasma levels of total cholesterol, triglyceride and LDL in treated animals and could therefore, serve as templates for anti-obesity drug development.

REFERENCES

1. Gupta D, Bhardwaj R, Gupta RK. In vitro antioxidant activity of extracts from the leaves of *Abies pindrow* Royle. Afr J Tradit Complement Altern Med. **2011**; 8(4):391–7. https://doi.org/10.4314/ajtcam.v8i4.8 PMID: 22654216.

2. Overweight and obesity. Division of Nutrition, Physical Activity, and Obesity, National Center for Chronic Disease Prevention and Health Promotion June **2020**.

3. Mahnaz K, Che WJ, Geoffrey AC, Iman Y. Potential of Traditional Medicinal Plants for Obesity: A review. 2012 International Conference on Nutrition and Food Sciences IPCBEE **2012**, 39, *IACSIT Press*, Singapore.

4. Yun JW. Possible anti-obesity therapeutics from nature—a review. Phytochemistry. **2010** Oct; 71(14-15):1625–41. https://doi.org/10.1016/j.phytochem.2010.07.011 PMID: 20732701.

5. Kiess W, Petzold S, Töpfer M, Garten A, Blüher S, Kapellen T, et al. Adipocytes and adipose tissue. Best Pract Res Clin Endocrinol Metab. **2008**; 22(1):135–53. https://doi.org/10.1016/j.beem.2007.10.002 PMID: 18279785.

6. Roberts CK, Berger JJ, Barnard RJ. Long-term effects of diet on leptin, energy intake, and activity in a model of diet-induced obesity. J Appl Physiol. **2002**; 93(3):887–93. https://doi.org/10.1152/japplphysiol.00224.2002 PMID: 12183482

7. Frederich RC, Hamann A, Anderson S, Löllmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med. **1995**; 1(12):1311–4. https://doi.org/10.1038/nm1295-1311 PMID: 7489415

8. Ichihara S, Yamada Y. Genetic factors for human obesity. Cell Mol Life Sci. **2008**; 65(7-8):1086–98. https://doi.org/10.1007/s00018-007-7453-8 PMID: 18097636

9. Vojtech H. Comparative Efficacy and Safety of Pharmacological Approaches to the Management of Obesity. Diabetes Care. **2011**; 34(2):349-54.

10. Kumar P, Bhandari U. Common medicinal plants with antiobesity potential: A special emphasis on *fenugreek*. Anc Sci Life. **2015**; 35(1):58–63. https://doi.org/10.4103/0257-7941.165629 PMID: 26600669

11. Patra S, Nithya S, Srinithya B, Meenakshi SM. Review of Medicinal Plants for Anti-Obesity Activity. Transl Biomed. **2015**; 6(3):3. https://doi.org/10.21767/2172-0479.100021.

12. Tobe H, Raven PH. Embryology of the Irvingiaceae, a family with uncertain relationships among the Malpighiales. J Plant Res. **2011**; 124(5):577–91. https://doi.org/10.1007/s10265-010-0393-7 PMID: 21116833

13. Oyedele T. A and Fatoki, A. Nutritional Composition of Irvingia gabonensis mycoflora with Extracts of Selected Allium Spices. Sci. Eng. Perspectives. **2017**; 12:9–16.

14. Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S. Agroforestree Database: A free reference and selective guide version 4.0 2009, 1-5 Assessed online 6 June **2017** (http://www.worldagroforestry.org/sites/free/feedatabases.asp)

15. Abalude FO, Ogunkoya MO, Akinjagunla YS. Phytochemical Screening of Leaves and Stem of Cashew Tree Anacardium Occidentale. Electr J. Environ. Agric. Food Chem. **2010**; 9:815–9.

16. Onimawo I, Oteno F, Orokpo G, Akuber PI. Physiochemical and Nutrient Evaluation of African Bush Mango *(Irvingia gabonensis*) seeds and Pulp. Plant Foods Hum Nutr. **2003**; 58:1–6.

17. Obianime AW, Uche FI. Effects of Aqueous Extracts of *Irvingia gabonensis* Seed on the Hormonal Parameters of Male Guinea Pigs. Asian Pac J Trop Med. **2010**; 12:200–4.

18. Adnyana IK, Elin YS, Yuniarto A, Finna S. Anti-obesity Effects of Pomegramate Leaves Ethanol Extract (*Punica granatumi*) in High Fat Diet Induced Mice. Int J Pharm Pharm Sci. **2014**; 6:626–31.

19. Cercato C, Fonseca FA. Cardiovascular risk and obesity. Diabetol Metab Syndr. **2019**; 11(1):74. https://doi.org/10.1186/s13098-019-0468-0 PMID:31467596

20. Van Gaal L, Dirinck E. Pharmacological Approaches in the Treatment and Maintenance of Weight Loss. Diabetes Care. **2016**; 39 Suppl 2:S260–7. https://doi.org/10.2337/dcS15-3016 PMID:27440841

21. Yuniarto A, Sukandar EY, Fidrianny I, Crystalia AA, Adnyana IK. Zebra Fish Model of Obesity: Relevance to Metabolic Syndrome. Int. J. Green Pharm. **2019**; 13:175–9.

22. Kim Y, Lee H, Kim YK, Park S, Kim J, Yun JH, et al., Association of Metabolites with Obesity and Type 2 Diabetes Based on FTO Genotype. PLoS ONE, **2016**; 11:29. e0156612.

23. Ngondi JL, Oben JE, Minka SR. The effect of *Irvingia gabonensis* seeds on body weight and blood lipids of obese subjects in Cameroon. Lipids Health Dis. **2005**; 4(1):12. https://doi.org/10.1186/1476-511X-4-12 PMID: 15916709

24. Kim GW, Lin JE, Blomain ES, Waldman SA. Antiobesity pharmacotherapy: new drugs and emerging targets. Clin Pharmacol Ther. **2014**; 95(1):53–66. https://doi.org/10.1038/clpt.2013.204 PMID: 24105257

25. Lee CM, Huxley RR, Wildman RP, Woodward M. Indices of abdominal obesity are better discriminators of cardiovascular risk factors than BMI: a meta-analysis. J Clin Epidemiol. **2008**; 61(7):646–53. https://doi.org/10.1016/j.jclinepi.2007.08.012 PMID: 18359190

26. Kuyooro SE, Abam EO, Agbede EB. Hypolipidemic Effects of *Irvingia gabonensis*-Supplemented Diets in Male Albino Rats. Biochem Anal Biochem. **2017**; 6(2):21–5. https://doi.org/10.4172/2161-1009.1000316.

27. Nderitu KW, Mwenda NS, Macharia NJ, Barasa SS, Ngugi MP. Antiobesity activities of methanolic extracts of *Amaranthus dubius, Cucurbita pepo*, and *Vigna unguiculata* in progesterone-induced obese Mice. Evid Based Complement Alternat Med. **2017**; 2017:4317321. https://doi.org/10.1155/2017/4317321 PMID: 28947909

28. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S, Kaushal C, et al. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res. **2011**; 2(4):236–40. https://doi.org/10.4103/2231-4040.90879 PMID: 22247890

29. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: an overview. Scientific World Journal. **2013**; 2013:162750. https://doi.org/10.1155/2013/162750 PMID: 24470791

30. Chang E, Kim CY. Natural products and obesity: a focus on the regulation of mitotic clonal expansion during adipogenesis. Molecules. **2019**; 24(6):1157. https://doi.org/10.3390/molecules24061157 PMID: 30909556

31. Amacher DE. Serum transaminase elevations as indicators of hepatic injury following the administration of drugs. Regul Toxicol Pharmacol. **1998**; 27(2):119–30. https://doi.org/10.1006/rtph.1998.1201 PMID: 9671567

32. Agahi A, Murphy KG. Models and strategies in the development of antiobesity drugs. Vet Pathol. **2014**; 51(3):695–706. https://doi.org/10.1177/0300985813492801 PMID: 23860008.

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Effect of Formulation and Process Variables on the Material and Release Properties of Metronidazole Tablets Using Enterolobium Gum as Binder

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ABSTRACT

Using the 2^3(=8) factorial design to determine the effects of excipient modification and process techniques, on mechanical and release properties of tablets containing *Enterolobium cyclocarpum* gum.

Properties of the native (NG), physically modified (PMG) and chemically modified (CMG) gums were evaluated. Metronidazole tablets were prepared from formulations containing NG, PMG, CMG or HPMC. Individual and interacting effects of the parameters were evaluated using the factorial design.

Formulations showed drug release of 81 – 99%. Nature and concentration of polymer, and processing technique (X_1, X_2 and X_3 respectively) had the highest effect on disintegration time. Interaction between X_1 and X_3 had the greatest effect on the mechanical properties; interaction between X_1 and X_2 had the greatest effect on drug release.

Enterolobium gum produced tablets with high drug release which was sustained for 12 hours. Gum modification and other formulation variables need to be selected to achieve desired formulation objectives.

Keywords: Enterolobium gum, tablet properties, processing techniques, drug release

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INTRODUCTION

The three major methods employed in the production of tablets are direct compression, dry granulation and wet granulation. Each of these methods have different requirement in term of materials, concentration of the materials and procedure. The mechanical and release properties of tablets could be affected by these varying parameters.

Polymeric materials such as gums and starches have been widely used as binders in tablet formulations; however, locally sourced natural materials have advantages over the synthetic polymers[1]. Binders are employed in pharmaceutical tablet formulation to promote the bonding between the different components of a powder mix in a formulation thereby enhancing the strength of the eventual tablet produced. They are usually selected on basis of previous experience, particular product needs, literature or vendor data or the preference of the individual or manufacturing unit [2]. Binders are added either dry (in direct compression) or wet (in wet granulation) to form cohesive compact or granules.

According to Samia *et al.*, (2009)[3], exudate gums are formed as a result of microbial infection on the plants and the plants in turn synthesize the liquid substances as a defence mechanism to seal off the wound and prevent further invasion of the tissue. Natural gums are known for their physical properties and employed as excipients in pharmaceutical preparations where they are used as thickeners, emulsifiers, viscosity enhancers, drug release modifiers and binders. Gums are known to have binding properties; they impart cohesiveness in to the powder mass and convert them to granules [4]. Due to excellent properties of exudate gum such as solubility, viscosity, binding, stabilizing, thickening and emulsifying, they are used in adhesive industries, beverages, confectionaries, cosmetics, paint, paper, pharmaceutical and food industries [5].

The physicochemical properties of gums in their native forms have been improved through various forms of modification. Agar and guar gums were modified by heat treatment at various temperatures in a hot air oven along with co-grinding of both materials. The modified gum was used as disintegrating agent [6]. Ayorinde and Odeniyi (2014) [7] investigated the effect of modification by microwave irradiation on the disintegrant properties of a starch and a gum in metronidazole tablet formulations.

Modification of gums can be done through derivatization of functional groups, grafting with polymers, cross-linking with ions etc. The techniques for modification of gums have been broadly classified into four categories namely: [8] Physical, chemical, enzymatic, and genetic modifications. These modifications aim at

producing various new derivatives with improved physicochemical properties along with useful structural attributes [9, 10].

The plant, *Enterolobium cyclocarpum* is one of the largest trees in the dry forest formation reaching up to 40 m in height and 3 m in diameter, with a huge spreading crown. It is commonly known as Guanacaste, Mexican walnut, Carocaro or Elephant-ear tree. It is species of flowering tree in the pea family, Fabaceae (Mimosoideae) that is native to tropical regions of America from central Mexico south to northern Brazil and Venezuela. In North America, it is often called elephant-ear tree due to the shape of the seedpods. It is evergreen or briefly deciduous for 1-2 months during the dry season. They have very fragrant flowers and their fruits are large, glossy dark brown indehiscent and spirally-organized pods shaped like orbicular disks.

Enterolobium gum is usually a transparent dark brown gum, partially soluble in water. The gum also called Mexican walnut gum is obtained from incised trunk of *Enterolobium cyclocarpum* tree. The gum has been found to have potentials for use as a film forming polymer [11].

This present study is therefore aimed at evaluating the native and modified Enterolobium gums as binders in metronidazole tablet formulations. The individual and interaction effects of various formulation parameters on mechanical and release properties of the tablets will also be investigated.

METHODOLOGY

Materials

The materials used in this study are *Enterolobium cyclocarpum* gum (obtained from Botanical garden, University of Ibadan, Nigeria), Metronidazole (Vision pharmaceutical Co Ltd, China), Hydroxylpropylmethyl cellulose (HPMC). All other reagents used were of Analar grade.

Methods

Collection, Extraction and Characterization of Enterolobium Gum

The collection and extraction process of Enterolobium gum have been reported in our previous studies [12, 1].

Modification of Enterolobium Gum

Modification was carried out using physical and chemical modification. In physical modification, a 70% w/v of the gum was prepared at 70 ˚C, thoroughly mixed with 20.5 g ethylene glycol and then allowed to cool at room temperature. The obtained slurry was dried at 60 ˚C in an oven (Model DHG-9053A,

Ocean Medical, England) and pulverized to a uniform particle size. The chemical modification was carried out by dispersing 20 g of the gum in 100 mL distilled water. Stirring was carried out for 30 minutes and the pH was adjusted to 8.0 with sodium hydroxide. Approximately 1.2 g of acetic anhydride was added to the slurry and the reaction was allowed to proceed for another 5 min. The pH was adjusted to 4.5 with 0.5 M hydrochloric acid and then filtered. The residue was washed four times with ethanol, dried in the oven and pulverized.

Determination of physicochemical properties

The following physicochemical properties were determined for both the native and modified gums:

Viscosity

Aqueous dispersion (2% w/v) of each gum sample was made and left for one hour to hydrate.

The viscosities were then determined using a Brookfield viscometer model DV-11+ Pro at

50 rpm and 100 rpm using a spindle with code 04.

Swelling Index

Quantity (2.5 g) of each gum sample was poured into a 100 mL measuring cylinder and the volume was read as V_1. Distilled water was then added gradually with the dispersion shaken thoroughly for 5 minutes. The dispersion was allowed to stand for 24 hours before the sedimentation volume was obtained and read as V_2.[13] The swelling capacity was calculated thus:

Swelling capacity = V_2/V_1 ..... (1)

V_1 = volume occupied by the gum prior to hydration.

V_2 = volume occupied by the gum after the hydration.

Density Measurements

Bulk and tapped densities were determined thus: Approximately 10 g of each gum sample was weighed and poured at an angle of 45° through a funnel into a 100 mL glass measuring cylinder and the volume occupied by the sample was calculated using the height (h) of the sample in the measuring cylinder and the radius (r) of the cylinder. Bulk density was calculated using the equation:

Bulk density $= \frac{Mass}{\pi r^2 h}$ ..... (2)

The tapped density was measured by applying 250 taps to 10 g of the gum samples in a measuring cylinder at a standardized rate of 25 taps per minute. The height of the powder bed and radius of the measuring cylinder were used in the calculation of volumes obtained and determinations were done in triplicates.

Carr's Index

The Carr's compressibility index was calculated from the results of bulk and tapped densities by using the equation below:

$$\text{Carr's Index} = \frac{Tapped\ density - Bulk\ density}{Tapped\ density}\ X\ 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (3)$$

Angle of repose

This was determined using an open-ended cylinder placed on a wooden cork of known diameter. Approximately 10 g of the gum powder was allowed to flow through a funnel under the force of gravity into the previously placed cylinder. The content in the open-ended cylinder was then released to form a conical heap. The height of this heap was measured and the angle of repose was then calculated using the equation:

$$\text{Tan}\ \theta = h/r \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (4)$$

Where h is the height of powder and r is the radius of the base of the cone which is the radius of the wooden cork. The angle of repose was calculated from a mean of three determinations.

Particle Size

The particle size of the native and modified gums were determined using optical microscope (MT 3300EXII, MictotracBel, Japan). The photomicrographs were taken at 20 Kv.

