ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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Founded in 1953 by Kasım Cemal GÜVEN

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Contents

Aims and Scope of Acta Pharmaceutica Sciencia

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

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

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

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK
Editor

INSTRUCTIONS FOR AUTHORS

Manuscripts must be prepared using the manuscript template

Manuscripts should contain the following elements in the following order :

Title Page

Abstract

Keywords

Introduction (Without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

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

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

Abstracts should not be separated into categories, it should be written in a paragraph format. Keywords: Max. 5

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

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

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

Figure captions: A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves

to a minimum but explain all symbols and abbreviations used. Figure captions should be written on the bottom.

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

References in the text should be identified using Arabic numerals. Years of the references should not be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters “a”, “b”, “c”, etc., placed after the year of publication. References should conform to Vancouver style and be numbered consecutively in the order in which they are cited in the text.

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

1. Scope and Editorial Policy

1.1. Scope of the Journal

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

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

All manuscripts has to be written in clear and concise English.

Starting from 2016, the journal will be issued quarterly both in paper and online 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. Review articles will not be accepted.

Research Articles are definitive accounts of significant, original studies. They are expected to present important new data or provide a fresh approach to an established subject.

1.3. Prior Publication

Authors should submit only original work that has not been previously published and is not under consideration for publication elsewhere. Academic theses, including those on the Web or at a college Web site, are not considered to be prior publication.

1.4. Patents and Intellectual Property

Authors need to resolve all patent and intellectual property issues. Acceptance and publication will not be delayed for pending or unresolved issues of this type. Note that Accepted manuscripts and online manuscripts are considered 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 au-

thors. Studies submitted for publication approval must present evidence that the described experimental activities have undergone local institutional review assessing safety and humane usage of study subject animals. In the case of human subjects authors must also provide a statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the 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 New Roman". Other fonts, particularly those that do not come bundled with the system software, may not translate properly. Ensure that all special characters (e.g., Greek characters, math symbols) are present in the body of the text as characters and not as graphic representations. Be sure that all characters are correctly represented throughout the manuscript—e.g., 1 (one) and l (letter l), 0 (zero) and O (letter o).

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

2.1.1 Articles of all kind

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

marized. Code numbers may be used once in the abstract. After the abstract, a section of Keywords not more than five has to be given. Be aware that the keywords, chosen according to the general concept, are very significant during searching and indexing of the manuscripts.

Keywords: instructions for authors, template, journal

2.2.3 Introduction

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

2.2.4. Methodology

Materials, synthetic, biological, demographic, statistical or experimental methods of the research should be given detailed in this section. The authors are free to subdivide this section in the logical flow of the study. For the experimental sections, authors should be as concise as possible in experimental descriptions. General reaction, isolation, preparation conditions should be given only once. The title of an experiment should include the chemical name and a bold Arabic identifier number; subsequently, only the bold Arabic number should be used. Experiments should be listed in numerical order. Molar equivalents of all reactants and percentage yields of products should be included. A general introductory section should include general procedures, standard techniques, and instruments employed (e.g., determination of purity, chromatography, NMR spectra, mass spectra, names of equipment) in the synthesis and characterization of compounds, isolates and preparations described subsequently in this section. Special attention should be called to hazardous reactions or toxic compounds. Provide analysis for known classes of assay interference compounds.

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

2.2.5 Results and Discussion

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

2.2.6 Ancillary Information

Include pertinent information in the order listed immediately before the references.

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

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

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

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

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

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

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

References

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

• References are listed in numerical order, and in the same order in which they are cited in text. The reference list appears at the end of the paper.

• Begin your reference list on a new page and title it 'References'.

• The reference list should include all and only those references you have cited in the text. (However, do not include unpublished items such as correspondence.)

• Use Arabic numerals (1, 2, 3, 4, 5, 6, 7, 8, 9) as a superscripts.

• Abbreviate journal titles in the style used in the NLM Catalog.

• Check the reference details against the actual source - you are indicating that you have read a source when you cite it.

• Use of doi URL at the end of reference is strongly advised.

Examples

For printed articles

• Article with 1-6 authors:

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

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

• Article with more than 6 authors:

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

Electronic journal article:

Author AA, Author BB. Title of article. Abbreviated title of Journal [Internet]. Date of publication YYYY MM [cited YYYY Mon DD];volume number(issue number):page numbers. Available from: URL

Electronic journal article with DOI:

Author AA, Author BB, Author CC, Author DD, Author EE, Author FF. Title of article. Abbreviated title of Journal [Internet]. Year of publication [cited YYYY Mon DD];volume number(issue number):page numbers. Available from: URL DOI

Books and book chapters

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

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

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

2.2.8 Tables

Tabulation of experimental results is encouraged when this leads to more effective presentation or to more economical use of space. Tables should be numbered consecutively in order of citation in the text with Arabic numerals. Footnotes in tables should be given italic lowercase letter designations and cited in the tables as superscripts. The sequence of letters should proceed by row rather than by column. If a reference is cited in both table and text, insert a lettered footnote in the table to refer to the numbered reference in the text. Each table must be provided with a descriptive title that, together with column headings, should make the table self-explanatory. Titles and footnotes should be on the same page as the table. Tables may be created using a word processor's text mode or table format feature. The table format feature is preferred. Ensure each data entry is in its own table cell. If the text mode is used, separate columns with a single tab and use a return at the end of each row. Tables may be inserted in the text where first mentioned or may be grouped after the references.

2.2.9 Figures, Schemes/Structures, and Charts

The use of illustrations to convey or clarify information is encouraged. Structures should be produced with the use of a drawing program such as ChemDraw. Authors using other drawing packages should, in as far as possible, modify their program's parameters so that they conform to ChemDraw preferences. Remove all color from illustrations, except for those you would like published in color. Illustrations may be inserted into the text where mentioned or may be consolidated at the end of the manuscript. If consolidated, legends should be grouped on a separate page(s). Include as part of the manuscript file.

To facilitate the publication process, please submit manuscript graphics using the following guidelines:

1. The preferred submission procedure is to embed graphic files in a Word document. It may help to print the manuscript on a laser printer to ensure all artwork is clear and legible.

2. Additional acceptable file formats are: TIFF, PDF, EPS (vector artwork) or CDX (ChemDraw file). If submitting individual graphic files in addition to them being embedded in a Word document, ensure the files are named based on graphic function (i.e. Scheme 1, Figure 2, Chart 3), not the scientific name. Labeling of all figure parts should be present and the parts should be assembled into a single graphic.

EPS files: Ensure that all fonts are converted to outlines or embedded in the graphic file. The document settings should be in RGB mode. NOTE: While EPS files are accepted, the vector-based graphics will be rasterized for production. Please see below for TIFF file production resolutions.

3. TIFF files (either embedded in a Word doc or submitted as individual files) should have the following resolution requirements:

- Black & White line art: 1200 dpi

- Grayscale art (a monochromatic image containing shades of gray): 600 dpi

- Color art (RGB color mode): 300 dpi

· The RGB and resolution requirements are essential for producing high-quality graphics within the published manuscript. Graphics submitted in CMYK or at lower resolutions may be used; however, the colors may not be consistent and graphics of poor quality may not be able to be improved.