Preparation of Tablets

Metronidazole tablets were prepared from formulations containing native Enterolobium gum, modified Enterolobium gum or HPMC at different concentrations as binders. Direct compression and wet granulation methods were used in preparing the tablets, using the formula:

Metronidazole	50%
Native gum/physically modified gum/chemically modified gum/HPMC	4% or 8%
Corn starch	10%
Lactose to	100%

In the direct compression method, a basic mixture of the above formula was prepared by dry mixing them in a mortar and pestle and then in a rotor mixer (Forster equipment Co. Ltd. whet stone, Leicester, England) for 10 minutes. From the powder mass, approximately 0.40 g was weighed out then compressed into tablets using a single punch carver hydraulic hand press (model c, carver Inc., Menomonee falls, Wisconsin, USA), a die of 10.5 mm in diameter at four different pressures (0.5, 0.75, 1 and 1.25 Nm^{-2}). A 1 %w/v dispersion of magnesium stearate in acetone was used to lubricate the punch and die surfaces before each compression.

Using the wet granulation method, A 4 or 8 % dispersion of native gum, physically or chemically modified gum or HPMC was made and used as granulating fluid in the above stated formula. The granules formed were dried in the oven (Model DHG-9053A, Ocean Medical, England) at 50 °C, allowed to cool and then stored in air-tight containers. Tablets were prepared from the granules following the methods described in direct compression.

Evaluation of Tablets

1. Tablet Properties

Uniformity of weights, thickness, crushing strength, tensile strength and friability were evaluated to determine the tablet properties.

2. Release Properties

(a). Disintegration test

The disintegration time for the tablets from each pressure in every batch was determined in distilled water at 37.0 ±0.2 °C using the DBK disintegration testing apparatus (DBK Instruments Mumbai, India). The time taken for the tablet to completely break down and pass through the mesh was recorded with determinations in triplicates.

(b). Dissolution rate test

The dissolution rate of the tablets was determined in 0.1N HCl using the DBK dissolution test apparatus (Type 40DRT01, DBK Instruments Mumbai, India). A calibration curve was first plotted for the metronidazole powder. Each tablet was placed in a cylindrical basket of stainless steel wire mesh which was attached to a rotor that can be regulated to varying speed and suspended in a glass beaker containing 500ml of 0.1N HCL. The glass beaker is in turn immersed in a water bath controlled at a temperature of 37±2 °C. The 0.1N HCl was also heated and maintained at this temperature. The apparatus was then

set to rotate at 100rpm and 5 mL of the dissolution medium was removed at specific time intervals as well as replaced simultaneously with an equal volume of fresh dissolution medium to maintain a sink condition. The withdrawn sample (1 mL) was diluted to 10mls and the absorbance of the diluted samples was measured using the ultraviolet/visible spectrophotometer (Spectrumlab 752S12078, Bicotek Ningbo Ltd., China)) at a wavelength of 265 nm and the drug concentration determined from the calibration curve.

The dissolution rate kinetics was calculated using the SolverDD, a Microsoft Excel add-in program. In order to determine the mechanism of drug release, the release data was fitted in a Korsemeyer-Peppas equation: [14]

$$\text{Log } (Mt/Mf) = \text{Log } k + n\text{Log } t \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

This equation describes drug release behaviour from polymeric systems. Mt is the amount of drug release at time t, Mf is the amount of drug release after infinite time; k is a release rate constant incorporating the structural and geometric characteristics of the dosage form and n is the diffusional exponent, which indicates the mechanism of drug release. For a cylinder-shaped matrix, the value of $n = 0.45$ indicates Fickian (case I) release; > 0.45 but < 0.89 for non-Fickian (anomalous) release; and > 0 indicates a super case II type of release. The case II mechanism refers to the erosion of the polymer and anomalous transport (non-Fickian) refers to a combination of both diffusion and erosion-controlled drug release.

Factorial Experimental Design

A 2^3 factorial experiment design, which required eight batches to be prepared, was used to determine the individual and interacting effects of the selected formulation variables on the mechanical and release properties of the tablets. This has been found very useful in determining the effect of various formulation factors on the characteristics of drug formulations [15, 16]. Three independent process parameters: nature of polymer, concentration of polymer and processing technique were applied at two different levels. These parameter levels are summarized in Table 1. The sequence of the eight experiments was randomized. The purpose of using a full factorial experimental design was to carry out a complete study of the effects of the process parameters and their interactions, with the aid of a computer and suitable statistical software (Minitab© 14.2). Table 1 shows the Independent process parameters and their levels. Lower Level was coded -1 while higher level was coded +1. Nature of polymer was X_1, Concentration of polymer was X_2 and processing technique was X_3.

Table 1: Factor combinations and values of Friability (F), Tensile strength (T) and Disintegration time (Dt) for tablets in the factorial experimental design

Batch number	Nature of Polymer (X_1)	Concentration of Polymer (X_2)	Processing technique (X_3)	Tensile Strength (KgF)	Friability (%)	Disintegration Time (min)
1	-1	-1	-1	0.43	0.95	10
2	1	-1	-1	0.63	1.97	15
3	-1	1	-1	0.49	0.87	12
4	1	1	-1	0.63	1.99	15
5	-1	-1	1	0.51	0.92	4.8
6	1	-1	1	0.9	0.99	14
7	-1	1	1	0.61	0.97	9.36
8	1	1	1	0.94	0.98	13.87

Key: -1: Low values / +1: High values

The effects of the various parameters on the mechanical and release properties of the tablets were compared by Student's t-test. At the 95% confidence level, a p value lower than or equal to 0.05 was required for significance.

RESULTS and DISCUSSION

Physicochemical Properties

Particle size

Particle size has been postulated to affect absorption rate, uniformity of content, dissolution rate and stability of pharmaceutical formulations. [17] The particles of the native gum were smaller and more irregularly shaped than the physically and chemically modified gums. The results therefore suggest that modification of the gums imparts increase in particle size with chemical modification having a greater increase (Table 2). Flow of materials is influenced by particle size because of the adhesion and cohesion phenomena which occur on the surface; small sized particles with high surface-to-mass ratio are cohesive and have poor flow while coarse particles are bigger in sizes and have better flow. The small particle size of the native gum could be of advantage to the formulation scientist, in the sense that it offers opportunity for modification according to the desired size.

Table 2: Physicochemical Properties of the Polymers (Mean±s.d, n=3)

Materials	NG	PMG	CMG	HPMC
Viscosity (cp)	44.60 ± 1.34	20.35 ± 2.45	9.20 ± 1.22	25.55 ± 4.10
Bulk Density (g/cm^3)	0.61 ± 0.05	0.45 ± 0.09	0.53 ± 0.12	0.64 ± 0.20
Tapped Density (g/cm^3)	0.78 ± 0.09	0.52 ± 0.03	0.65 ± 0.08	1.45 ± 0.07
Particle density	1.71 ± 0.51	1.48 ± 0.09	1.40 ± 0.55	1.25 ± 0.06
Carrs' Index (%)	22.60 ± 1.65	13.96 ± 3.45	18.62 ± 5.50	14.19 ± 2.25
Hausner's Ratio	1.29 ± 1.01	1.16 ± 0.88	1.23 ± 0.54	1.17 ± 0.45
Angle of repose (^{0})	51.76 ± 1.65	49.60 ± 2.50	52.72 ± 1.05	56.05 ± 1.58
Swelling Index (%)	3.17 ± 0.85	1.18 ± 0.54	1.59 ± 0.25	2.58 ± 2.45
Particle size (µm)	18.01 ± 3.65	120.32 ± 5.15	210.10 ± 4.45	25. 60 ± 1.70

Angle of repose

Angle of repose is a characteristic of the internal friction or cohesion of the particles. The value of the angle of repose will be high if the powder is cohesive and low if the powder is non-cohesive. [18] It is an indirect method of quantifying powder flowability because of their relationship with interparticulate cohesion and has been used in several branches of science to characterize the flow properties of solids. It is worthy of note that angle of repose is not an intrinsic property of the powder i.e. it is to a large extent dependent upon the method used to form the cone of the powder. Angle of indicates excellent flow; represents good flow; indicates fair flow; represents passable flow; indicates poor flow while indicates very poor flow and indicates very very poor flow property. [19]

From the results, it can be observed that the gums in both native and modified forms have high values of angle of repose which indicates poor flow properties. The ranking was HPMC > chemically modified gum > native gum > physically modified gum (Table 2). This indicates that physical modification could slightly improve the flow.

Density Measurements

The bulk and tapped densities give an insight of the packing and arrangement of the particles and the compaction profile of a material. Various factors can affect the bulk and tapped densities of a material such as cohesiveness due to surface forces, particle size and distribution, particle shape and true density of solids [19, 20]. It is an important property in packaging and powder handling. Powders with

large bulk density are heavy powders while powders with low bulk density are light powders. In a free-flowing powder, the inter -particulate interactions are less significant and the bulk and tapped densities will be close in value. For poor flowing powder, inter-particulate interaction is greater and a great difference is noticed between the bulk and tapped density. The rank order for bulk density is native gum > chemically modified gum > physically modified gum (Table 2). Tapped density also followed this order. The difference in the bulk and tapped density of native and chemically modified gum was higher than that of physically modified gum showing that the former had a higher inter-particulate interaction hence a poor flowing powder. This shows that the chemically modified gum was more compact than the physically modified gum and less compact than the native gum.

Swelling Properties

Natural gums are often known for their good swelling properties and their ability to reach a steady swelling state in a short time by the entrapment of large amounts of water between their chains and branches. [18, 21] The rank order of swelling index among the polymers was native gum > HPMC > chemically modified gum > physically modified gum with values of 3.17 > 2.58 > 1.59 > 1.18, respectively (Table 2). This shows that modification of the gum reduced the swelling of the gum. This could be of importance in the formulation of sustained release delivery systems when reduced swelling may delay disintegration and subsequently prolong drug delivery.

Viscosity

The viscosity profile of the gum both native and modified ones showed an increase in the viscosity with an increase in the revolution per minute (rpm) used which are 50 and 100rpm. There was also a decrease in viscosity with increase in time. The order of viscosity is native gum > HPMC > physically modified > chemically modified gum (Table 2). The modified gums had a low swelling index (capacity) due to poor interaction between their particles and water molecules; this could have been responsible for the low viscosity when compared with native gum [21, 22].

Organoleptic Properties

The extracted gum was observed to be brown in colour and with a pleasant odour. However, the physically modified gum had a darker shade of brown colour while the chemically modified polymer was greyish. These properties suggest acceptability, both in term of colour and odour. The change in colour from brown to grey after chemical modification is probably due to the acetylation process.

Tablet Properties

Crushing Strength

Crushing strength provides a measure of the mechanical strength of the tablets and there are official requirements for compressed tablets [23] but there are no limits for the acceptance or rejection of tablet formulation due to crushing strength; this is probably because the desired crushing strength is largely dependent on the intended use of tablet. [23, 24] Results obtained showed that increase in concentration of the gums (native and Modified) resulted in an increase in the crushing strength of the respective tablets and this increase corresponds to the increase in compression pressure used (Table 3& 4).

Table 3: Properties of Tablets Prepared by Direct Compression (Mean±s.d, n=3)

Polymer/ Concentration (%)	Compression Pressure MNm^{-2}	Mean Weight (g)	Thickness (mm)	Crushing Strength (KgF)	Friability (%)	Disintegration Time (sec)
NG /4	0.50	0.19 ± 0.03	2.13 ± 0.26	6.00 ± 1.02	1.04 ± 0.13	8.00 ± 6.35
	1.25	0.20 ± 0.02	1.99 ± 0.11	15.12 ± 2.86	0.91 ± 1.55	11.00± 8.62
NG/8	0.50	0.19 ± 0.01	2.11 ± 0.10	15.98 ± 3.29	0.98 ± 1.10	10.00 ± 4.65
	1.25	0.21 ± 0.12	2.08 ± 0.11	16.55 ± 2.55	0.92 ± 0.18	12.00± 8.39
PMG/4	0.50	0.19 ± 0.11	2.03 ± 0.09	15.25 ± 0.72	0.75 ± 1.18	11.00 ± 5.19
	1.25	0.19 ± 0.22	2.00 ± 0.14	18.94 ± 5.05	0.72 ± 1.58	14.00 ± 3.35
PMG/8	0.50	0.21 ± 0.05	2.02 ± 1.25	16.20 ± 0.82	0.78 ± 1.20	13.00 ± 7.13
	1.25	0.20 ± 0.09	2.01 ± 0.85	17.98 ± 0.55	0.70 ± 1.18	15.00 ± 4.25
CMG/4	0.50	0.20 ± 0.10	2.05 ± 1.45	15.23 ± 0.15	1.98 ± 1.50	13.00 ± 1.55
	1.25	0.20 ± 0.15	2.00 ± 0.18	21.52 ± 0.24	1.99 ± 2.21	15.00± 1.83
CMG/8	0.50	0.19 ± 0.21	2.08 ± 1.45	17.62 ± 0.11	1.92 ± 1.90	14.00 ± 4.15
	1.25	0.21 ± 0.16	2.02 ± 0.62	23.02 ± 0.14	1.99 ± 5.21	15.00± 6.12
HPMC/4	0.50	0.19 ± 0.15	2.00 ± 1.85	15.20 ± 0.82	0.92 ± 2.90	13.00 ± 3.35
	1.25	0.21 ± 0.10	1.97 ± 0.12	17.88 ± 0.15	0.99 ± 4.21	15.00± 3.06
HPMC/8	0.50	0.20 ± 0.30	1.99 ± 1.15	15.65 ± 0.22	0.87 ± 2.10	13.00 ± 2.35
	1.25	0.20 ± 0.19	1.96 ± 0.20	16.98 ± 0.12	0.86 ± 5.11	15.00± 1.62

Table 4: Properties of Tablets Prepared by Wet Granulation (Mean±s.d, n=3)

Polymer/ Concentration (%)	Compression Pressure MNm^{-2}	Mean Weight (g)	Thickness (mm)	Crushing Strength (KgF)	Friability (%)	Disintegration Time (sec)
NG/4	0.50	0.20 ± 0.10	2.50 ± 0.16	12.00 ± 7.12	0.95 ± 6.15	249.00 ± 3.75
	1.25	0.20 ± 0.20	2.22 ± 5.35	22.12 ± 8.60	0.94 ± 5.51	292.00± 7.22
NG/8	0.50	0.19 ± 0.11	2.31 ± 0.17	15.12 ±9.10	0.91 ± 3.33	525.00 ± 6.15
	1.25	0.21 ± 0.42	2.08 ± 0.22	26.15 ± 7.51	0.96 ± 8.32	570.00 ± 9.70
PMG/4	0.50	0.20 ± 0.15	2.45 ± 5.18	14.05 ± 0.28	0.82 ± 5.68	588.00 ± 12.70
	1.25	0.21 ± 0.22	2.10 ± 0.16	22.16 ± 5.75	0.92 ± 5.18	615.00 ± 7.80
PMG/8	0.50	0.20 ± 0.15	2.40 ± 1.70	16.80 ± 7.12	0.89 ± 2.50	640.00 ± 9.70

The increase in hardness might be due to the fact that stronger inter-particulate bonds were formed with increasing polymer concentration. Ranking order among the formulations was CMG8 > CMG4 > PMG8 > PMG4 > HPMC8 > HPMC4 > NG8 > NG4. Crushing strength was generally high in tablets prepared by wet granulation than those prepared by direct compression method. This suggests that the addition of water in wet granulation allows for wetting, thereby producing greater penetration of the polymer. The gums would therefore serve a better binding agent in the presence of water than in their dry states.

Tensile Strength

Tensile strength is the resistance of a material to breaking under tension; it is a characteristic of internal friction or cohesion of particles and calculated from crushing strength i.e.

$T = 2F/\Pi dt$..... (7)

Where F is the crushing strength, d and t are the tablet diameter and thickness respectively.

Tensile strength provides a more fundamental measure of the mechanical strength of the compacted material and takes into account the geometry of the tablet. If the tablets fail in tension, the breaking force can be used to calculate the tensile strength. It is a measure of mechanical property of tablet. [25] The trend observed in the calculated tensile strength of the tablets was same with the crushing strength.