· Most graphic programs provide an option for changing the resolution when you are saving the image. Best practice is to save the graphic file at the final resolution and size using the program used to create the graphic.

4. Graphics should be sized at the final production size when possible. Single column graphics are preferred and can be sized up to 240 points wide (8.38 cm.). Double column graphics must be sized between 300 and 504 points (10.584 and 17.78 cm's). All graphics have a maximum depth of 660 points (23.28 cm.) including the caption (please allow 12 points for each line of caption text).

Consistently sizing letters and labels in graphics throughout your manuscript will help ensure consistent graphic presentation for publication.

2.2.10 Image Manipulation

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

2.3 Specialized Data

2.3.1 Biological Data

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds

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

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

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

2.3.3 Confirmation of Structure

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

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

3. Submitting the Manuscript

3.1. Communication and log in to Author's Module

All submissions to Acta Pharmaceutica Sciencia should be made by using e-Collittera (Online Article Acceptance and Evaluation) system on the journal main page (www. actapharmsci.com)

3.2. Registration to System

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

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

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

3.3 Submitting A New Article

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

3.1.1. 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.

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3.3.5. Submitted Manuscripts Information related to articles can be followed through the Submitted Manuscripts line. Here you can find the information such as the article's number assigned by system, title, sending date and transaction situation. The Manuscript Details and summary files can be reached by clicking the title of the article and the Processing Status part makes it possible to follow the evaluation process of the article.

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ORIGINAL ARTICLES

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Investigation of Hidden Crisis of Prescription Drug Abuse in Turkey: Pregabalin Monitoring

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ABSTRACT

This is a cross-sectional retrospective observational national study. Abuse/misuse of the pregabalin and related parameters analyzed. Pregabalin was mostly prescribed by general practitioners (26.2%). Pregabalin prescribing frequencies of other physicians were as follows, respectively: neurology (14.1%); mental health and illness (12%); physical therapy and rehabilitation (8.8%); internal medicine (4.8%). Accordingly, pregabalin (811.954box) and paracetamol, combinations excl. psycholeptics (1,131,069box) were the first two sales. In total cost of sales, pregabalin (57,721,322.00 TL) and methylphenidate (41,915,196.00 TL) were the top two sales rankings. According to the disposal results of ILAYS pharmaceuticals in 2020, pregabalin (2693box per year) and morphine (862box per year) were the most disposed drugs. It was determined that the frequency of pregabalin was followed by gabapentin. In conclusion, our study reveals the extent of abuse of pregabalin in Turkey with its data.

Key words: controlled drug pharmaco-economy, drug abuse, pregabalin, prescription drug monitoring

INTRODUCTION

In Turkey administrative regulations are made to ensure the rational use of drugs and to prevent drug abuse. In Turkey, red-colored prescriptions (opioids, methylphenidate etc.) are generally for the prescription of narcotic opioid drugs, while green-colored prescriptions (benzodiazepines, barbiturates, pre-

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gabalin etc.) are generally for the prescription of psychotropic drugs, including pregabalin. Apart from these, there are also prescription drugs in white-controlled prescription (gabapentin), subject to control. After coloured-controlled paper prescriptions, the electronic colour-controlled prescription system became operational in Turkey. Electronic prescription data provides both pharmaco-epidemiological and pharmaco-economic benefits, considering the patient's comorbidity and literature data, which are thought to be predisposing factors in the disease, electronic records were used in this study.

The abuse of drugs is among the irrational drug parameters, and it is stated by the World Health Organization (WHO) that drugs should be prescribed and used rationally[1].

According to WHO estimates, more than 50% of medicines are improperly prescribed, procured or sold. Half of all patients cannot use their medications properly[2].

Drug abuse is defined as the use of drugs other than for medical purposes, especially when the physician does not see it necessary, on their own will or on the recommendation of unauthorized persons[3].

Pregabalin is an antagonist of voltage gated Ca^{2+} channels and specifically binds to alpha-2-delta subunit of the channel. It has antiepileptic and analgesic actions. It safe and efficous enough to be first line therapeutic agent for neuropathic pain. Pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid, is a pharmacologically active S-enantiomer of a racemic 3- isobutyl gamma amino butyric acid analogue. It is the first frug to be approved by FDA for diabetic neuropathy and post-herpetic neuralgia[4].

Pregabalin is a GABA analogue, and this has direct or indirect effects on dopaminergic reward system. That is why pregabalin is a good candidate to be abused by the patients. The physicians must be aware of previous abuse history of the patients before prescription pregabalin[5].

Explaining the pharmacoeconomics and pharmacoepidemiology of prescription drug abuse will facilitate monitoring. Prescription drug abuse can be prevented by including results in electronic decision support mechanisms. It is aimed to attract attention of the pregabalin abuse and the role of health professionals including clinical pharmacists in prevention and awareness of the drug abuse.

METHODOLOGY

This national study is an observational retrospective research study based on electronic prescriptions of all individuals who have been declared to have abuse-controlled drug prescription drugs in Turkey between the years of 2017 and 2020.

All electronic records of the people who were mentioned in the announcements of the Ministry of Health, which they have made drug abuse for five years between 2016-2020, about the need to be careful against abuse to health institutions, until 31 December 2020, were examined.

The extent of this abuse has been examined from the perspective of pregabalin drug with active ingredient, for which special restrictions have been imposed due to the unusual increase in abuse across Turkey.

Detailed 18-month sales reports of one of Turkey's three largest pharmaceutical distribution warehouses and ILAYS (national licensed disposal center), that is, the year 2020, 12-month report of drugs that pharmacies are liable to destroy were examined.

The first of the 216 announcements, which were detected to have abused drugs and distributed to health institutions/organizations in Turkey, was on 24.02.2016 and the last one was on 04.09.2020. When missing/wrong information, duplicate person/articles were accepted as exclusion criteria in these articles, 199 articles were evaluated. The limitations of our study are the distribution of only 216 abuse detection official announcements across Turkey, between the years 2016-2020, the deficiencies/inaccuracies in 17 articles, and the context problems between electronic prescriptions. These prescriptions are excluded.

The Microsoft Excel document of our study was determined to be 5,506 kilobytes, consisting of approximately 700 thousand cells.

Statistical program was used for statistical analysis. In our study, "$p<0.05$ value" was accepted as a significant value.

RESULTS and DISCUSSION

199 people were included in the study as abusers. After the first warning letter belonging to this patient, the electronic prescription records of the person were examined with all the prescribing details since 2017 (the start of e-prescription in Turkey). 725 health institutions in 76 different provinces; It was determined that a total 6610 prescriptions were prescribed, 3262 green colour-controlled-controlled prescriptions 680 of which were red colour-controlled-controlled

prescriptions, and prescriptions were dispensed from 1222 different pharmacies in 69 different cities.