Friability

Friability is another mechanical property which evaluates the ability of tablets to withstand abrasion in packing, handling and transporting. It is regarded as attrition resistance method in determining the mechanical property of a tablet. Unlike crushing strength that is a bulk deformation of the tablet, friability is a surface deformation of the tablet which may be enhanced by the morphology of the tablet. The rougher the surface of the tablet, the more friable it will be [25, 26]. For compressed tablets, the percentage loss in weight of less than or equal to 1% is usually considered acceptable [23].

From the results obtained (Tables 3 and 4), tablets prepared with wet granulation method generally passed the friability. However, tablets containing chemically modified polymers (CMG 4 and CMG 8) had friability values of greater than 1%, while the values were less than 1% in all other formulations. This is an indication that irrespective of the form of the polymer, wet granulation method will produce tablets of better mechanical strength. However, if direct compres-

sion is the desired method of tablet production, physical modification should be considered. Furthermore, it was observed that the percentage weight loss generally reduced with increase in binder concentration and compression pressure; this is probably due to more binder molecules being made available together with the fact that stronger compacts are formed with higher compression pressure, thus producing tablets with less rough surfaces.

Release Properties

Disintegration Time

Disintegration is a crucial step in release of drugs from immediate release dosage forms. It is useful for assessing the potential importance of formulation and process variables on the biopharmaceutical properties of the tablet and as a control procedure to evaluate the quality reproducibility of the tablet during production [26]. The rate of disintegration is influenced by the rate of influx of water into the tablets which is also dependent on the porosity of the tablets. Disintegration time measures the time required for the tablet to crumble into particles and it is a necessary condition for dissolution and could be the rate determining step in the process of drug absorption. The British Pharmacopoeia stipulated a disintegration time of not more than 15 minutes for uncoated tablets. [23] From the result obtained, it was observed that the tablets containing native gum, modified gums and HPMC had disintegration time less than fifteen minutes and thus fell within the acceptable limit specified in the British Pharmacopeia. The ranking was native gum < Physically modified gum < chemically modified gum > HPMC and the disintegration time were observed to increase with increase in the compression pressure and also with increase in the concentration of the binder. The results (Tables 3 and 4) therefore suggest that the gum, both in its native and modified form could produce tablets of acceptable disintegration time. The disintegration time of tablets produced by wet granulation is generally longer than those produced by direct compression ($p < 0.05$); this is probably due to the water influx which occurred in wet granulation, thereby leading to creation of stronger bonds between the materials [27].

Drug Release

The dissolution of a drug from the dosage form is one of the important parameters that determine bioavailability of that drug [1]. Fundamentally, this process is controlled by the relative affinities between the molecules of the solid substance and those of the solvent. The factors that affect dissolution include type and concentration of binder, mechanical properties of tablets, surface area, distance of diffusion, solubility of the drug, manufacturing processes[18]. Dissolution rate

test is therefore used to evaluate the potential effect of formulation and to ensure the performance of the preparation under in-vivo condition. The dissolution rate profiles of the formulations are shown in Figures 1 and 2.

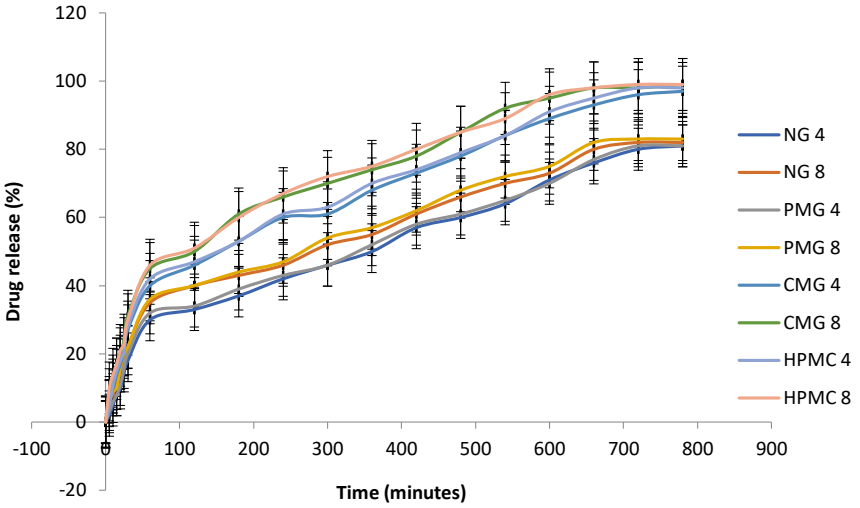

Figure 1: Release Profile for Tablets Produced by Direct Compression (mean values plotted; n=3, with standard error bars on the plots)

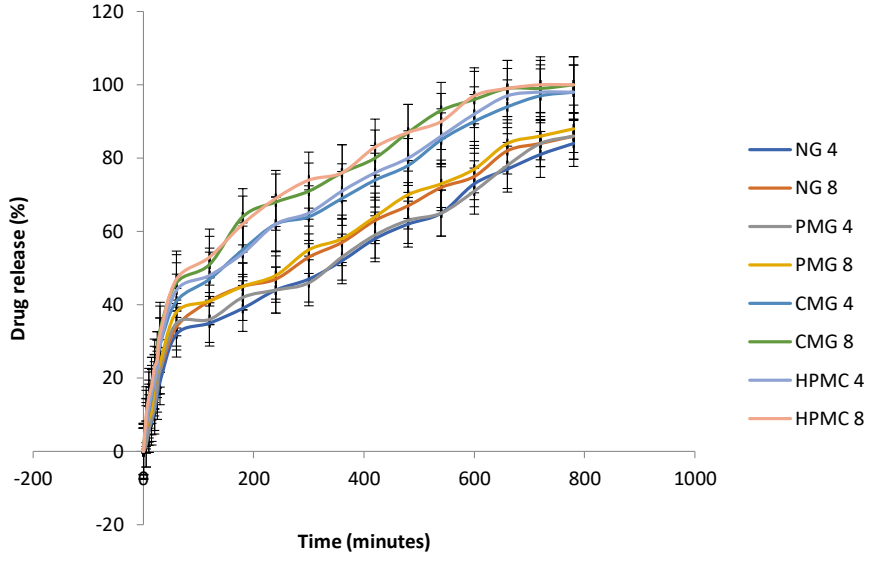

Figure 2: Release Profile for Tablets Produced by Wet Granulation (mean values plotted; n=3, with standard error bars on the plots)

All the formulations exhibited similar trends in their drug release; an initial gradual release up to 30 minutes followed by a sustained release for more than 12 hours. Similar release profile was observed in a previous study on Enterolobium gum [1]. All the formulations showed a high drug release of 81 – 99%. These results suggest that the gum in its native and modified forms has similar release profile and capable of sustaining the drug for up to 12 hours. The chemically modified gum formulations (CMG 4 and CMG 8) showed the highest drug release, almost same with formulations containing HPMC; the ranking was HPMC 8 > HPMC 4 > CMG 4 > CMG 8 > PMG 4 > > PMG 8 > NG 4 > NG 8. The difference between drug release in formulations containing native and physically modified gum is not significant while a significant difference was observed between formulations containing the chemically modified and those containing the native and physically modified gum. This implies that chemical modification had a significant effect on the drug release profile of the gum while physical modification does not. The drug release profiles of tablets prepared with direct compression (Fig. 1) and wet granulation (Fig. 2) were similar; this shows that tablet processing may not significantly affect drug release profile of tablets prepared with the gum.

Mechanism of Drug Release

The physicochemical properties of the drug and the polymer have been shown to govern the release of drug from formulations and this which could affect their release kinetics. The drug release kinetics of the different formulations were fitted into zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg, Korsmeyer-Pepppas [28, 29, 30, 14].

The constants of release kinetic and coefficient of correlation (R^2) were obtained from slopes of plots by linear regression analysis. All the formulations have the highest value of R^2 in the in the Korsmeyer-Pepppas equation (Tables 5 and 6). This indicates that the drug- release from all the formulations is not concentration dependent. In order to determine the mechanism of drug release, the release data was fitted in a Korsemeyer-Peppas equation, which is suitable for the description of drug release from polymeric materials. From the equation, the value of n for each formulation was taken. When n is 0.430 or less, it indicates release is by diffusion mechanism. When n is 0.85, the mechanism of release is swelling controlled and when n is between 0.430 and 0.85, the mechanism of drug release is by both diffusion and swelling controlled mechanisms and this is termed anomalous. The values of n for formulations containing the gum (native or modified) at 4% concentration range between 0.432 and 0.485 (Tables 5 and 6); which indicates that the mechanism of release from these formulations is by

both diffusion and swelling. When the gum (native or modified) was used at the 8% concentration, values of n for the formulations were less than 0.430 (Tables 5 and 6), indicating that the mechanism of release is by diffusion.

Table 5: In vitro Release Kinetics from Formulations Prepared by Direct Compression

Direct Compression							
Batch	Zero order	First order	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer	
	R^2	R^2	R^2	R^2	R^2	R^2	N
NG 4	0.765	0.869	0.976	0.840	0.869	**0.976**	0.485
NG 8	0.649	0.805	0.964	0.761	0.805	**0.977**	0.425
PMG 4	0.730	0.846	0.970	0.813	0.846	**0.973**	0.465
PMG 8	0.656	0.815	0.966	0.771	0.815	**0.978**	0.427
CMG 4	0.667	0.869	0.972	0.818	0.869	**0.982**	0.432
CMG 8	0.614	0.876	0.964	0.815	0.876	**0.982**	0.412
HPMC 4	0.655	0.866	0.969	0.814	0.866	**0.981**	0.428
HPMC 8	0.610	0.880	0.966	0.819	0.880	**0.986**	0.410

Table 6: In vitro Release Kinetics from Formulations Prepared by Wet Granulation

Direct Compression							
Batch	Zero order	First order	Higuchi	Hixson-Crowell	Hopfenberg	Korsmeyer	
	R^2	R^2	R^2	R^2	R^2	R^2	N
NG 4	0.745	0.859	0.972	0.827	0.859	**0.973**	0.474
NG 8	0.644	0.809	0.968	0.763	0.809	**0.982**	0.421
PMG 4	0.686	0.815	0.955	0.778	0.815	**0.961**	0.447
PMG 8	0.624	0.793	0.956	0.746	0.793	**0.974**	0.414
CMG 4	0.620	0.853	0.966	0.794	0.853	**0.983**	0.444
CMG 8	0.588	0.873	0.959	0.809	0.873	**0.983**	0.402
HPMC 4	0.622	0.854	0.964	0.796	0.853	**0.982**	0.414
HPMC 8	0.584	0.878	0.961	0.814	0.878	**0.986**	0.400

Factorial Experimental Design

The individual effects of the three parameters used in the factorial experiment; nature of polymer (X_1), concentration of polymer (X_2) and processing technique (X_3) on the response variables are presented in Table 7. A positive qualitative effect signifies that the response variable has increased in magnitude, while a negative effect shows a decrease [15, 16].

Table 7: Individual Coefficients of Process Parameters for Tablet Properties

Factor	Coefficient	Tensile Strength (KgF)	Friability (%)	Disintegration Time (min)
X_1	Effect	0.265	0.5550	5.427
X_2	Effect	0.050	-0.0050	1.607
X_3	Effect	0.195	-0.4800	-2.493

Nature of polymer (X_1) had the highest effect on disintegration time. The ranking for the effect of X_1 on the response variables was Disintegration time > Friability > Tensile strength. Values of the effects were positive on all the variables showing that as nature of polymer changed from native gum to modified gum, values of tensile strength, friability and disintegration time increased. It can also be inferred that modification of the gum would improve the mechanical properties of tablets. However, the difference in the disintegration time of formulations containing modified and native gum was not significant.

The effect of concentration of polymer was highest on disintegration time; ranking was Disintegration time > Tensile strength > Friability. Positive effects were obtained in disintegration time and tensile strength while the effect on friability was negative. These results show that increasing the concentration of gums from 4% to 8% improved the mechanical strength of the tablets. The negative value observed for friability reinforced the increased binding effect of increased polymer concentration leading to decreased tablet friability.

The ranking of effect of Processing technique (X_3) on the response variables was Disintegration time > Friability > Tensile strength. The effect was positive on tensile strength and negative on both disintegration time and friability. This suggests that changing the processing technique from direct compression to wet granulation produced tablets of higher tensile strength due increased bonds formed. The water influx which enhances stronger bonds in wet granulation could also be responsible for the reduction in the time taken for the tablet to disintegrate [29, 30].

Tensile strength was affected most by the parameter X_1 (Figures 3 and 4). The rank order for effect of the parameters was $X_1 > X_3 > X_2$ ($p < 0.05$). Similar trend was observed in friability and disintegration time. This indicates that as parameters changed from low to high level (native gum to modified gum; 4% to 8% polymer concentration; direct compression to wet granulation) the values of tensile strength, friability and disintegration time increased. This shows that when the parameters are applied at the high levels, improve tensile strength and friability of tablets with prolonged disintegration time may be obtained. It

is noteworthy that the prolonged disintegration time is within the officially acceptable limit for compressed tablets [23].

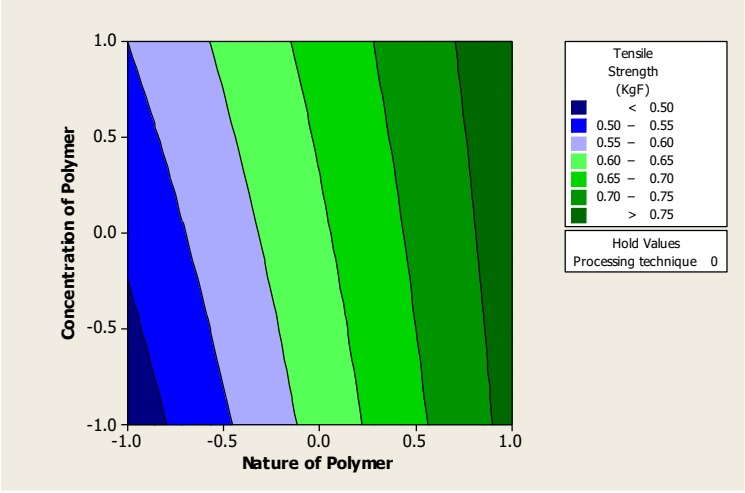

Figure 3: Contour plot of the effect of Concentration and Nature of Polymer on the Tensile strength of tablet formulations

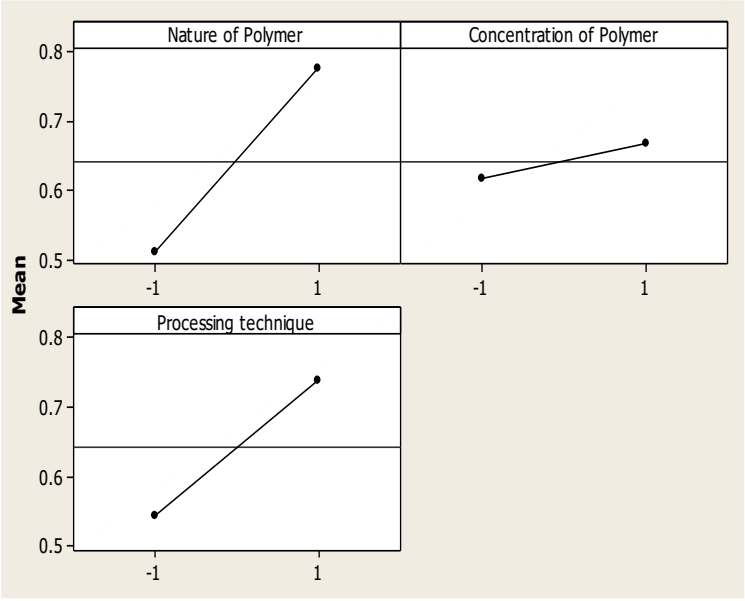

Figure 4: The main effect plots of the different variables on the tensile strength of the tablet formulations

Values of the interaction coefficient are shown in Table 8. Rank orders for the interaction effects are $X_1X_3 > X_1X_2 > X_2X_3$ for tensile strength; $X_1X_3 > X_2X_3 > X_1X_2$ for friability and $X_1X_2 > X_1X_3 > X_2X_3$ for disintegration time. The results indicate that interaction between nature of polymer and processing technique had the greatest effect on the mechanical properties of the tablets while the interaction between nature and concentration of polymer had the greatest effect on the release property.

Table 8: Interaction Coefficients of Process Parameters for Tablet Properties

Factor	Coefficient	Tensile Strength (KgF)	Friability (%)	Disintegration Time (min)
X_1X_2	Effect	-0.030	0.010	-1.672
X_1X_3	Effect	0.095	-0.515	1.428
X_2X_3	Effect	0.020	0.025	0.608

Furthermore the results show that the highest level of factor-factor interaction occurred between X_1 and X_2. The nature of a polymer has been reported to influence greatly its plastoelastic properties [31] and this could in turn affect the mechanical properties of tablets. In the work of Ayorinde and Itiola (2010), [16] it was shown that the effect of concentration of binder is strongly related to the nature of binder employed in the formulation. Thus, the interaction between the nature and concentration produced the greatest effect on the mechanical properties of tablets.

The suitability of Enterolobium gum as a formulation excipient in metronidazole tablet formulations has been determined. Gum modifications, concentration of polymer and the processing technique affect the mechanical and release properties of tablets. This type of study will be valuable in appropriately selecting and combining formulation variables in order to achieve desired formulation objectives.

CONFLICT OF INTEREST:

The authors hereby declare that they do not have any conflict of interest.

REFERENCES

1. Ayorinde JO, Odeniyi MA, Bansal AK. Evaluation of two novel plant gums for bioadhesive microsphere and sustained- release formulations of metformin hydrochloride. Polim Med. **2017**; 47(1): 13 – 23.

2. Adetogun EA, Alebiowu G. Properties of Delonix regia seed gum as a novel tablet binder. Acta pol Pharm drug. **2009**; 66(4): 433 - 438.

3. Samia EA, Babitar EM, Karamalla. Analytical studies on gum exudates of Angeissus leiocarpus. Pakistan nutr. **2009**; 8(6): 782 – 786.

4. Ayorinde JO, Itiola OA, Odeku OA, Odeniyi MA. Influence of binder type and process parameters on the compression properties and microbial survival in diclofenac tablet formulations. Braz J Pharm Sci. **2011**; 47(4): 845 – 854.

5. Ayorinde JO, Odeniyi MA. Evaluation of the suspending properties of new plant gum in Sulphamethoxazole formulations. Int J Pharmacol Pharm Tech. **2012**; 1(2): 47-50.

6. Jani GK, Goswami JM, Prajapati VD. Studies on formulation and evaluation of new superdisintegrants for dispersible tablets. Int J Pharm Excip. **2005**: 2; 37-43.

7. Ayorinde JO, Odeniyi MA. Disintegrant properties of native and modified polymers in metronidazole tablet formulations. Afri J Biomed Res. **2014**; 17(3): 143- 152.

8. Kavlani N, Sharma V, Singh L. Various technique for the modification of starch and the applications of its derivatives. Int Res J Pharm. **2012**; 3 (5): 7 – 12.

9. Vatanasuchart N, Naivikul O, Charoenrein S. Molecular properties of cassava starch modified with different uv irradiatons to enhance baking expansion. Carbohydr Polym, **2005**; 61: 80 – 87.

10. Khan MA, Bhattacharia SK, Kader MA. Preparation and characterization of ultraviolet (uv) radiation cured biodegradable films of sago starch/PVA blend. Carbohydr Polym. **2006**; 63: 500 – 506.

11. Ayorinde JO, Effiong DE, Odeniyi MA. Design and evaluation of oral dissolving films of chlorpheniramine from native and modified Enterolobium cyclocarpum gum. Afr J Biomed Res. **2018**; 21: 175 – 182.

12. Ayorinde JO, Odeniyi, MA. Solid state characterization of two novel gums from Cedrela odorata and Enterolobium cyclocarpum. J Pharm Invest. **2017**; 48(4): 487 – 496.

13. Ayorinde JO, Balogun-Agbaje O, Odeniyi MA. Formulation and evaluation of oral dissolving films of amlodipine besylate using blends of starches with hydroxypropyl methyl cellulose. Polim Med. **2016**: 46(1): 45 – 51.

14. Korsmeyer RW, Gurny RE, Doelker EP, Buri NA, Peppas NA. Mechanisms of solute release from porous hydrophilic polymers. Int J Pharm. **1983**; 15: 25-35.

15. Odeniyi MA, Jaiyeoba KT. Optimization of ascorbic acid tablet formulations containing hydrophilic polymers. Farmacia. **2009**; 57 (2): 157-166.

16. Ayorinde JO, Itiola OA. Individual and interacting effects of formulation variables on the tensile strength and microbial survival in diclofenac tablets. Archiv Pharm Res. **2010**; 33(3): 395 – 403.

17. Ebere IO, Anthony OO, Kunle OO. Solid state characterization of Anacardium occidentale gum. Res J Sci Eng Tech. **2012**; 4(19): 3709-3719.

18. Warsi MH, Ahmad FJ, Rankell AS, Lieberma HA, Schiffmann RF. Drying. In: Khar RK,

Vyas SP, Ahmad FJ, Jain GK, editors. In The Theory and Practice of Industrial Pharmacy. 4th ed. India: CBS Publishers and Distributors; **2013**. p. 89-107.

19. Arul K, Palanisamy S, Rajasekran A. Evaluation of Cassia roxburghiiseed gum as binder in tablet formulations of selected drugs. Int J Pharm Sci Nanotech. **2010**; 2(4): 726-732.

20. Ayorinde, JO, Odeniyi MA, Oyeniyi YJ. Material and compression properties of native and modified plantain starches. Farmacia. **2013**; 60(3): 574 – 590.

21. Franceschini F, Selmin S, Monghetti F, Cilurzo F. Nanofiller for mechanical reinforcement of maltodextrin orodispersible films. Carbohydr Polym. **2016**; 135: 676–681.

22. Odeniyi MA, Oyedokun BM, Bamiro OA. Native and microwave-modified Terminalia mantaly gums as sustained-release and bioadhesive excipients in naproxen matrix tablet formulations. Polim Med. **2017**; 47(1): 35–42.

23. British Pharmacopoeia. The pharmaceutical press, Her Majesty's Stationary office: London, **2010**.

24. Ghule BV, Darwhekar GD, Jain DK, Yeole PG. Evaluation of binding properties of Eulophia campestris wall mucilage. Ind J Pharm Sci. **2006**; 68 (5): 566-569.

25. Odeku OA, Itiola OA. Evaluation of the effects of khaya gum on the mechanical and release properties of paracetamol tablets. Drug Dev Ind Pharm. **2003**; 29: 311 – 320.

26. Adeleye OA, Femi-Oyewo MN, Odeniyi MA, Babalola CO. Compaction and mechanical properties of Cissus populnea gum. Trop J Nat Prod Res. **2018**; 2(10): 447-451.

27. Rippi M, Antikainem O, Niskanen T, Yiruusi J. The effect of compression force on surface structure, crushing strength, friability and disintegration time of erythromycin acistrate tablets. Eur J Pharm Biopharm. **1998**; 46(3): 339-345.

28. Adjei FK, Osei YA, Kuntworbe N, Ofori-Kwakye K. Evaluation of the disintegrant properties of native starches of five new cassava varieties in paracetamol tablet formulations. J Pharm [Internet]. **2017**; Article ID 2326912, https://doi.org/10.1155/2017/2326912.

29. Hixson AW, Crowell JH. Dependence of reaction velocity upon surface and agitation. J Ind Eng and Chem. **1931**; 23: 923-931.

30. Higuchi T. Rate of release of medicaments from ointment bases containing drugs in suspension. J Pharm Sci. **1961**; 50: 874 - 875.

31. Adeleye AO, Odeniyi MA, Jaiyeoba KT. The influence of cissus gum on the mechanical and release properties of paracetamol tablets – a factorial analysis. J Bas Appl Pharm Sci. **2010**; 31 (2): 131-136.

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A Niosomal Gel of Cefoperazone Sodium for Topical Application

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ABSTRACT

The present study endeavors to prepare a niosomal gel of Cefoperazone sodium (CFS), as a novel dermal delivery for the treatment of skin infections. CFS loaded niosomes were prepared using different molar ratio of Tween 80 and Cholesterol by ether injection method using experimental design. The optimized formula was evaluated for DSC, XRPD and AFM. A niosomal gel with the optimized formulation was prepared in Carbopol 934 and were evaluated for gelling properties, *in-vitro* release, *ex-vivo* permeation and skin irritation study on rats. Quality by design was successfully executed to get stable (Zeta potential -30mV), nano sized (365.3 nm) niosomal vesicles. The niosomal gel of CFS showed a pH around 5.5, and a viscosity of 84.13±0.25 cps, enhanced permeation and no skin irritation. Hence, the study depicts that a superior site-specific delivery of CFS can be achieved with a niosomal gel of the drug in the treatment of skin infections.

Keywords: Cefoperazone sodium, custom design, niosomes, niosomal gel, *ex-vivo* evaluation

INTRODUCTION

Niosomes are hydrated non-ionic vesicles of surfactant having the unique potential to entrap both hydrophilic and lipophilic drugs. They are unilamellar or multilamellar vesicles of surfactants with cholesterol or its derivatives, enclosed by an aqueous compartment[1]. The self-assembling properties of surfactants on hydration are responsible for formation of shapes like micelles or planar lamellar bilayer of microscopic and nanoscopic vesicles[2]. They are osmotically stable, non-immunogenic, biocompatible, biodegradable, and act as permeation en-

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hancers[3]. They can easily adsorb or fuse with stratum corneum and can pass through the intra epidermal channels, and diffuse deep into the skin to produce systemic effects. They can increase the fluidity of the skin membrane and results in enhancement in the permeability of drugs when applied topically[4].

Cefoperazone sodium, a semi synthetic broad-spectrum antibacterial drug used in the infections of skin caused by *Pseudomonas aeruginosa, Streptococcus pyogenes* and *Staphylococcus aureus* [5]. It is administered in divided doses of 2 to 4 gm intravenously per day depending on the severity. The drug has low serum half-life (2hours) and bound mostly in the plasma protein which is also reported to be dose dependent[6]. Therefore, an approach to develop a topical formulation of cefoperazone sodium could be beneficial in the treatment of skin infections considering localization of the drug at the site of action with improved patient compliance. As per the reported data, the drug has a low log P value[6], therefore, encapsulation of the drug in the niosomal vesicle could improve permeation of the drug through the skin. The drug can be encapsulated in the aqueous core of the nonionic vesicles. Cholesterol which is a common component in biological membrane, can influence the permeability and fusion of the vesicles through the stratum corneum, can be added to improvise the bilayer property of the nonionic surfactant vesicle[7]. Several studies have reported that the residence time of drug in stratum corneum was enhanced through niosomal delivery as it altered the horny layer properties[8] and enhance the stability of the entrapped drug[9].

Hence, the present study discusses on the development and evaluation of niosomal gel of cefoperazone sodium with targeting of the drug at the site of infection to provide better efficacy and patient compliance over the conventional dosage forms.

METHODOLOGY

Materials

Cefoperazone sodium (CFS) was gifted from Aurobindo Pharma Ltd, Hyderabad, India. Cholesterol was obtained from Loba Chemie, Mumbai, India. Carbopol-940 was purchased from SD Fine Chemicals Limited, Mumbai, India. Rest of the used chemicals and reagents were of analytical grade.

Methods

Employment of custom design in the methods of preparation of niosomes

The niosomes were prepared by ether injection method. The various process parameters like rate of injection, volume of injection and the property of the

materials can affect the encapsulation efficiency and the vesicular size of niosomes[10]. Presence of cholesterol plays an important role in bilayer stability of niosome and entrapment of drug[1]. The software JMP version 13 was availed to estimate the effect of surfactant and cholesterol ratio, and process parameters on the response on niosomes vesicle size, and entrapment efficiency with twelve experimental runs through custom design. A two level (low (-1) and high level (+1)) testing of each variable was done against the responses. From the initial screening study and review of reported literature the composition of surfactant: cholesterol molar ratio (X1) was varied from 6:4 to 8:6, and the process parameters, the rate of injection(X2) and the hydration volume(X3) were varied from 0.5 to 1.5 mL/min, and 10mL to 30mL respectively.

Ether Injection Method for preparation of cefoperazone loaded niosomes (CFS-NIO)

A solution with appropriate molar ratio of cholesterol and surfactant (Tween 80) in ether was prepared. An aqueous solution of the drug (0.067%w/v) was prepared in phosphate citrate buffer at pH 3. The organic phase was slowly injected into the preheated aqueous solution of the drug maintained at 60 °C through a syringe pump. The vaporization of ether leads to the spontaneous formation of lamellar vesicles of the surfactants containing a drug[11]. The resultant drug-loaded niosome formulations (CFS-NIO) were equilibrated at room temperature and stored overnight at 4°C in the refrigerator prior taken for further evaluation.

Evaluation of niosomes

Particle size distribution

Horiba SZ-100 nanoparticle size analyzer was used to determine the particle size of the niosomes. After suitable dilution with double distilled water, the sample of niosomal dispersion was placed in disposable cuvettes for particle size measurements at a scattering angle of 90° at 25.2°C[12]. Three trials were done for determining the average particle size of each formulation.

Estimation of zeta potential

The zeta potential of all the CFS-NIOs was measured in Horiba SZ100. After dilution of the samples with doubled distilled water, three measurements were carried out for each sample at 25.2°C [12].

Estimation of drug entrapment efficiency

Entrapment efficiency of all the CFS-NIO formulations were determined by centrifugation method. Niosomal formulations were centrifugated at 14000 rpm

for 40 min at 4°C. The supernatant layer was separated to estimate the unentrapped drug (F_{drug}). A 0.22µm AST syringe filter was used to filter the supernatant layer. It was suitably diluted with phosphate buffer pH 5.5 and analyzed by UV-visible spectroscopy at λ max 286nm to estimate the free drug. The analytical method for assay was validated prior by establishing a linearity range between 5-25µg/mL with a regression coefficient value of (R^2) 0.9991. The method was found to be accurate, precise, and robust with relative standard deviation (RSD) of less than 2%. The total drug (T_{drug}) was estimated by lysing the equal volume of niosomal dispersion in methanol followed by centrifugation and analyzed spectrophotometrically at 286nm. A blank niosomal dispersion of each formulation was treated in the similar way and used as blank to nullify the effect of excipients in absorbance.

The entrapment efficiency was determined using the following formula [8,13]. Each result represents an average of three trials with standard deviation.

$$\%\ Drug\ entrapment\ efficiency = (Tdrug - Fdrug).100/Tdrug$$

Atomic force microscopy (AFM)

The surface morphology of optimized CFS-NIO was analyzed by atomic force microscopy (AFM). The sample was diluted with deionized water to make a nano dispersion. A drop of this dispersion was placed over a glass slide and covered with a coverslip. The sample was air dried to remove moisture from the sample. AFM was carried out by using Park systems NX-10 AFM at 80 kV . The images were recorded in 2D and 3D scales [14].

Freeze drying of optimized niosomes formulation

Lyophilization was done for the optimized drug loaded niosomal suspension. The lyoprotectants mannitol (15%w/w) and dextrose (5% w/w) were added to the niosomal suspension to yield a sugar: lipid ratio of 3:1. The suspension was subjected to freezing at -80'C for 24 h in a deep freezer (REMI ULT-90) followed by freeze drying in (LABCONCO freeze-dryer, Free-Zone 4.5, USA) at a preset condition of vacuum pressure maintained at a 54×10^{-3} bar and surface temperature held at -54'C for 8 h[15]. The freeze-dried samples were collected and stored in a tightly closed glass vials in a desiccator. The lyophilized sample (LYP-CFS-NIO) were further taken for thermal analysis, x-ray powder diffraction, and surface morphology study.