Table 1. Grouping of controlled drugs in our study according to ATC codes and number of patients/prescriptions

ATC	ATC NAME	ATC CODE	PRESCRIBED ABUSE (n)		ABUSED PRESCRIPTION (n)	
Benzodiazepine derivatives	clonazepam	N03AE01	65	109	907	1.819
	alprazolam	N05BA12	66		659	
	diazepam	N05BA01	36		227	
	lorazepam	N05BA06	21		106	
Other antiepileptics (pregabalin)	pregabalin	N03AX16	98	98	548	548
Tertiary amines	biperiden	N04AA02	36	36	400	400
Opium alkaloids and derivatives	combinations (codein+dionin)	R05DA20	4	4	44	44
Drugs used in opioid dependence	buprenorphine, combinations	N07BC51	11	11	86	86
Barbiturates and derivatives	phenobarbital	N03AA02	2	2	22	22
Opioids	tramadol	N02AX02	23	40	305	818
	pethidine	N02AB02	24		218	
	morphine	N02AA01	8		119	
	oxycodone	N02AA05	12		96	
	fentanyl	N02AB03	11		88	
	tramadol and paracetamol	N02AJ13	11		23	
	hydromorphone	N02AA03	1		1	
Centrally acting sympathomimetics	methylphenidate	N06BA04	4	4	28	28
Benzodiazepine related drugs	zopiclone	N05CF01	4	4	7	7

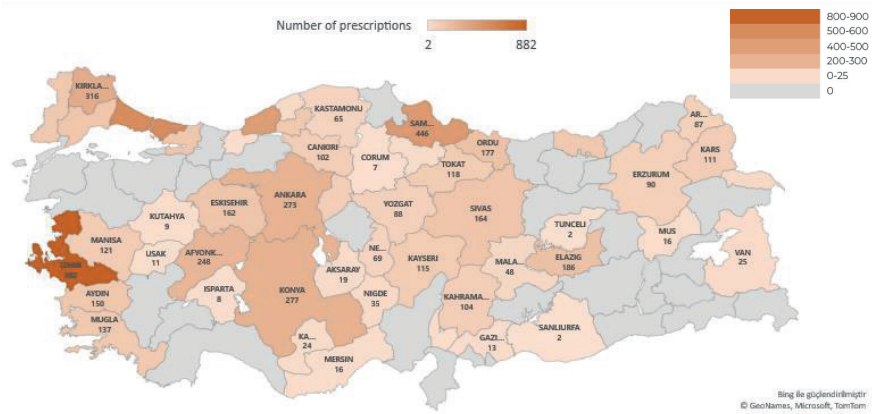

Figure 1. Number of abuse prescriptions by province

All drugs taken by abusers for approximately 4 years (17.3.2017- 31.12.3020) are limited according to ATC group and active substance name and code. Benzodiazepines are followed by opioids, followed by pregabalin. Considering that pregabalin is not a drug group but only an active ingredient, abuse seems to be much greater.

It is aimed to collect relevant data for the contribution of the e-prescription system to drug abuse as the stages of pregabalin in e-prescription systems:

• Neither colour-controlled nor e-prescription

• E-prescription but not colour-controlled

• Both colour-controlled and e-prescription

While the active ingredient of Pregabalin (ATC: N03AX16) was in the system as a controlled drug as of February 1, 2018, it was included in the colour-controlled prescription class as of 01.04.2019. While the e-prescription requirement was on 17.03.2017, this requirement for pregabalin started on 01.04.2019. In terms of prescription history, prescriptions were examined according to drug ATC names.

In addition, the obligation to write pregabalin in the e-prescription system is different from other coloured-controlled prescription drugs. In other words, while other drugs were already controlled, they had to be in the system as soon as the e-prescription system started, but this was not the case for pregabalin, and it was included in the system approximately 2 years after the e-prescription system started.

The active ingredient of pregabalin can be used as an index-marker for controlling drug abuse and waste, in a way, for the control of the e-prescription system in Turkey, due to the above-mentioned features. Because this drug with active substance was passed in all layers of the prescription monitoring system (it was not in the system, it was subject to control in the system, it was as a green prescription in the system) with certain date intervals.

Unlike other coloured-controlled prescriptions, the fact that pregabalin is in all the following stages provides the opportunity to use it as an indicator:

• While neither colour-controlled nor e-prescription (n=18), the average prescription date is September 12, 2017 (±98,073 days)

• Coloured-controlled but non-e-prescription (n=175) while the average prescription date was September 20, 2018 (±110,84 days)

• Average January 27, 2020 (±191,57 days) for both e-prescription and colour-controlled (n=364)

The differences in dates between the prescription phases of the active ingredient of pregabalin were examined by one-way ANOVA. Since the equality of variances could not be achieved, Tamhene test was applied for post hoc for pairwise comparisons. Accordingly, the difference between all paired groups was found to be significant ($p<0.001$).

The following results were found to be statistically significant for the other mean prescription differences of pregabalin:

• Both e-prescription and colour-controlled prescription date averages - 866 days more than neither colour-controlled nor e-prescription date average

• Both e-prescription and colour-controlled prescription date average - 493 days more than the colour-controlled but non-e-prescription date average

• Colour-controlled but non-e-prescription date average - 372 days more than neither colour-controlled nor e-prescription date average

By taking the date of April 2019, the relevant figure was created so that a general view would be more accurate so that the course of all drugs that are taken by patients who abuse drugs and that are required to be registered in the system.

The decision to take urgent measures for pregabalin was given by the scientific advisory commission with the announcement of the Department of Pharmacovigilance and Controlled Substances of the Ministry of Health[6]. In our study, we have created the following figure for a visual overview in order to take a holistic view of the drug course taken by individuals who abuse drugs according to drug pregabalin.

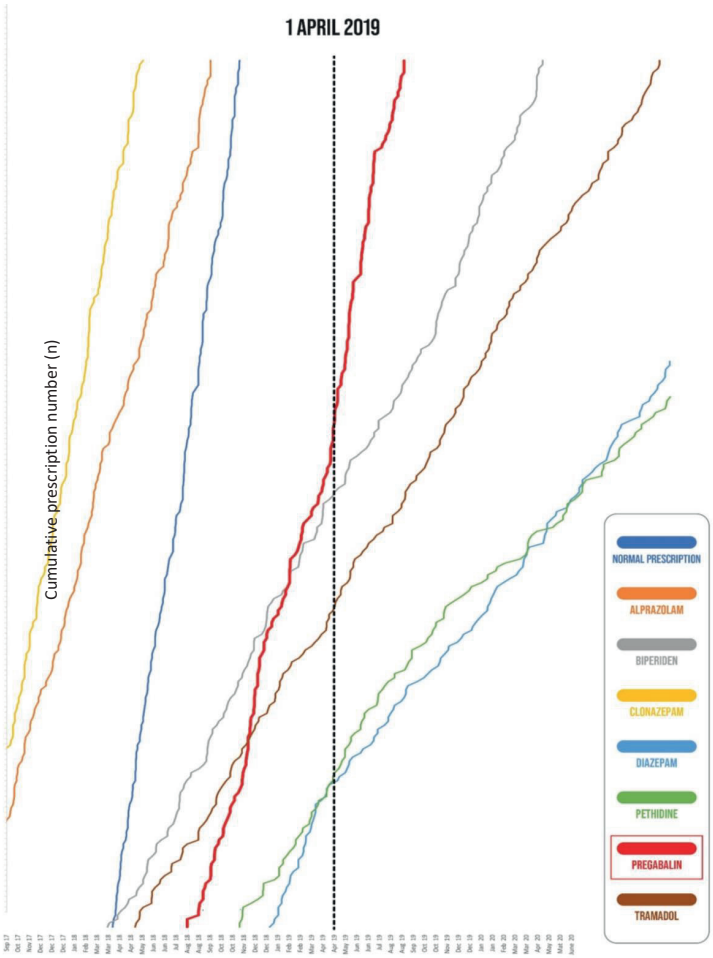

Figure 2. Pregabalin, cumulative prescription number graph by other drugs in the e-prescription system between September 2017 and June 2020

It was understood that the decision of the Ministry of Health's scientific committee was correct, but since there was no decrease in the slope of the pregabalin curve before the decision, we can say that we have concluded that the e-prescription system is not sufficient to prevent drug abuse, and perhaps it is sufficient to register the prescription.

The fact that it almost intersects with the perpendicular line on the block just after 1 April date may have shown the accumulation in the relevant dates, but the fact that this hunger does not go away even though months have passed reveals the seriousness of the problem.

As a result, we can say that monitoring is not enough. However, since the literature detailed below mentions that this may not be easy, we can emphasize that a reaction should be taken according to the relevant literature from now on.

Monitoring has an important place in the identification, evaluation and treatment of drug abuse or misuse. All monitoring tools have some limitations. At the beginning of these constraints are the ignorance of age and cultural differences. The healthcare industry and patients benefit greatly if abuse and misuse can be detected with monitoring tools. Monitoring tools are cost-effective in identifying patients at risk, and the use of these tools provides therapeutic benefits[7].

However, these monitoring tools should be age and population compatible[8].

With the use of monitoring tools, early diagnosis of substance use can be made, so that young people can be prevented from encountering larger problems in the future[9].

The use of computerized monitoring tools is both speed and cost-effective[10].

In our study, these three aspects were supported and explained. It is thought that we can get better results with better opportunities.

For example, we can say that our study, which we started to work on before the publication of the following study and which is Turkey-wide, shows results in line with the literature, but it is more comprehensive than those in the literature. Rose et al. The risk factors of abuse in the study, in which data on more than three million adults were examined, are similar to those in our study and support our study. These risk factors include frequent visits to the pharmacy and frequent visits to the same doctor[11].

In order to prevent the abuse of prescription drugs in the world, laws are made on how to collect and dispose of prescription drugs[12].

In our country, the destruction of these drugs can be given as examples of licensed disposal centers with the determination of the Ministry of Health officials. The Pharmaceutical Waste Management System (ILAYS), managed by pharmacists, is one of them, and first of all, a list of drugs is created for drug disposal, and transactions are carried out by pharmacists. As a result of the delivery of the drugs subject to control, together with the drug lists and the report prepared in the Health Directorates, they are directed to the disposal centers[13].

In our study, detailed 18-month sales reports of one of Turkey's three largest pharmaceutical distribution warehouses and ILAYS, that is, the year 2020, 12-month report of the drugs that pharmacies are obliged to dispose were examined.

Accordingly, pregabalin (811,954box for 18 months) and paracetamol, combinations excl. psycholeptics (1,131,069box for 18 month) are the first two sales. In total cost of sales, pregabalin (57,721,322.00 TL for 18 months) and methylphenidate (41,915,196.00 TL for 18 months) were the top two sales rankings.

According to the drug dispose report of ILAYS, pregabalin (2693box per year) and morphine (862 box per year) were the most destroyed drugs in 2020. According to the same report, when the costs of disposed controlled drugs for 2020 were calculated, pregabalin and morphine were determined as two values.

It has been observed that the most common active ingredients of clonazepam, alprazolam, pregabalin and biperiden are included in the prescriptions of these abusers. The fact that these patients continue to use drugs that may be the subject of abuse reveals that the e-prescription system in our country is not sufficient to follow these patients.

Pregabalin was the highest number of general practitioners (26.2%), respectively; neurology (14.1%); mental health and illness (12%); physical therapy and rehabilitation (8.8%); internal medicine (4.8%); It was prescribed by family medicine specialist (2.3%).

In accordance with the decisions taken by the Scientific Advisory Commissions, "Medicines Containing the Active Substance of Pregabalin", which is in the status of a drug subject to monitoring that must be given with a normal prescription, will be included in the list of "Green Drugs to be Given by Prescription" as of 01.04.2019. In addition, provided that it is valid from the specified date, "Medicines Containing Pregabalin Active Substance" can be prescribed only by the relevant specialist physicians within the therapeutic indications specified in the Approved Short Product Information, in case it is desired to be prescribed without a Drug Use Report[6].

However, both addicts and abusers put pressure, including violence, on healthcare professionals to take this drug. According to the results of the second half of 2020, people who abused prescription drugs in our study took gabapentin after pregabalin. We think that the reason for this is that after pregabalin was counted as a controlled drug, abusers turned to gabapentin. Because gabapentin shows similar effects to pregabalin[14].

Considering the fact that overuse of gabapentin leads to deaths in an article published in JAMA in 2022, it will be seen from another perspective that the magnitude of the prescription drug crisis in Turkey are much larger[15].

As a result, our study revealed that pregabalin cannot be adequately controlled in Turkey and it would be beneficial to incorporate the data in this publication in e-prescription decision-making processes immediately.

STATEMENT OF ETHICS

Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee date is 25/04/2018 and number is 272. This investigation is ethically approved.

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Determination of Digitoxin by High-performance thin layer chromatography in Digitalis purpurea

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ABSTRACT

A quantitative HPTLC method has been established for quantification of digitoxin in chloroform extract of *Digitalis purpurea* leaves. For HPTLC quantification method, the plate used was of pre-coated silica gel 60 F254 and mobile phase was Ethyl acetate: Methanol: Water in the ratio of volume (8.0:1.5:1.0, v/v/v) and UV detection was performed at 219nm. Four samples of mother tincture were used for the study, in-house-mother tincture (A) of *Digitalis purpurea* and market samples (B, C and D) of renowned brands. Result shows that the mother tinctures prepared by authenticated plant sample showed maximum amount of digitoxin as compared to the mother tinctures procured from the market. The present study suggested that the mother tincture of *Digitalis purpurea*, contained a cardiac glycosides (digitoxin) justifying its medicinal usage in Homeopathy. This is the reason for cure and healing property of leaves of *Digitalis purpurea*.