Differential scanning colorimetry (DSC)

DSC was used for thermal analysis of pure drug (CFS) and LYP-CFS-NIO formulation. The instrument used was DSC-60, Shimadzu, Japan. Each sample was placed in an aluminum pan, sealed with pierced lids, and heated at 5 °C/min over a temperature range of 30–300 °C under a nitrogen purging of 40 mL/min[16].

X-ray powder diffraction (XRPD)

XRPD patterns of the pure drug (CFS) and LYP-CFS-NIO formulation were obtained using an X-ray diffractometer (X' Pert3 powder-Malvern Panalytical). A Cu-Ka radiation was used to analyze the sample between 4° and 100° 2θ with a scan rate of 4°/min. Voltage and current were maintained at 40 kV and 30 mA, respectively[16].

Preparation of niosomal gel CFS-NIO-gel

The LYP-CFS-NIO equivalent to 0.01%w/w drug was incorporated into a gel base of Carbopol 934(2%w/w). The required quantity of Carbopol 934 in a small amount was dispersed in distilled water and hydrated for 4 h. Propylene glycol (7%w/w) was added to the hydrated base. Triethanolamine solution (1%w/v) was used to adjust the pH of the base to 5.5. Finally, distilled water was added to adjust the gel weight to 10g[17]. A gel containing pure drug of the equivalent quantity was prepared in the same manner for comparative evaluation.

Evaluation of gel

pH of gel

Digital pH meter (Digisun Electronics System) was used to determine pH of **CFS**-NIO-gel. It was calibrated before its use. The pH measurement was made in triplicates[18].

Viscosity of gel

The viscosity of CFS-NIO-gel was determined at 25°C by using brook field viscometer. The niosomal gel (20g) was rotated at 10 rpm with spindle 2. Three trials were made for the estimation of viscosity[18].

Drug content for drug-loaded niosomal gel

A known quantity of the CFS-NIO-gel was taken in the Eppendorf tube and diluted with methanol and kept for vortex mixing for 10minutes. An aliquot was withdrawn, filtered with syringe filter, diluted suitably with phosphate buffer pH 5.5, and estimated for drug content spectrophotometrically at 286nm[19,20]. Three trials were run to confirm the estimation.

Spreadability

Spreadability was determined based on slide and drag method. An excess of the CFS-NIO-gel was placed on a glass slide. Another slide was placed over it. To spread the gel uniformly on slide, a weight of 500g was placed on the top of the slides for few minutes. Spreadability was determined by measuring the time to drag a fixed distance after placing a weight of 100 g on the slides[21,22]. Spreadability was calculated by

$$Spreadability = \frac{Mass\ on\ the\ slide\ X\ length\ of\ slide}{Time\ taken\ for\ complete\ separation\ of\ slides}.$$

In-vitro drug release and kinetic study

The *in-vitro* drug release of niosomal gel was carried out in Franz diffusion cell apparatus using 0.22µm dialysis membrane from Himedia. The dialysis membrane was soaked in phosphate buffer pH 7.4 overnight prior use. The receptor compartment was filled with 45mL of phosphate buffer of pH 7.4. A quantity of 1 g gel was placed in the donor compartment. The whole assembly was kept over magnetic stirring and the temperature of the assembly was maintained at 37°0.5°C. An aliquot of 1mL was withdrawn at a suitable time interval and replenished with equal volume of fresh media to maintain sink conditions. The study was carried out for 8h. The aliquots after suitable dilution were analyzed spectrophotometrically at 286nm. The % cumulative drug release was calculated[23]. The release kinetics data were analyzed for zero order, first order, Higuchi, Korsmeyer-Peppas model through linear regression analysis

Ex-vivo diffusion study

The *ex-vivo* studies were executed using the abdominal skin of albino Wistar rats weighing between 250-300gms. To conduct the study an approval from the institutional ethical committee vides the approval number KCP/IAEC/PCEU/39/2019 was procured in advance. The rats were euthanized using excess of carbon dioxide, the abdominal skin was depilated, and rinsed thoroughly with phosphate buffer of pH 7.4. A section of the skin was cut and tied to donor compartment of the Franz diffusion cell such that the dorsal side of the skin projecting the donor compartment. The receptor compartment was filled with 45mL phosphate buffer of pH 5.5. The LYP-CFS-NIO-gel and gel of pure drug containing equivalent drug were taken for permeation study. The receptor compartment was under magnetic stirring. The temperature of the assembly was maintained at 32°0.5°C. The samples were withdrawn at constant interval of time for 8h, the same volume of fresh solution was replaced to maintain sink condition. The withdrawn samples were suitably diluted and analyzed spectro-

photometrically at 286nm[24]. From the data the permeation constant (Kp) and the steady state permeation flux (Jss) were determined.

Skin irritation test

The skin irritation study of LYP-CFS-NIO-gel was performed on six wistar albino rats. The dorsal and ventral side of the rats were shaved to remove the hairs. Marking was done on both sides. Ventral and the dorsal side served as control and test, respectively. Gel was applied once a day for three consecutive days. The skin irritation was recorded by observation for any skin sensitivity reactions like swelling, redness, and skin rash[25,26].

RESULTS and DISCUSSION

Optimization of the custom design through evaluation of particle size, zeta potential and % entrapment efficiency of CFS-NIO

The custom design constitutes a radical approach to find the possibility of investigating a high number of variables at different levels with minimal experimentation. The use of center points in the design increased the confidence level and helped to minimize the errors on experimentation. The experimental runs of the twelve formulations resulted the responses %entrapment efficiency (%EE), and particle size (PS) as shown in table 1.

Table 1: Factors and observed responses for the custom design

Formulation code	Surfactant: cholesterol (Molar ratio) (X1)	Rate of injection (mL/min) (X2)	Hydration volume (mL) (X3)	Particles size(nm) (PS)	Drug entrapment efficiency (%) (EE)
F1	1	1	1	317.4± 5.09	98.93±0.001
F2	-1	-1	1	416.5± 4.04	98.93±0.003
F3	0	0	0	499± 0.05	98.89±0.009
F4	1	-1	-1	339.8± 3.08	98.56±0.052
F5	-1	1	-1	433.5± 6.08	98.76±0.001
F6	-1	1	1	453.9±7.03	99.11±0.025
F7	1	-1	-1	361.3± 9.05	98.41±0.01
F8	1	-1	1	644.3± 507	98.81±0.085
F9	0	0	0	412.1±9.44	98.92±0.096
F10	1	1	-1	290.4±8.06	98.98±0.034
F11	1	1	1	249±6.01	98.99±0.047
F12	-1	-1	-1	378.5±8.08	98.94±0.011

Regression analysis was done to generate a relationship between factors and responses using JMP V13 software. The response surface diagrams depicted the significant effect of composition, hydration volume and rate of injection on entrapment efficiency. The particle size of the niosomes were greatly affected with rate of injection and hydration volume. The three-dimensional (3D) response surface graphs represented the most statistically significant variables on the evaluated responses as shown in figure 1.

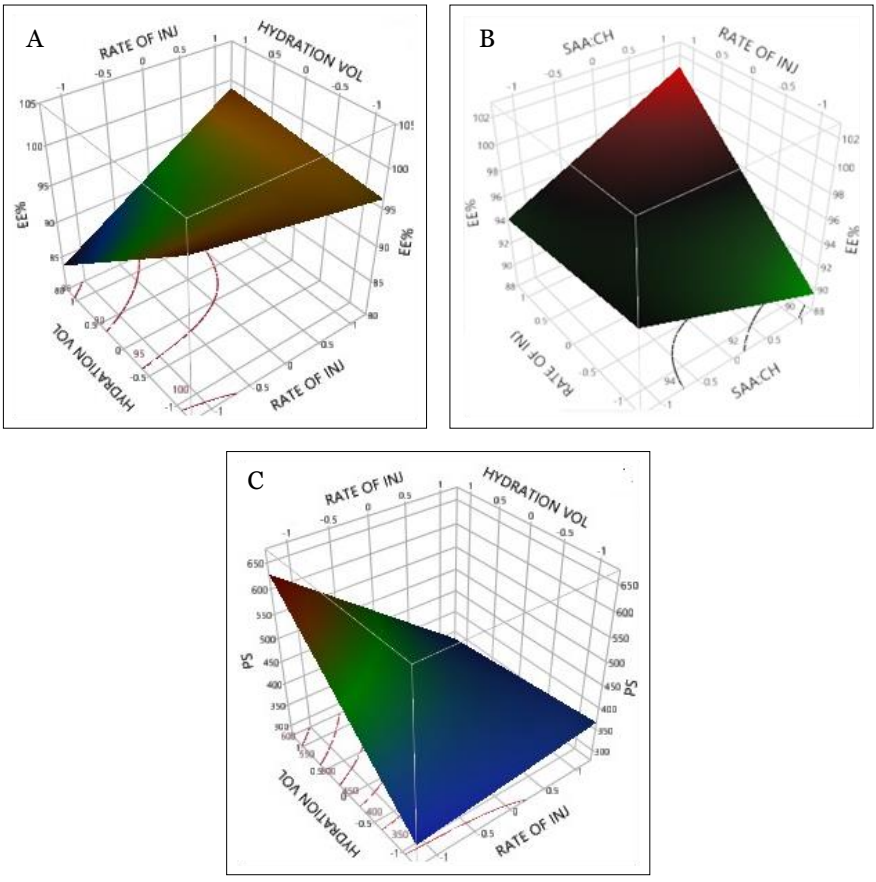

Figure 1: Response surface diagram of variables on the three responses A: %EE, B: %EE C:PS

The response surface diagrams revealed that higher the hydration volume the higher the EE. The particle size was significantly affected by the rate of injection. The effect of the various factors on the responses were estimated through parameter sensitivity analysis and listed in table 2.

Table 2: Parameter sensitivity analysis

Factors	Prob> t	
	% Entrapment Efficiency (EE)	Particle Size (PS)
SAA:CH	0.042*	0.088
Rate of injection	0.036*	0.013*
Hydration	0.010*	0.011*

*indicates significance

The best-fitted model for the design was found to be the quadratic model when the validation was carried out at a significance level of P<0.05. The ANOVA study showed the model was significant for estimation of the effects of compositions and process parameters on entrapment efficiency and particle size as shown in figure 2 in the predicted vs observed graph. The regression coefficient value of more than 0.9 proved further the significance of the model with respect to all the responses.

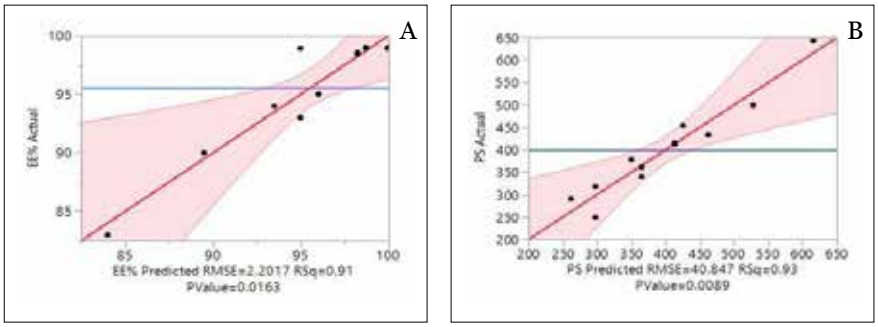

Figure 2: Actual vs predicted plot of variables on the two responses A: %EE, B: PS.

The model optimization was carried out at desirability of 0.78 and an optimized condition was predicted at a SAA/CH molar ratio of 7:5, rate of injection 1 mL/min and a hydration volume of 20 mL to yield a drug loaded niosome with high EE and small PS. The optimized formulation was prepared with the predicted parameters and evaluated for the responses. The listed experimental values of the optimized product prepared are shown in table 3. The responses of the optimized product were close to the predicted values with low percentage bias, suggesting the rationality and reliability of the model.

Table 3: Comparison of the predicted and experimental values of the optimal condition

Responses	Predicted	Experimental	%Bias
%Entrapment efficiency	96.65	94±0.66	2
Particle size (nm)	374	365.3±0.82	2.94

The zeta potential of the optimized formulations was found to be -30mV. The particle size and the zeta potential of the optimized formulations are shown in figure 3.

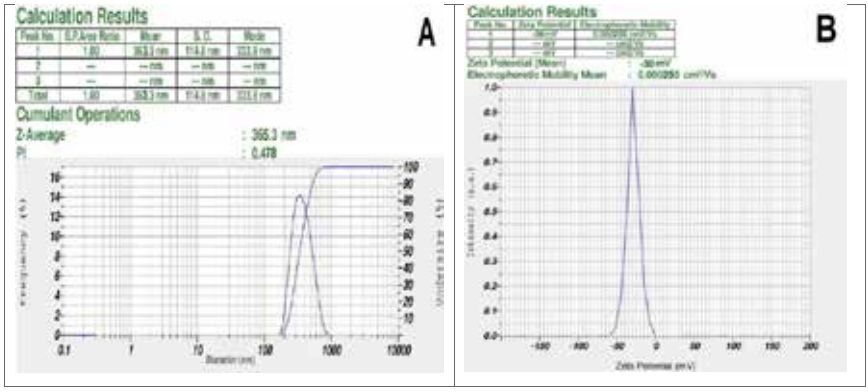

Figure 3: Particle size (A) and zeta potential (B) of optimized formulation

A stable vesicular system was predicted from the study of surface charge[27]. Therefore, the proposed design was capable to produce CFS loaded stable nano sized niosomes.

Atomic force microscopy (AFM)

The surface morphology of the niosomes were shown in figure 4, which revealed the formation of spherical and smooth surface niosomes. The AFM images in different scales further revealed the formation of spherical nano sized particles of similar size range as predicted by the design.

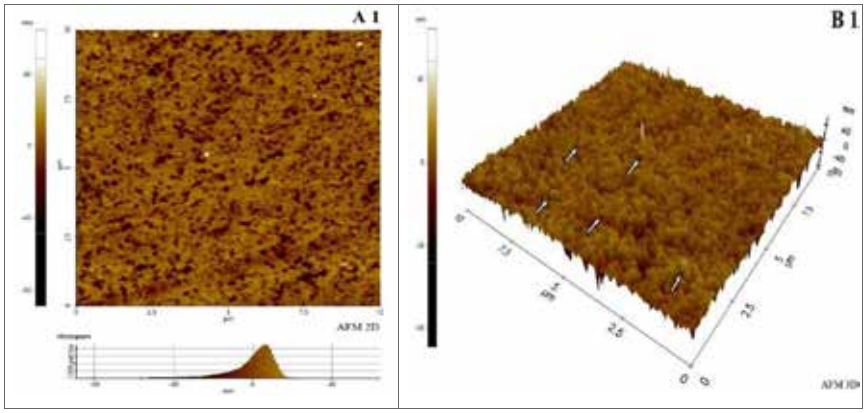

Figure 4: AFM images of optimized drug-loaded niosomes formulation (A1, 2D image B1, 3D Image)

Differential scanning colorimetry (DSC)

The DSC thermogram (figure 5) of the pure drug showed an endothermic peak corresponds to its melting point 180°C, whereas the freeze-dried formulation (LYP-CFS-NIO) revealed a shift in the peak to 149 °C, showing a decrease of phase transition temperature and heat (ΔH), which was an indication of the localization of drug inside the bilayer of lipids and surfactant. This agrees with the reported studies that the presence of cholesterol affects the gel liquid transition temperature of the vesicles[28]. Presence of long alkyl chain and hydrophilic moiety in tweens showed greater entrapment efficiency in niosomes while presence of cholesterol ensures greater bilayer stability[29].