Keywords: *Digitalis purpurea,* HPTLC quantification, digitoxin, Homoeopathic drug

INTRODUCTION

Digitalis purpurea is commonly known as Foxglove[1] is a biennial plant belongs to the family Plantaginaceae. It is indigenous to the part of western and south-western Europe. In India, it is found in Nilgiri hills of Tamil Nadu, southern

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Sikkim and eastern Himalayan region. It is herbaceous biennial shrub grows in the colder region of Himalayan. Its leaves contain both primary and secondary glycosides. Among primary glycosides, purpurea glycoside A, purpurea glycoside B[2]and among secondary glycosides, digitoxin, gitoxin and getaloxin are most pronounced. Its leaves contain flavones, anthraquinones, saponins, degalactotigonin and F-gitonin[3]. Digitoxin is the main active constituent of *D. purpurea* plant which is used as cardiac glycosides in medicines.

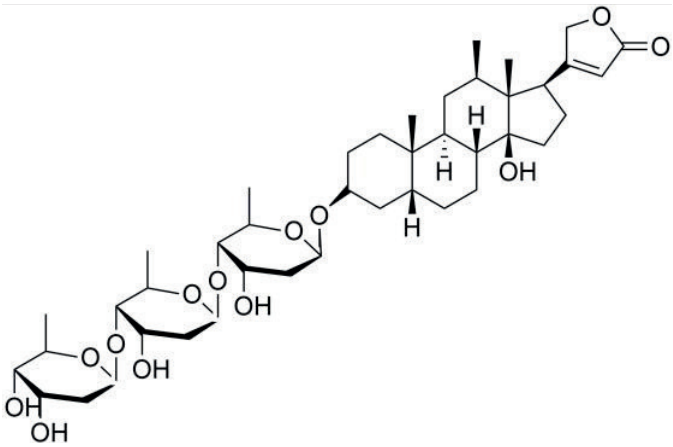


Figure 1. Structure of Digitoxin

Digitoxin (Figure 1) is generally known as highly toxic by-product[4]. In Homeopathy *D. purpurea* is mainly used for the treatment of heart related disease where the heart is primarily involved where the pulse is weak, irregular and intermittent, abnormally slow and dropsy of external and internal parts. Weakness and dilatation of the myocardium and in other symptoms of heart disease such as great weakness, sinking of strength, faintness, coldness of skin and irregular respiration, cardiac irritability, jaundice from in duration and hypertrophy of the liver. In female during labor pain in abdomen and back before menses and for uterine haemorrhage[5] *D. purpurea* homeopathic medicine is recommended in Homeopathy. As per the information given in homeopathic book Materia Medica Pura, *D. purpurea* is mainly used for disease where heart is primarily involved[6], such as in case of atrial flutter, atrial fibrillation and in case of congestive heart failure conditions. In case of congestive heart failure[7] situation, our heart is unable to pump blood effectively at the rate that meet the need of the metabolizing tissues. Our muscles weakly perform the contraction and weakly force the blood out of heart which reduces the contraction rate of heart output and increase rate of heart input leading to increase in the heart

blood volume. Because of this reason our heart feels congested. Cardiac glycosides[8] such as digitoxin present in the leaves of *D. purpurea*[9] helps to prevent congestive heart failure by increasing the force of contractions of heart in the body. In previous suggested studies digitoxin present in the leaves of *Digitalis* has highest gastrointestinal (GI) absorption 90-100% with half-life of 4-5 days[10] which is greater than other commercially available cardiac steroids such as Digoxin, Deslanoside and Ouabain11. Due to rich in active constituents *D. purpurea* shows cardiovascular, cytotoxic[12], antioxidant, anti-diabetic[13], insecticidal, immunological, cardio protective, hepatoprotective and neuroprotective effect and has greater importance in Homeopathy like alternative system of medicine[5]. The purpose of this study is to determine the concentration of digitoxin in *D. purpurea* Homeopathic mother tincture by High performance thin layer chromatography.

METHODOLOGY

Collection of Plant materials

The plant material of *D. purpurea* was collected and authenticated by staff at Center of Medicinal Plants Research in Homoeopathy (CMPRH), Emerald, Tamil Nadu. The voucher specimen was deposited in the herbarium and in the laboratory of DDPR CRI (H), Noida, Uttar Pradesh, India, for future reference with collection number 9649. Authentic plant material was used to prepare mother tincture. Digitoxin ($C_{41}H_{64}O_{13}$, Melting Point 240°C with purity by HPLC>92% w/w purchased from Sigma Aldrich, USA. Solvents used were ethanol, methanol, HPLC water and chloroform of analytical grade purity (Merck Ltd., India).

Physicochemical studies for raw drug standardization

Loss on drying

Loss on drying method used for determination of moisture content as per methods recommended in Homoeopathic Pharmacopeia of India[14].Percentage loss on drying was calculated[15].

Foreign matter determination

For foreign matter determination, 100 g of plant raw material has been taken and outspread it in thin-layer. Sample examined by 6x lens or with unaided eye, the foreign organic matter was picked manually. Ratio of total foreign matter weighed and the weight of drug taken gave the % of foreign matter[15].

Total Ash value determination

In drug, the impurity present in the form of organic matter was determined with the help of total Ash value. For its determination, 2 g of the raw drug was weighed in powdered form in a pre- weighed silica crucible. Incinerated the sample in silica crucible by gradually increasing the temperature up to 450°C for 4 hours or until it became carbon free. The crucible was cooled and weighed until constant weight was obtained[14]. Percent of total ash value was then calculated[14]by taking the ratio of loss in weight to weight of sample taken.

Acid-Insoluble ash value determination

After total ash value determination, 25 mL of 5 M hydrochloric acid was added in the dried ash and boiled on water bath for 10 minutes. Concentrated the solution till its color changed to yellow. Acid insoluble matter was filtered using Whatman paper 1 followed by washing with distilled water. The paper was again ignited in crucible at a temperature not more than 450°C for 4hours, after which crucible was kept in a desiccator, cooled and weighed[14].With reference to the originally taken air dried powdered drug, % of acid insoluble ash value calculated[15].

Water -soluble extractive value determination

For determination of water extractive value, 2 g of accurately weighed, air dried powdered drug was put in a conical flask with 100 mL water added in it. The solution was allowed to stand for 24 hours with intermittent shaking of flask after every 4 hours. The water-soluble extractive was filtered using Whatman filter paper. 25 mL of this filtrate was completed dried on a pre-weighed petri plate at 105°C. The increase in weight of petridish was noted to calculate the water-soluble extractive value determination[14].With reference to originally taken air dried powdered drug, % of water-soluble extractive value calculated[15].

Alcohol-soluble extractive value determination

For determination of alcohol soluble extractive value accurately weighed 2 g air dried powdered drug put it in a conical flask and 100 mL absolute alcohol added in it. Keep the whole solution for 24 hours. In each six hours frequently shake the solution for complete mixing and stand for 18 hours. On next day filtered the solution with the help of Whatman filter paper by taking precaution against loss of alcohol. Weighed the empty flat-bottomed Petri dish. For drying heat, the Petri dish with 25 mL of filtrate at 105°C and cooled the Petri dish in a desiccator and weighed[14]. With reference to originally taken air dried powdered % of alcohol-soluble extractive value calculated[15].

Qualitative Phytochemical screening

Phytochemical tests were performed on crude extract for qualitative estimation of cardiac glycosides with all respective testing procedures include glycosides tests, Keller Kiliani test, Raymond's test, Liebermann's test[16] as described in the text book of JB Harborne[17].

Preparation and Standardization In-house mother tinctures/crude extract

100 g of coarsely dried powdered *D. purpurea* leaves were taken, in which 532mL purified water and 468 mL alcohol was added to make one thousand milliliters of the mother tincture[18] using the percolation method[19] (as per Homoeopathic Pharmacopoeia of India). This tincture was transferred to a tightly packed amber glass container and stored for further study. Standardization of mother tincture was conducted to identify the organoleptic and physicochemical properties of mother tincture[20].

Preparation of standard Digitoxin

Dissolved 5 mg of Digitoxin in 5mL ethanol in volumetric flask, and sonicated for 10 minutes to prepare working standard of Digitoxin with concentration 1mg/mL.

Preparation of chloroform extract

25 mL of Mother Tincture (A) and three market sample B, C and D were taken in a 50mL beaker. To remove the ethanol, solution was evaporated on water bath and extracted three times with 20 mL chloroform. Combined and concentrated chloroform extract up to 2 mL volume. Carried out the TLC of chloroform extract of A, B, C and D with reference standard digitoxin on silica gel 60 F254 pre-coated plate.

HPTLC fingerprinting profile study

For HPTLC fingerprinting study a densitometric HPTLC Camag Linomat 5 (Switzerland) system, was used. In HPTLC system, Camag Linomat 5 was used as sample applicator; for development of mobile phase, a saturating chamber Camag Twin Trough glass chamber was used. Camag TLC Scanner and software vision CATS were used. HPLC grade solvents were used for all the extracts solution. Spots were made on silica gel 60 F254 pre-coated plate (Merck) 20 × 10 cm plate with an aid of sampling machine and solvent front was run up to 70mm height. Volume applied for standard 1 to 6 µL and for sample 5-10 µL. Mobile phase used was ethyl acetate: methanol: water (8:1.5:1, v/v/v) and

TLC spots were visualized after illumination at 254 and 366 nm. Anisaldehyde-sulfuric acid reagent solution was used as derivatizing agent for HPTLC profiling. Digitoxin was used as reference standard.

RESULTS and DISCUSSION

Results of Physiochemical and Phytochemical studies:

Phytochemical glycoside tests performed on the crude extract of leaves of *Digitalis purpurea* showed positive results for Keller-Kiliani test, Raymond's test and Liebermann's test. The results obtained for physiochemical studies of raw drug were tabulated in **Table1**. Organoleptic observations of the prepared in-house mother tincture indicated formation of a clear brown solution with characteristic tincture odor. The physicochemical properties of the tinctures of in-house drug sample (A) and comparison with three procured market samples (B, C and D) for parameters like Sediments, pH, total solids, alcohol content and weight per mL were analyzed and tabulated in Table 2.

Table 1. Results of Physiochemical studies of raw drug material

Name of Physiochemical parameter studied	% composition in raw drug
Foreign matter	2.00
Loss on drying	2.73
Water-soluble extractive value	40.00
Alcohol-soluble extractive value	15.00
Total ash value	5.00
Acid-insoluble ash value	0.60

Table 2. Results of physicochemical properties of the tinctures

S.No.	Parameter	In-house tincture	Market tincture samples		
		A	B	C	D
	Sediments	Nil	Nil	Nil	Nil
	pH	4.80	4.92	4.77	5.94
	Total solid	5.36 % w/v	3.36 % w/v	3.84 % w/v	2.01 % w/v
	Wt./mL	0.943 g	0.955 g	0.959 g	0.942 g
	Alcohol content	43.50 % v/v	41.70 % v/v	41.40 % v/v	44.20 % v/v

Result of HPTLC study:

Based on extensive literature reviews, various combinations of solvent systems were studied with an aim to have an appropriate mobile phase composition for best and efficient HPTLC chromatographic separation of digitoxin in *D. purpurea* chloroform extract. In mobile phase chloroform: methanol (9:1, v/v), toluene: ethyl acetate: formic acid (7:5:1, v/v/v), ethyl acetate: methanol: water (8.0:1.1:0.8, v/v/v) no appropriate resolution of band observed whereas in mobile phase ethyl acetate: methanol: water (8.0:1.5:1.0, v/v/v) efficient band resolution of digitoxin observed with improved $R_{f.}$ value of 0.65. Among all the mobile phase combination studied, ethyl acetate: methanol: water in the ratio of volume (8.0:1.5:1.0, v/v/v) was finalized to be ideal one for evaluation of digitoxin in *D. purpurea*. Thus, it was finalized the best appropriate mobile phase composition for entire HPTLC method development study. Table 3 recorded various mobile phase combination used for preliminary screening study for best possible separations.

Table 3. Various mobile phase combinations used for preliminary screening study for best possible chromatographic separations of Digitoxin.

Used Mobile phase combinations for evaluation of Digitoxin	$R_{f.}$ value	Observations
Chloroform, Methanol (9:1, v/v)	0.49	Poor resolution of band
Toluene, Ethyl acetate, Formic acid (7:5:1, v/v/v)	0.12	Poor resolution of band
Ethyl acetate, Methanol, Water (8.0:1.1:0.8, v/v/v)	0.64	No appropriate resolution of band
Ethyl acetate, Methanol, Water (8.0:1.5:1.0, v/v/v)	0.65	Efficient band resolution with improved $R_{f.}$

Qualitative HPTLC study of in-house mother tincture and market samples

HPTLC study of *D. purpurea* chloroform extract of in-house mother tincture (A), three market samples (B, C and D) and standard digitoxin by using selected mobile phase ethyl acetate: methanol: water in the ratio of volume (8.0: 1.5: 1.0, v/v/v) at U.V light 254nm showed very light brown spots at $R_{f.}$ 0.63 (Figure 2) but no spot was visualized at the same $R_{f.}$ at 366 nm illumination as evident in (Figure 3). For better resolution, anisaldehyde sulfuric acid reagent was used as derivatizing agent. On spraying the plate with anisaldehyde

sulfuric acid reagent, a clear brown spot of digitoxin standard was observed at $R_{f.}$ 0.63 as well as in in-house mother tincture (A) and market samples (B, C and D) (Figure 4). The result of HPTLC fingerprinting profile study confirms the presence of cardiac glycoside digitoxin in in-house mother tincture (A) as well as in commercial market samples (B, C and D). However, by analyzing the separated bands observed in in-house mother tincture (A), commercial market samples (B, C and D) and quantification of bands by studying their densitogram, a quantitative picture was obtained.