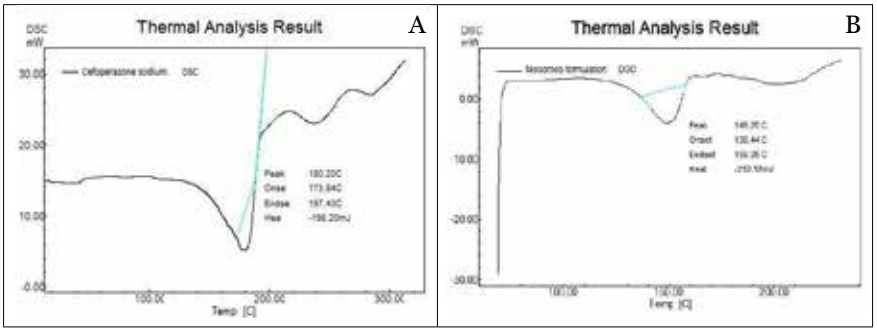

Figure 5: DSC thermograms of a pure drug(A) and Formulation (B).

X-ray powder diffraction (XRPD)

The peak intensities of pure drug and the optimized freeze-dried formulations at various diffraction angles are represented in figure 6. The pure drug showed high intensity peaks whereas the peak intensities of the same peak were reduced in the formulation graph. The high-intensity peaks of the pure drug represented its crystalline nature, while the optimized formulation showed the appearance of same peaks with low intensities. The low intensities of the peak area were attributed to the localization of the drug in the lipid and surfactant matrix and was an indication of high entrapment Which was in confirmation with DSC thermograms[16].

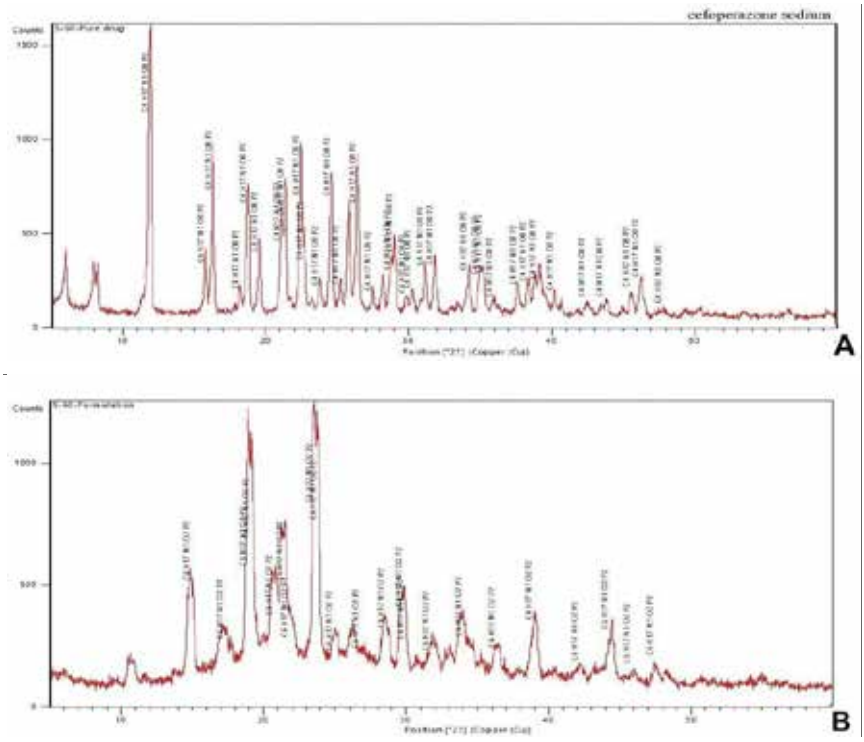

Figure 6: PXRD patterns of a pure drug (A) and formulation (B)

Evaluation of gel

The LYP-CFS-NIO-gel showed a pH around 5.5 optimal to the skin conditions, a viscosity, drug content and spreadability of 84.13±0.25 cps, 95±0.52%, and 7.63 ± 0.125gm.cm/sec respectively. Therefore, the gel was found to be suitable for dermal application based on its pH, mechanical and rheological properties.

In-vitro release and kinetics study

The *in-vitro* release of the LYP-CFS-NIO-gel revealed a slow release of drug upto 86% in 8 hours as presented in figure 7. The release kinetics followed Higuchi model as per the highest regression coefficient value(R^2). The Korsmeyer-Peppas modelling yielded a release component "n" value of 0.57, indicated that the drug release followed quasi-Fickian diffusion model with a matrix swelling and diffusion of the drug from the formulation.

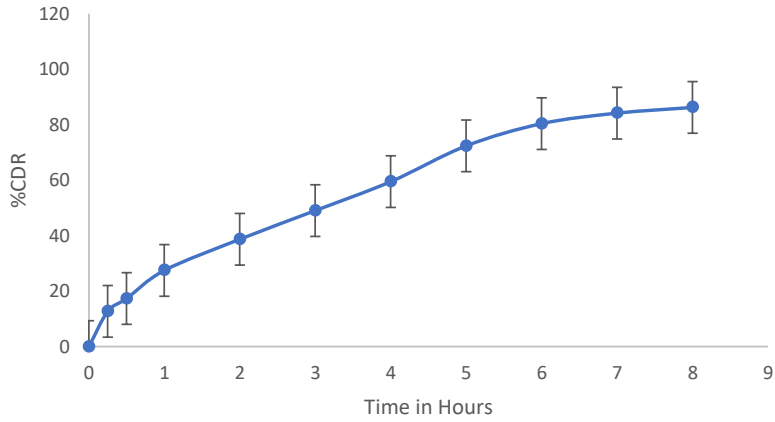

Figure 7: *In-vitro* release study of niosomal gel of CFS.

Ex-vivo permeation study

The *ex-vivo* permeation study unveiled that the niosomal gel showed higher penetration than the non niosomal gel of pure drug as shown in figure 8. It was further proved by the calculation of permeation parameters as mentioned in table 4. The niosomal gel had a 3.28 times higher flux value than the non-niosomal gel of cefoperazone sodium. This observations attributes to the formulation characteristics, as niosomes can be effectively used as permeation enhancer.

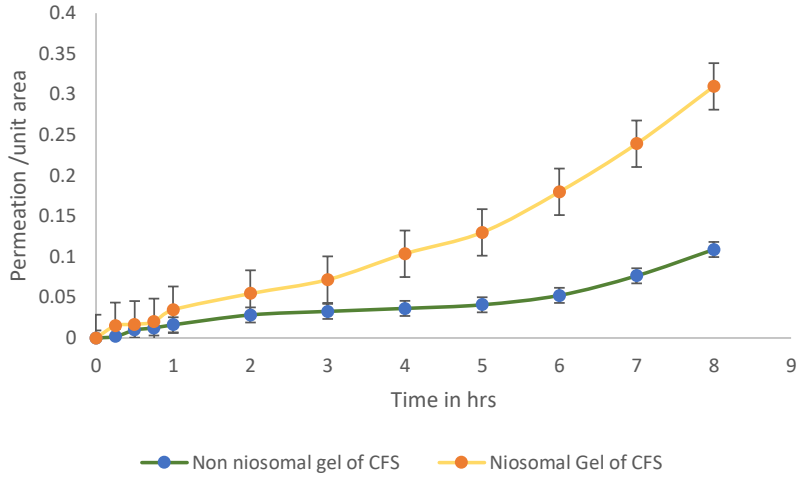

Figure 8: *Ex-vivo* permeation study of niosomal gel and non niosomal gel of CFS.

Table 4: Permeation parameters

Formulation	Steady state flux(mg/cm^2/hr)	Permeation constant (Kp) cm/hr	Drug permeation at 8 hours (mg/cm$^{2)}$
LYP-CFS-NIO-gel	0.032	0.016	0.309
Pure drug gel	0.009	0.005	0.108

Skin irritation study

The results of the skin irritation study revealed that following three days application of CFS-NIO- gel, there was no signs of skin irritation, no evidence on patchy or severe erythema associated with edema. Therefore, the gel was found to be suitable for dermal application.

The present study was designed to develop a novel delivery of cefoperazone sodium in niosomal gel for the treatment of skin infections. A highly permeable gel was prepared with niosomal cefoperazone sodium in Carbopol base, by screening of factors with custom design using JMP V13 software. The CFS-NIO were characterized by their particles size, drug entrapment efficiency and surface charge. The optimized formulation was evaluated for AFM, DSC, XRPD studies and revealed the formation of nanosized stable vesicles with high encapsulation of drug. The CFS-NIO loaded Carbopol 934 gel showed its good mechanical and rheological property. The *in-vitro* release study showed a quasi Fickian release of the drug. The *ex-vivo* permeation and skin irritation study proved the improved penetrability and suitability of the gel for dermal appli-

cation, respectively. Therefore, an effective transdermal delivery of CFS can be made with the prepared niosomal gel of CFS. These findings can create a paradigm for future studies for superior delivery of cefoperazone sodium in the treatment of skin infections.

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REFERENCES

1. Moghassemi S, Hadjizadeh A. Nano-niosomes as nanoscale drug delivery systems: An illustrated review. J Control Release. **2014**;185: 22-36.

2. Mahale NB, Thakkar PD, Mali RG, Walunj DR, Chaudhari SR. Niosomes: novel sustained release nonionic stable vesicular systems—an overview. Adv Colloid Interface sci. **2012**; 183:46-54.

3. Jiao J. Polyoxyethylated nonionic surfactants and their applications in topical ocular drug delivery. Adv Drug Deliv Rev. **2008**; 60:1663-73.

4. Fathalla D, Abdel-Mageed A, Abdel-Hamid F, Ahmed M. *In-vitro* and *in-vivo* evaluation of niosomal gel containing aceclofenac for sustained drug delivery. Int J Pharm Sci Res. **2014**; 1:105.

5. https://www.glowm.com/resources/glowm/cd/pages/drugs/c028.html.

6. https://www.drugbank.ca/drugs/DB01329.

7. Liu T, Guo R, Hua W, Qui J. Structure behaviors of hemoglobin in PEG6000/Tween 80/Span 80/H2O niosome system. Colloid Surf A Physicochem Eng Aspects. **2007**; 293: 255-61.

8. Ibrahim MM, Shehata TM. The enhancement of transdermal permeability of water soluble drug by niosome-emulgel combination. J Drug Deliv Sci Technol. **2012**;22(4):353-59.

9. Di Marzio L, Esposito S, Rinaldi F, Marianecci C, Carafa M. Polysorbate 20 vesicles as oral delivery system: In vitro characterization. Colloids and Surfaces B: Biointerfaces. **2013**;104:200-06.

10. Nahar S, Islam S, Khandaker S, Nasreen W, Hoque O, Dewan I. Formulation and Evaluation of Metoprolol Tartrate Loaded Niosomes Using 23 Factorial Design. J Pharm Res Int. **2018**;22(6):1-17.

11. Srinivas S, Kumar YA, Hemanth A, Anitha M. Preparation and evaluation of niosomes containing aceclofenac. Dig J Nanomater Bios. **2010**;5(1):249-54.

12. Bhattacharyya S, Sogali B. Application of Statistical Design to Assess the Critical Process Parameters of Ethanol Injection Method for the Preparation of Liposomes. Dhaka Univ J Pharm Sci. **2019**;18(1):103-11.

13. Kumbhar D, Wavikar P, Vavia P. Niosomal Gel of Lornoxicam for Topical Delivery: In vitro Assessment and Pharmacodynamic Activity. AAPS Pharm Sci Tech. **2013**;14(3):1072-82.

14. Gogoi H, Mani R, Bhatnagar R. A niosome formulation modulates the Th1/Th2 bias immune response in mice and also provides protection against anthrax spore challenge. Int J Nanomed. **2018**;13:7427-40.

15. Jin Y, Wen J, Garg S, Liu D, Zhou Y, Teng L, Zhang W. Development of a novel niosomal system for oral delivery of Ginkgo biloba extract. Int J Nanomed. **2013**;8:421-30.

16. El-Sayed MM, Hussein AK, Sarhan HA, Mansour HF. Flurbiprofen-loaded niosomes-in-gel system improves the ocular bioavailability of flurbiprofen in the aqueous humor. Drug dev ind pharm. **2017**;43(6):902-10.

17. Shirsand S, Kanani K, Keerthy D, Nagendrakumar D, Para M. Formulation and evaluation of Ketoconazole niosomal gel drug delivery system. Int J Pharm Investig. **2012**;2(4):201-07.

18. Shilakari Asthana G, Asthana A, Singh D, Sharma P. Etodolac Containing Topical Niosomal Gel: Formulation Development and Evaluation. J Drug Deliv. **2016**;2016:1-8.

19. Acharya A, Dhakal P and Khadka D: Formulation and Evaluation of Transdermal Gel of Lornoxicam and its Delivery by Passive and Inotophoresis Method: A Comparative Study. Int J Pharm Sci Res **2016**; 7(2):810-18

20. Narayana RC, Harish NM, Gulzar A M, Prabhakara P, Singh AK, Subrahmanyam EV. Formulation and in vitro evaluation of in situ gels containing secnidazole for vaginitis. Yakugaku Zasshi. **2009**;129(5):569-74.

21. Singh S, Parashar P, Kanoujia J, Singh I, Saha S, Saraf SA. Transdermal potential and antigout efficacy of Febuxostat from niosomal gel. J drug deliv sci technol. **2017**; 39:348-61.

22. Hardia A, Jamindar D, Mahajan A, Hardia A. Formulation and In Vitro and Skin Permeability Evaluation of Dexamethasone Loaded Niosomal Gel. Asian J Pharm Res Dev. **2017**;5(2):1-9.

23. Payyal SP, Rompicherla NC, Sathyanarayana SD, Shriram RG, Vadakkepushpakath AN. Microemulsion Based Gel of Sulconazole Nitrate for Topical Application. Turk J Pharm Sci. **2020**;17(3):259-64.

24. Arafa MG, Ayoub BM. DOE Optimization of Nano-based Carrier of Pregabalin as Hydrogel: New Therapeutic &Chemometric Approaches for Controlled Drug Delivery Systems. Sci Rep. **2017**; 7:41503.

25. Shirsand SB, Kumar GR, Keshavshetti GG, Bushetti SS, Swamy PV. Formulation and evaluation of cotrimoxazole niosomal gel for topical application. RGUHS. J Pharm Sci. **2015**;5(1): 32-8.

26. Patel D, Dasgupta S, Dey S, Roja Ramani Y, Ray S, Mazumder B. Nanostructured lipid carriers (NLC)-based gel for the topical delivery of aceclofenac, preparation, characterization, and in vivo evaluation. Sci Pharm. **2012**;80(3):749-64.

27. Detroja C, Chavhan S, Sawant K. Enhanced antihypertensive activity of candesartan cilexetil nanosuspension: formulation, characterization and pharmacodynamic study. Sci Pharm. **2011**;79(3):635-51.

28. Kumar GP, Rajeshwarrao P. Nonionic surfactant vesicular systems for effective drug delivery—an overview. Acta Pharm Sin B. **2011**;1(4):208-19.

29. Yeo LK, Olusanya TOB, Chaw CS, Elkordy AA. Brief Effect of a Small Hydrophobic Drug (Cinnarizine) on the Physicochemical Characterisation of Niosomes Produced by Thin-Film Hydration and Microfluidic Methods. Pharmaceutics. **2018**;10(4):185.

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Physical Performance Enhancing Effects of The Aqueous Extract of *Alysicarpus Ovalifolius* in Mice

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ABSTRACT

Alysicarpus ovalifolius (fabaceae) is an herb used as ingredient of tea that is consumed as a tonic in Northern Nigeria. This study evaluated effects of aqueous extract of the aerial parts of *Alysicarpus ovalifolius* (AO) on physical performance in Swiss albino mice. Phytoconstituents were identified by HPLC. Performance was assessed by exhaustive swim, rotarod, four limb-hanging and hypoxia test. Effects on diazepam-induced sleep was also evaluated. HPLC detected presence of caffeic (60.34%) and chlorogenic acids (3.44%) and rutin (0.19%). The extract (100 – 400mg/kg) produced significant ($p<0.05$) dose-dependent increase in swimming time; increased time spent on the rotarod and also increased duration in the four-limb hanging test. No significant effect was observed in endurance to hypoxic condition. Diazepam-induced sleeping time was significantly decreased in AO-treated animals. These observations were comparable to caffeine (15mg/kg). The aqueous extract of *Alysicarpus ovalifolius* increased physical performance in mice by enhancing endurance, muscle strength and co-ordination.