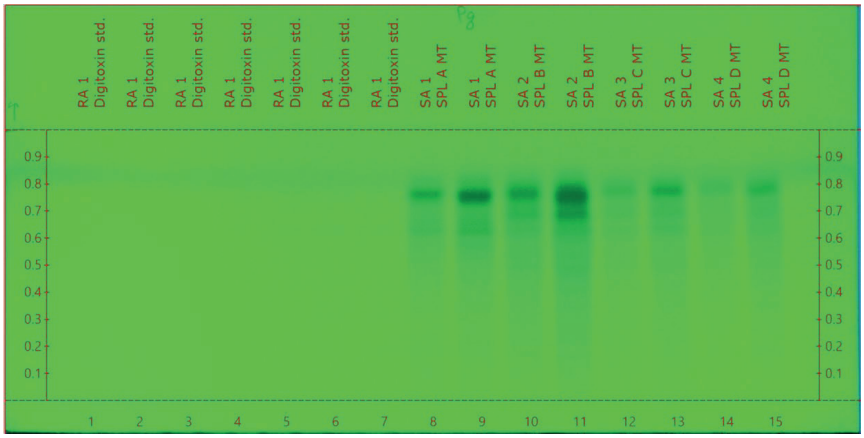


Figure 2. High-performance thin layer chromatography fingerprints of *D. purpurea* at UV 254nm. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

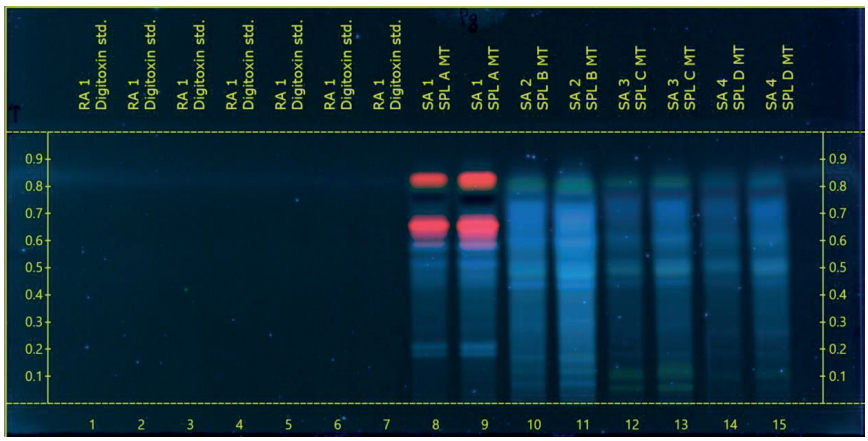


Figure 3. High-performance thin layer chromatography fingerprints of *D. purpurea* at UV 366nm. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

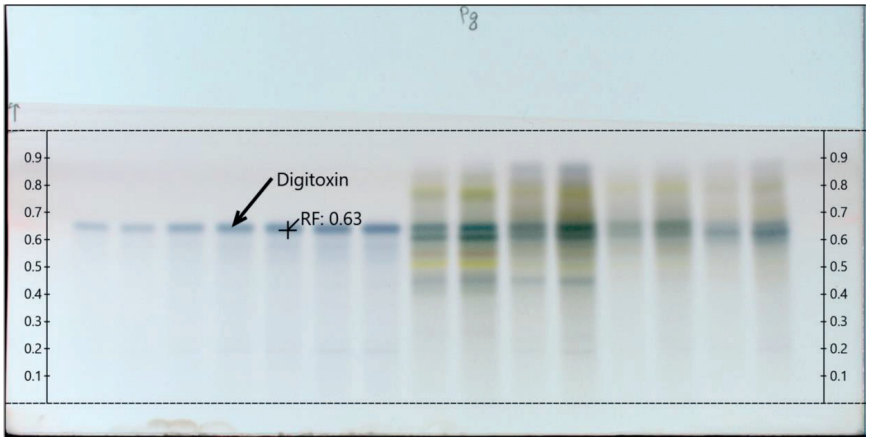


Figure 4. High-performance thin layer chromatography fingerprints of *D. purpurea* after derivatization with anisaldehyde sulfuric acid reagent. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

HPTLC Quantitative study

To check the linearity response, the volume of the in-house sample was optimized to10 µL for quantification. Different concentrate on range 1000-6000 ng/spot of digitoxin reference standard i.e. 1 to **6µL** consecutively applied on TLC plate. The method was found to be linear with a correlation coefficient (R) = 0.995 in the concentration range 1000-6000 ng/spot. The amount of digitoxin was calculated in in-house mother tincture and available market samples and summarized in Table 4. First seven spots marked were for standard digitoxin (track 1-7), next two spots consecutively applied for chloroform extract of in-housemother tinctures (8-9) followed by three different market samples chloroform extracts (10-15). For comparative study, 10 µLin-house mother tinctures spots applied along with other three available mother tinctures on same plate(Figure 2-4). For monitoring and selection of optimum wavelength, multi wavelength (MWL) scan mode was selected during the scanning process. At 219 nm optimum wavelength showing absorption maxima of digitoxin was observed (Figure 6). For quantification and spectral match, the entire plate was further scanned and summarized. With the help of characteristic spectra, fractions of in-house mother tincture and other marketed samples were matched. In spectral scanning maximum absorbance of each fraction were observed then the plate was scanned with the selected wavelength in MWL mode. On comparison of the peaks pattern of in-house mother tincture and market samples a linearity response for various concentrations of standard digitox-

in in the range of 1000-6000ng with correlation coefficient of (R) 0.995 and relative standard deviation (RSD%) 5.21% was obtained. Thus the developed HPTLC method validation performed as per ICH Q2 R1 guidelines[21]hence the developed method once validated. The quantification of digitoxin in in-house mother tincture and market mother tincture samples was achieved. This study helped in comparison study of in-house mother tincture and market for active principles. (Figure 5-7). Results of this study helped to determine the concentration of digitoxin present in in-house mother tincture (A, track 8-9) and market samples (B, C and D, track 10-15) summarized in Table 4.

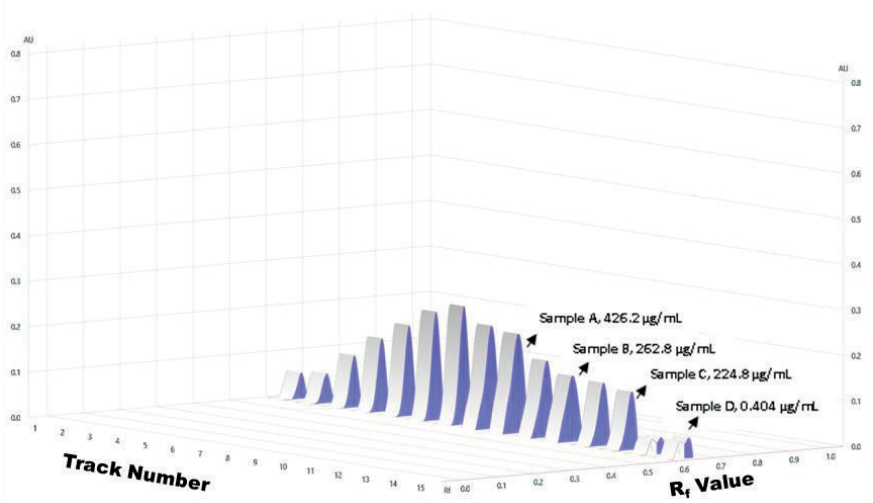


Figure 5. 3D diagram of HPTLC densitogram of chloroform extract of *D. purpurea* in-house sample and market samples with respective standard.