Keywords: *Alysicarpus ovalifolius*, endurance, swim, muscle strength

INTRODUCTION

Psychotropic drugs have been defined as substances used mainly to relieve

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symptoms of mental or emotional origin without impairing consciousness[1] and psychoactive stimulants are psychotropic substances that can stimulate the central nervous system to cause excitation, increased alertness and elevated moods[2]. They are used clinically for the management of neurological conditions[1]. These agents are used in clinical settings for the management of mood and attention disorders such as depression and attention deficit hyperactivity disorders (ADHD), as well as for relieve of nasal congestion and also in orthostatic hypotension and postural orthostatic tachycardia syndrome[3,4,5].

The use of these substance in healthy individuals is an increasingly alarming global phenomenon. An earlier study showed the category of users cutting across the different strata of society[6]. Stimulant use has been reported globally amongst students as well as the adult population irrespective of gender, educational, financial or employment status[7,8,9,10].

Non-medical reasons that have been given for use of psychoactive stimulants by healthy individuals include enhanced physical and mental alertness, improved memory and attention, increased sociability, euphoria, increased arousal, to loosen inhibitions, improve moods, motivation, relief of pain, inhibition of fatigue thus improving endurance and productivity. They have also been applied to cosmetics and for weight loss due to the ability to suppress appetite. These effects have been demonstrated in studies involving animal and human subjects[2,11,12,13]. As a result of these attributes of the CNS stimulants, there is high propensity for misuse of these substances for recreational purposes with resultant deleterious effects on the normal functions on the individuals that use them. Identified harmful effects may include headache, decreased appetite, psychological and physical dependence and addiction[9,14,15].

A wide range of substances can be included in this category ranging from substances with mild effects such as caffeine in coffee and energy drinks, methylxanthines as occurring in tea, nicotine which is present in tobacco and kolanut; to prescription analgesics and other substances like cocaine and amphetamines that have profound activities on the physiological processes of the organism[10].

Many plants containing phytochemical compounds have been reported to have stimulant effects and have been demonstrated to improve stamina[5]. 'Gadagi' is a type of herbal tea made using *Alysicarpus ovalifolius* alone or in combination with other plant parts. This is consumed by persons engaged in labour demanding activities to improve alertness, cognition and gain strength for enhanced performance especially at physically demanding tasks[16].

Alysicarpus ovalifolius (Schumach. & Thonn.) J. Leónard is an annual herb that is wide spread in West and East Africa as well as Asia and the United States. The weed is a protein-rich fodder for live-stock and has been reportedly used as wound medicine[17] (t'mannetje, 2002). The CNS stimulant action for this plant has been reported[18] as well as its use as stimulant for child birth[19]. This study was designed to evaluate the effects of the aqueous extract of *Alysicarpus ovalifolius* on physical performance and endurance using mice.

METHODOLOGY

Plant material

The aerial parts of *Alysicarpus ovalifolius* were collected from Suleja, Niger State, Nigeria, in the month of August 2017 by Mal Ibrahim Muazzam. The plant was identified and authenticated by the curator of the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD) Idu, Abuja, Nigeria, where a voucher specimen (NIPRD/H/6920) was prepared and deposited for future reference.

Collection and extract preparation

The aerial parts of the plant were collected by cutting with a pair of scissors about 2cm off the ground. Sand particles and other debris were shaken off. The plant material was then washed and air-dried under a shade until a constant weight was obtained. This was further cut with a pair of scissors and subsequently pulverized using a pestle and mortar to obtain a coarse material.

A decoction of the plant material was prepared by boiling one hundred and fifty grams (150 g) of the crushed plant material in water for 20 min. This was then allowed to cool overnight after which it was filtered using a muslin cloth and the marc pressed to strain out as much liquid as possible. The filtrate was further filtered using a Whatman filter paper No 1. This was then evaporated to dryness on a water bath to afford a dark-brown solid (the extract) with a yield of 11.65 % $^{w}/_{w}$ and stored in a refrigerator in an air-tight container.

Phytochemical Test

High Performance Liquid Chromatography (HPLC) Analysis

The bioactive constituents of the extract were analyzed by high performance liquid chromatography. The HPLC consisted of Ultra-Fast LC-20AB equipped with SIL-20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, 5µm VP-ODS C18 and dimensions (4.6 x 150 mm). The chromatographic con-

ditions included mobile phase: 0.2% v/v formic acid and acetonitrile (20:80); isocratic mode; flow rate 0.6 ml/min; injection volume 10 µl of 50 mg/ml solution extract in the mobile phase; detection UV 254 nm. Reference standards rutin, caffeic acid, ferulic acid and chlorogenic acid respectively were analyzed under the same condition as the extract. The HPLC operating conditions were programmed to give the following: solvent A: 80%, solvent B: 20%, column oven temperature was 40 °C and total run time of 20 minutes[20].

Animals

Swiss male albino mice (25 – 29 g), obtained from the Animal Facility Centre (AFC) of the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria were used for the studies. All animals were housed under ambient conditions and fed on standard rodent diet and clean drinking water ad libitum. Animals were approved for this study based on the experimental and animal handling conditions which were in compliance with the ethical requirements of the Institutional Animal Care and Ethics (ACE) Committee (NIPRD/05.03.05-08).

Swim Test

Mice were weighed and randomly placed into five groups of 6 mice each. Group 1 was administered distilled water (negative control), group 2 served as the positive control and received caffeine 15 mg/kg (standard drug), while groups 3 – 5 test groups were given graded doses of the extract at 100, 200 and 400 mg/kg (respectively). Extract/drug were administered orally by means of an orogastric cannula. Plastic containers with measurements of 32 x 50 x 35 cm were filled with water maintained at temperature of 33 ± 1°C to a marked level of a depth of 25 cm. Sixty minutes after treatment a weight equivalent to 10% of the body weight was attached to the tail of each mouse. The mice were then placed individually into the water with a stopwatch started simultaneously. The mice were allowed to swim to exhaustion (when the animals sank below the level of the water and could not come back up within 7 sec) after which the mice were removed, toweled and placed under a lamb to dry[21].

Rota-rod test

A rotarod device (Ugo Basile, 7560, Milano, Italy) was used for this study. Mice were pre-trained to remain on moving rods of 3 cm diameter. Mice that fell off the rod were immediately placed back on the rod. However, animals that fell up to three times during the trial period were removed. The animals were subsequently grouped into five groups (n = 6) and treated with distilled water, caffeine 15 mg/kg (o.p) or extract (100, 200 and 400 mg/kg). Sixty minutes

post-treatment, animals were simultaneously placed in separate lanes on the stationary rod. The motor was then turned on and the rod allowed to accelerate up to 20 rpm and held at that rate. The mice were allowed to remain until they fell off the rotating bars. Tests began when acceleration was started and ended when an animal fell off the rod. The procedure was repeated three times at 15-min interval. The latency to fall was recorded. Endurance was interpreted as increased duration of stay on the rotarod[22,23].

Four-limb hanging test

Treated mice were individually made to hang on all four limbs from the underside of a wire mesh such that the limb tension opposed their body weight. The mesh was held at a height of 30 cm and the table top lined with soft padding so as to cushion any mouse that fell off the grid. The timer was started when the mice held onto the mesh and stopped when the animals fell off or reached a maximum holding time of 20 min. Test was repeated three times for each mouse with an interval of 2 min[24]. Prior to the test, mice were trained to hang on the wire mesh. Mice that fell off were immediately placed back on the wire. Animals that fell up to three times during the trial period or jumped off were removed from the study.

Hypoxia Endurance test

Treated mice were placed individually in airtight containers with capacity of 300 ml. A timer was started immediately and then stopped at the onset of convulsion in the mice. The animals were subsequently exposed to air and allowed to recover. The latency to onset of convulsion was taken as the endurance to hypoxia[25].

Diazepam induced sleeping time

The test subjects were randomly placed into 5 groups of 7 animals each. The mice were treated with distilled water, caffeine 15 mg/kg (o.p) or extract (100, 200 and 400 mg/kg). Sixty min later, each animal received diazepam 25 mg/kg (i.p.). The time taken for loss of the righting reflex indicated the onset of sleep, while the time between the loss and recovery of the righting reflex for each mouse was taken as the duration of sleep. The period of onset and duration of sleep were calculated for each mouse[26].

Statistical analysis

Group data were presented as Mean ± SEM. The data on exhaustive swim, diazepam induced sleep and hypoxia endurance tests were analyzed by one-way ANOVA followed by a post hoc Dunnet's test and two-way ANOVA followed by

a post hoc Bonferroni test was used to compare the data in the rotarod and four limb hanging tests. Graphpad PRISM® 6.0 was used. The level for statistical significance was set at $p<0.05$.

RESULTS and DISCUSSION

The study was designed to investigate the effects of the aqueous extract of the aerial part of Alysicapus ovalifolius (AO) on physical activity using mice which had been subjected to exercise and identify the bioactive compounds that may be involved in the activity. The HPLC chromatogram (Figure 1) showed that nine peaks were detected with retention times in minutes of 3.67, 4.56, 6.44, 9.52, 10.54, 11.35, 14.12, 15.55 and 16.60. Identification of the phytoconstituents of the extract was achieved by comparing their retention times with those of the reference standards analyzed under the same experimental condition as the extract. Compound with retention time of 3.67, 4.56 and 6.44 minutes corresponded to chlorogenic acid, caffeic acid and rutin (Figure 1) which was equivalent to 3.44 %, 60.34% and 0.19 % respectively of the contents of the extract.

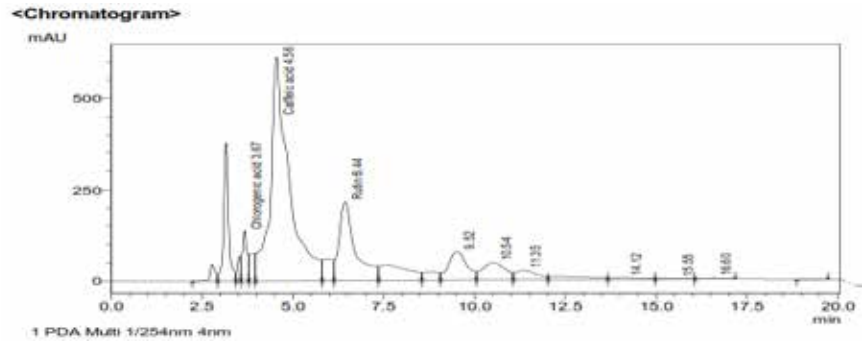


Figure 1: HPLC chromatogram of the aqueous extract of Alysicarpus ovalifolius (AO) extract with detection UV at 254 nm showing 9 peaks which include caffeic acid (60%), chlorogenic acid (3.44%) and rutin (0.19%).

Swim endurance and rotarod animal models test for fatigue in animals[27]. Treated animals subjected to exhaustive swim test, demonstrated significant dose-dependent increase in swimming time in all extract treated animals with 200 and 400 mg/kg exhibiting significant ($p<0.05$) increase (Table 1). The swimming endurance test is an experimental exercise model that assesses exercise durability[28]. Swim test when applied in laboratory animals directly measures fatigue thus evaluating endurance capacity. Prolonged swimming time connotes reduced susceptibility to fatigue and enhanced endurance[29]. The prolonged swim time exhibited by the extract suggests the extract's effect to delay the onset of exhaustion[30].

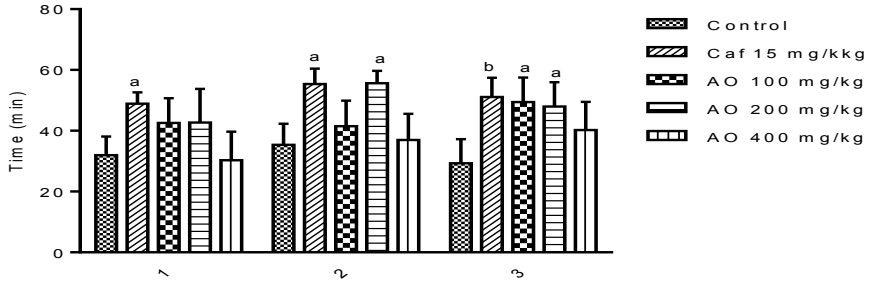
Table 1: Effect of aqueous extract of *Alysicarpus ovalifolius* (AO) on exhaustive swim test.

Treatment	Dose (mg/kg)	Duration of Swimming (min)
Distilled water	10 ml/kg	35.75 ± 16.87
Caffeine	15	119.20 ± 8.46[a]
A. ovalifolius	100	91.75 ± 24.30
A. ovalifolius	200	110.00 ± 19.65[a]
A. ovalifolius	400	138.90 ± 20.61[b]

Results are presented as mean ± SEM (n=6). [a]$p<0.05$, [b]$p<0.01$ significant difference between distilled water and treated groups.

In the rotarod test, all animals successfully completed 3 successive tests on the horizontal rotarod. Pre-treatment with the extract at 100 and 200 mg/kg prolonged the time animals stayed on the rotating rods, however time spent on the rods at 400 mg/kg group was observed to be lower compared to the 100 and 200 mg/kg groups. The increase in time spent on the rotating rod was significant ($p<0.05$) for the extract at 100 and 200 mg/kg and caffeine when compared to the distilled water group. The endurance period was highest in the 200 mg/kg treated group (Figure 2).

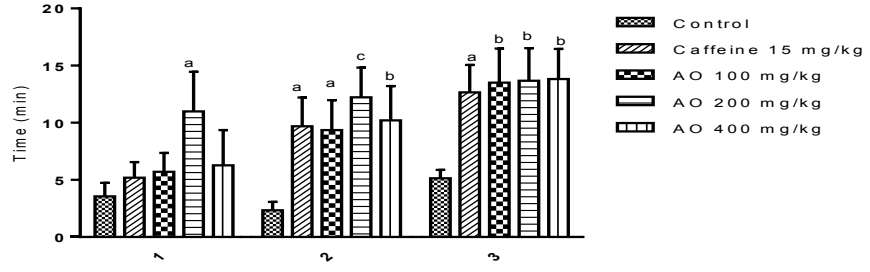
This test has been used to assess balance and coordination in motor performance and endurance in rodents[31]. The accelerating rotarod eliminates the need for extensive training or the introduction of maximal time limit for performance[32]. This model measures an animal's ability to maintain itself on a rod being turned at accelerating speed, thus it is used to test drugs that may interfere with skeletal muscle co-ordination and cause motor impairment[33,34]. Fatigue may impair muscle co-ordination thereby hampering performance at tasks that require motor skill[30]. The increase in the duration of the task performed by the mice on the rotarod may suggest an enhancement of motor co-ordination in animals after extract treatment or improvement of resistance to physical stress[35].



DW = Distilled water, CAF = Caffeine. Values are mean ± SEM (n = 6); [a]p<0.05, [b]p<0.01 significant vs control (Two-way ANOVA followed by Dunnet's post hoc test).

Figure 2: Effect of aqueous extract of *Alysicarpus ovalifolius* (AO) on rotarod endurance test.

In the four-limb hanging test, all animals successfully completed all the trials. The period that the animals hung on the mesh was prolonged on administration of the extract and caffeine. The hanging time increased with successive tests in all treated animals. The observed increase was significant (p<0.05) for the extract and caffeine on comparison to the negative control group. The four-limb hang time increased with subsequent trials, with 200 mg/kg group exhibiting the longer duration on all three sessions (Figure 3). This test assesses muscle strength in all four limbs of the mice and can be applied to demonstrate neuromuscular impairment and the lack of coordination or to study the effects of genetic or pharmacologic treatments on skeletal muscle functionality[24]. In this test, administration of the extract did not impair co-ordination rather skeletal muscle activity was enhanced thus implying that the extract possessed the potential to enhance muscle activity and endurance[36].



DW = Distilled water, Caf = Caffeine. Values are mean ± SEM (n = 6); [a]p<0.05 [b]p<0.01 significant vs control (Two-way ANOVA / Dunnet's post hoc test).

Figure 3: Effect of *Alysicarpus ovalifolius* extract (AO) on Four-Limb Hanging test.

In the hypoxia endurance test the time spent by mice in hypoxic condition was not significantly increased by treatment of animals with extract of *Alysicarpus ovalifolius* or caffeine. The results obtained in the hypoxia endurance study showed that administration of caffeine was devoid of significant effects on oxygen utilization as the latency to onset of convulsion was not significantly ($p<0.05$) different from the untreated control group (Table 2). The extract treated animals did not show significant resistance and endurance to hypoxic conditions on comparison to untreated control group. This suggested that the extract may not produce observable influence on oxygen uptake during physical activity or physical stress[37].

Table 2: Effect of aqueous extract of *Alysicarpus ovalifolius* (AO) on Hypoxia endurance test.

Treatment	Dose (mg/kg)	Latency to onset of convulsion (min)
Distilled water	10 ml/kg	35.00 ± 1.16
Caffeine	15	37.50 ± 1.75
A. ovalifolius	100	35.67 ± 0.80
A. ovalifolius	200	38.00 ± 0.26
A. ovalifolius	400	39.17 ± 1.97

Results are presented as mean ± SEM (n=6). No significant difference between distilled water and treated groups.

Oxidation is necessary in several living organisms for the production of energy that is required for regular biological processes. Reactive oxygen species (ROS) are generated by contracting skeletal muscles during physical activity. Exhaustive exercise enhances the generation of these free radicals induced by increased oxygen consumption of active muscles. Exercise or acute, intense physical activity can cause oxidative stress which is an imbalance between ROS and the organism's antioxidant defense systems. Excessive ROS levels can lead to stress-related tissue damage, muscle fatigue and contractile dysfunction[28,38,39]. Also, when mice are exposed to a condition of hypoxia, a state of oxidative stress is induced[25]. Earlier studies have shown that administration of antioxidants inhibited the increase in blood levels of muscle injury markers and mitigated muscle fatigue[40]. Bashir et al (2018) had previously reported the antioxidant effects of *Alysicarpus ovalifolius*. It is therefore possible that the extract's antioxidant effects may be contributing in the reduction of exercise induced oxidative stress with subsequent delay in the onset of fatigue[41].

In the diazepam induced sleep test, the results showed that administration of the plant extract caused delay in the onset of sleep and conversely decreased the duration of sleep when compared to control. However, the sleep duration increased with increasing dose of the extract (Table 3).

Table 3: Effect of aqueous extract of *Alysicarpus ovalifolius* (AO) on diazepam induced sleep in mice.

Treatment	Dose (mg/kg)	Onset of sleep (min)	Duration of sleep (min)
Distilled water	10 ml/kg	1.63 ± 0.20	245.70 ± 22.72
Caffeine	15	14.84 ± 2.50[d]	44.76 ± 3.53[d]
A.ovalifolius	100	4.74 ± 0.98	93.61 ± 13.05[c]
A.ovalifolius	200	3.61 ± 0.36	144.2 ± 10.01[a]
A.ovalifolius	400	4.49 ± 0.10	200.0 ± 41.22

Results are presented as mean ± SEM (n=7). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, [d]$p<0.0001$significant difference between distilled water and treated groups.

The results indicate that the extract may possess CNS stimulant effect as indicated by its ability to reduce the duration of diazepam-induced sleeping time at the doses tested[42]. Acute administration of psychoactive stimulants such as caffeine and amphetamine at low doses caused increased locomotion and enhanced physical activity. However, administration at high doses produced decreased motor activity[43,44]. Similar pattern was observed for AO extract as decreased performance at rotarod was recorded for the extract at 400 mg/kg. The explanation for this observation of the extract cannot be explained within the scope of this study.

Analgesics are used by athletes in order to suppress pain and inflammation from injury thereby enhancing exercise performance and giving competitive advantage[45]. Our previous study reported the pain-relieving property of the phytoconstituents of *A. ovalifolius*[46]. This activity may also be playing a role to increase tolerance to pain thus prolonging the duration at a physical task.

It has been proposed that phytochemicals mediate their pharmacological actions by multiple complementary mechanisms of different constituents[47]. HPLC analysis detected the presence of caffeic acid, chlorogenic acid and rutin in this plant extract. These compounds have shown antioxidant activity[48]. Compounds such as caffeic and chlorogenic acids have demonstrated ergogenic actions in other experiments[49]. However, further studies will be necessary to determine the effects of the extract on biochemical markers of fatigue and muscle injury.

In conclusion, the extract of *Alysicarpus ovalifolius* mitigated the effects of exercise induced exhaustion thus indicating that the extract may possess constituents with the potentials to enhance physical endurance thereby delaying fatigue and improve performance.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

AUTHOR CONTRIBUTIONS

LBJA conceptualization of the research, designed the study and was involved in laboratory work, collection, analysis and interpretation of data and wrote the manuscript. LKE was involved in laboratory work and data collection. SEO carried out the HPLC analysis and interpreted the spectra. AB did the critical analysis of the manuscript, contributed to the study design and is Head of the laboratory where this research work was carried out. All authors have read and approved the manuscript.

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REFERENCES

1. Olajide A, Mansfield M, Olubankole O, & Odoka O. Psychotropic drug prescription practice in psychiatric in-patients in Saskatchewan, Canada. J Neur Ment Health. 2016; 1(4): doi: 10.4172/2472-095X.100114.

2. Favrod-Coune T, & Broers B. The Health effects of Psychostimulants: a literature review. Pharmaceuticals. 2010; 3, 2333 – 2361.

3. Ilieva IP & Farah MJ. Enhancement stimulants: perceived motivation and cognitive advantages Fron Neurosci. 2013; doi.org/10.3389/fnins.2013.00198

4. Morgan CJA, Noronha LA, Muetzelfeldt M, Fielding A & Curran HV. Harms and benefits associated with psychoactive drugs: findings of an international survey of active drug users. J Herb Med. 2013; 27(6):497 – 506.

5. Mestry M, Bajaj A, Rane M. & Lalan K. Herbal CNS stimulants. Int J Herb Med. 2016; 4(6): 109-116

6. Adamson TA, Ogunlesi AO, Morakinyo O, Akinhanmi AO, Onifade PO, Erinosho O, et, al. Descriptive National survey of Substance use in Nigeria. J Addict Res Ther. 2015; 6:3. Doi: 10.4172/2155-6105.1000234.

7. Eegunranti BA, Fatoye FO & Morakinyo O. Stimulant use among secondary school students in Osogbo, Nigeria. Niger Postgrad Med J. 2009; 16(3):218-23.

8. Adayonfo EO. Pattern of stimulant use among Nigerian undergraduate students in Benin city. Nigeria. Ann. Biomed. Sci. 13(2):43 – 50.

9. Hildt E, Lieb K, Bagusat C & Franke AG. Reflections on addiction in students using stimulants for neuroenhancement: A preliminary interview study. BioMed Res Int. doi. org/10.1155/2015/621075.

10. Adamu H, Ahmad MM, Mudi K, Dakani KM & Bakare AT. Predictors of stimulants use among physicians in a Nigerian tertiary health institution in Sokoto, Northwest Nigeria. J Neurosci Behav Health. 10(2): 9-17.

11. Wood S, Sage JR, Shuman T & Anagnostaras SG. Psychostimulants and cognition: a continuum of behavioural and cognitive activation. Pharmacol Rev. 66(1): 193 – 221.

12. Justo CC, Carneiro-de-Oliveira PE, DeLucia R, Aizenstein ML & Planeta CS. Repeated exposure of adolescent rats to oral methylphenidate does not induce behavioural sensitization or cross-sensitization to nicotine. Braz J Med Biol Res. 2010; 43(7):651 – 656

13. Uddin S, Sufian MA, Hossain F, Kabir T, Islam T, Rahman M et al. Neuropsychological effects of caffeine: Is caffeine addictive? Psychol Psychother. 7:2. doi:10.1472/2161.1000295

14. Boys A, Marsden J & Strang J. Understanding reasons for drug use amongst young people: a functional perspective. Health Educ Res. 6(4): 457 – 469.

15. Stangor A, & Walinga J. Altering consciousness with psychoactive drugs. In: Introduction to Psychology 1st Canadian Edition. 2010; BCCampus Victoria. Canada.

16. Dukku, A. M. Emerging Perspectives on Drugs of Abuse: A Focus on Gadagi Consumption in Kano, Nigeria. Adv Psychol Neurosci. 2017; 2(2-1):7- 14.

17. Mannetje L. Alysicarpus ovalifolius (Schumach.) J. Leonard. (Internet) Record from PROTA4U. Oyen LPA & Lemmens RHMJ (Editors). 2002; PROTA (Plant Resources of Tropical Africa/ Ressources vegetales de L'Afrique tropicale), Wageningen, Netherlands. Available at: http://www.prota4u.org/search.asp.

18. Banwo OA. Phytochemical and some pharmacological studies of the aerial part of Alysicarpus ovalifolius Schumach & Thonn Family: Fabaceae. 2013; Ahmadu Bello University Zaria (MSc thesis) 44.

19. Bashir M, Uzair M & Ahmad B. Ethnobotanical, phytochemical and pharmacological aspects of genus Alysicarpus. Int J Pharm. 2018; 8(4):1-15

20. Ezenyi IC, Verma V, Singh S, Okhale SE & Adzu B. Ethnopharmacology-aided antiplasmodial evaluation of six selected plants used for malaria treatment in Nigeria J Ethnopharmacol. 2020; doi: 10.1016/j.jep.2020.112694.

21. Oh SL, Chang H, Kim HJ, Kim YA, Kim DS, Ho SH et al. Effect of HX108-CS supplementation on exercise capacity and lactate accumulation after high-intensity exercise. J Int Soc Sports Nutr. 2013; 10:21. doi:10.1186/1550-2783-10-21.

22. Xu Z. & Shan Y. Anti-fatigue effects of polysaccharides extracted from Portulaca olarecea L. in mice. Indian J Biochem Biophys. 2014; 51: 321 - 325

23. Dickinson M, Flenniken A, Ji X. Teboul L, Wong MD, White JK. et al. High-throughput discovery of novel developmental phenotypes. Nature, 2016; 537, 508–514

24. Bonetto A, Andersson DC, & Waning DL. Assessment of muscle mass and strength in mice. BoneKEy Reports 2015; 4, 732. doi:10.1038/bonekey.2015.101

25. Piato AL, Detanico BC, Linck VM, Herrmann AP, Nunes DS & Elisabetsky E. Anti–stress effects of the "tonic" Ptychopetulatum olacoides (Marapuama) in mice. Phytomedicine 2010; 17:248 – 253

26. Rakotonirina AH, Ngo Bum E, Rakotonirina A & Bopelet M. Sedative properties of the decoction of the rhizome of Cyperus articularis. Fitoterapia., 2001;72: 22 – 29.

27. Wang X, Qu Y, Zhang Y, Li S, Sun Y, Chen, Z, et. al. Antifatigue potential activity of Sarcodon imbricatus in acute excise-treated and chronic fatigue syndrome in mice via regulation of Nrf2-mediated oxidative stress. Oxid Med Cell Longev. 2018; 9140896. doi: 10.1155/2018/9140896.

28. Jung K, Kim I-H & Han D. Effect of medicinal plants extracts on forced swimming capacity in mice. J Ethnopharmacol. 2004; 93:75 – 81.

29. Kan NW, Ho, CS, Chiu YS, Huang WC, Chen PY, Tung YT, et.al. Effects of Resveratrol supplementation and exercise training on exercise performance in middle-aged mice. Molecules 2016; 21 (5):661Doi. 10.3390/molecles21050661

30. Li S & Chen Z. Evaluation of antifatigue effects of 20(S)-Ginsenoside Rg3 in forced swimming in mice. Ind J Pharm Sci. 2018; 80(3):510 – 515.

31. Deacon RMJ. Measuring motor coordination in mice. J Vis Exp. 2013; 75:2609.

32. Rustay NR, Wahlsten D, & Crabble JC. Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. Behav Brain Res, 2003; 141: 237 – 249.

33. Amos S, Chindo B, Abbah J, Edmond I, Binda L, Adzu B, et al. Neuropharmacological effects of the aqueous extract of Nauclea latifolia root bark in rats and mice. J Ethnopharmacol. 2005; 97: 53 – 57.

34. Garige BSR, Keshetti S & Vattikuti UMR. Invivo study on depressant effects and muscle coordination glauca stem methanol extract. Pharmacogn Res. 2016; 8(4):219 – 225.

35. Guo SS, Gao XF, Gu YR, Wan ZX, Lu AM, Qin ZH, et al. Preservation of cognitive function by Lepidium meyenii (maca) is associated with improvement of mitochondrial activity and upregulation of autophagy-related proteins in middle-aged mouse cortex. Evid-Based Complementary Altern Med. 2016; doi: 10.1155/2016/4394261.

36. Toda K, Hitoe S, Takeda S & Shimoda H. Black ginger extract increases physical fitness performance and muscular endurance by improving inflammation and energy metabolism. Heliyon. 2016; doi.org/10.1016/j.heliyon. 2016.e00115.

37. Guoquan J & Yueying L. Study of safflower on blood lactate concentration and exercise function of mice after exercise. Afr J Biotechnol. 2011; 19(45): 9148 – 9152.

38. Belviranli M. & Gokbel H. Acute exercise induced oxidative stress and antioxidant changes. Eur J Gen Med. 2006; 3(3): 126-131.

39. He F, Li J, Liu Z, Chuang CC, Yang W & Zuo L. Redox Mechanism of Reactive Oxygen Species in Exercise. Front Physiol. 2016; 7:486. doi:10.3389/fphys.2016.00486

40. Kawamura T & Muraoka I. Exercise induced oxidative stress and the effects of antioxidant intake from a physiological viewpoint. Antioxidants, 2018; 7:119. doi:10.3390/antiox7090119.

41. Vina J, Gomez-Cabrera MC, Lloret A, Marquez R, Minana JB, Pallardo FV et. al. Free radicals in exhaustive physical exercise mechanism of production and protection by antioxidants. Life. 2002; 50:271 – 277.

42. Owolabi OJ, Amaechina FC & Eledan AB. Central nervous system stimulant effect of the ethanolic extract of Kigelia Africana. J Med Plant Res. 2008; 2(2): 20 – 23.

43. Yates JW, Meiji JTA, Sullivan JR, Richtand NM & Yu L. Neurosci Lett. 2007; 427(1):66- 70.

44. Almosawi S, Mahdi S, Baksh H, Qareeballa A, Falamarzi F, Assaleh B et. al. Acute administration of caffeine: The effect on motor coordination, higher brain cognitive functions and the social behavior of BLC57 mice. Behav Sci. (Basel). 2018; 8(8):65. doi:10.3390/bs8080065.

45. Lundberg TR & Howatson G. . Analgesic and anti-inflammatory drugs in sports: Implications for exercise performance and training adaptations. Scand J Med Sci Sports. 2018; 28(11): 2252 – 2262. doi.org/10.1111/sms.13275.

46. John-Africa LB, Danborno AM, Tikinade MG & Kayinu N. . Anti-nociceptive effects of the hydro-ethanolic extract of Alysicarpus ovalifolius in rodents. J Med Plant Res. 2020; 14(5): 195 – 201.

47. Carmona F & Pereira AMS. Herbal medicines: old and new concepts, truths and misunderstandings. Rev Bras Farmacogn. 2013; 23(2):379 – 385.

48. Kaurinovic B & Vastag D. Flavonoids and phenolc acids as potential natural antioxidants. In Antioxidants. E Shalaby, Ed. IntechOpen. 2018; Doi 10.5772/intechopen.83731. pp 1-14.

49. Sellami M, Slimeni O, Pokrywka A, Kuvacic G, Hayes LD, Milic M et. al. Herbal medicine for sports: a review. J Int Soc Sports Nutr. 2018; 15,14. doi.org/10.1186/s12970-018-0218-y.

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