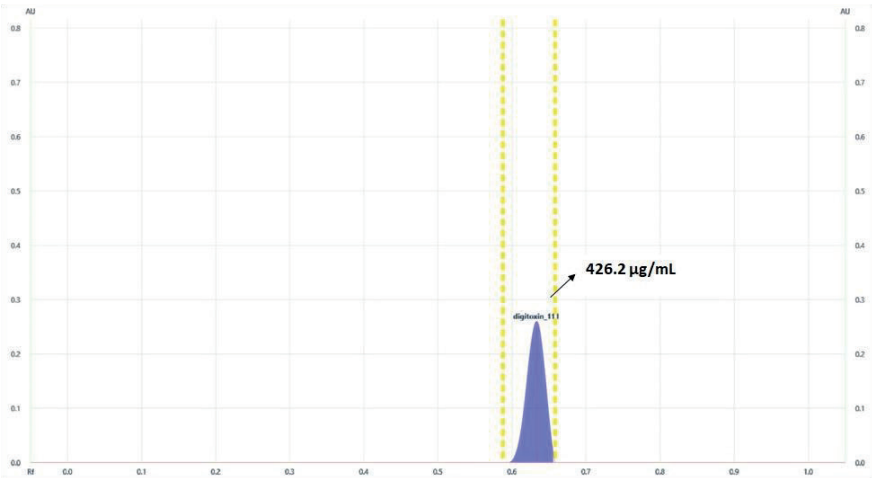


Figure 6. HPTLC peak densitogram display of standard digitoxin present in *D. purpurea* (R_f = 0.63).

Table 4. Concentration of Digitoxin found in in-house sample and market sample of *D. purpurea.*

S.No.	Sample UV detection at 219 nm	Volume applied (µL)	Concentration found in (µg/mL)
1.	Sample A	10	426.2
2.	Sample B	10	262.8
3.	Sample C	10	224.8
4.	Sample D	10	0.404

Method Validation

Linearity

For evaluation of Linearity response six different concentration of standard digitoxin were spotted in the range of 1000-6000ng. A linear relationship between the peak area and the concentration of digitoxin was observed with a good linearity response. The equation of linear regression curve obtained was $Y= mx + c = 1.472\times10^{-9}x +6.818\times 10^{-4}$, with a correlation coefficient (R) = 0.995and relative standard deviation (RSD %) = 5.21% (Table 5).

Limit of detection (LOD) and Limit of quantitation (LOQ)

Based on the standard deviation response and the slope obtained the detection limit may be expressed as:

LOD = (3.3*σ) / S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

Based on the standard deviation response and the slope obtained the quantitation limit may be expressed as:

LOQ = (10*σ) / S

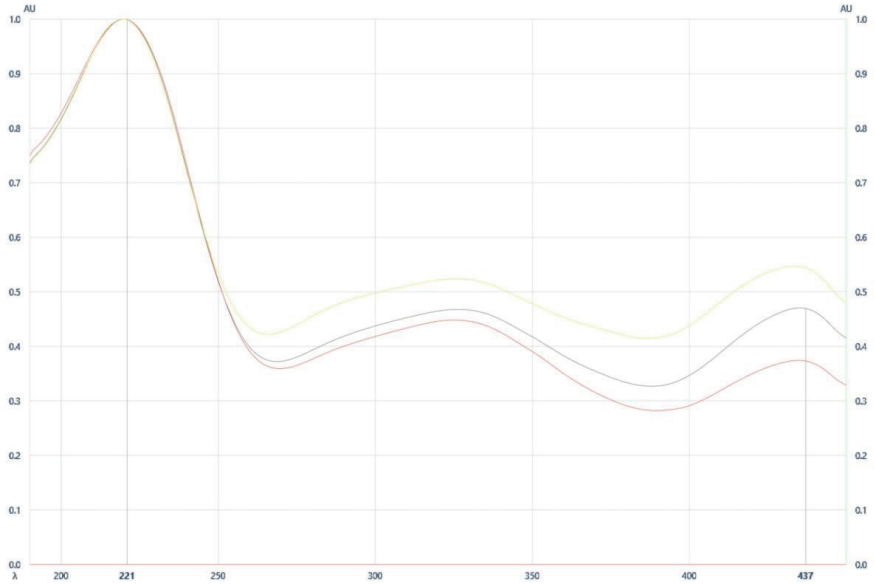
Where σ = the standard deviation of the response

S = the slope of the calibration curve

The signals to noise ratio 3:1 and 10:1 were used to measure LOQ and LOD, respectively. The LOQ and LOD of digitoxin were 1000 ng and 300ng/mL (Table 5).

Specificity

For specificity the plate was run with tracks of in-house sample A CRI (H), market sample B, C and D, standard digitoxin, solvent and mobile phase. The developed plate was scanned at lambda maximum value of standard digitoxin i.e. at 219nm. The observed peak area of standard digitoxin was integrated by using evaluation tab. The observed R_f. value of separated concerned standard of digitoxin matched with pure standard R_f. The observed area under integration of standard digitoxin peak contained no any other peak in all tracks i.e. tracks of mobile phase and samples. The developed method was found to be specific as no interfering or contaminating peaks were detected and was also evidence from the peak purity data (Figure 7, Table 5)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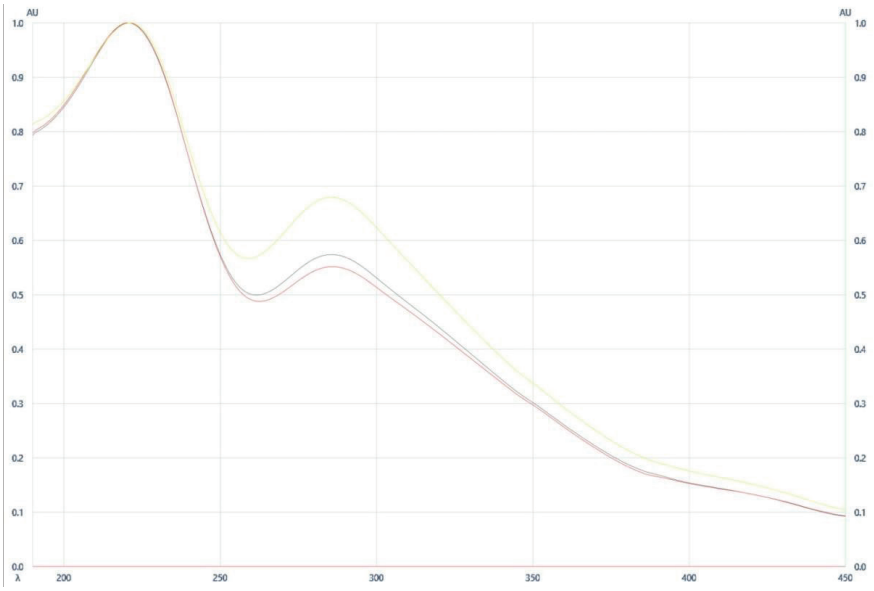
(a)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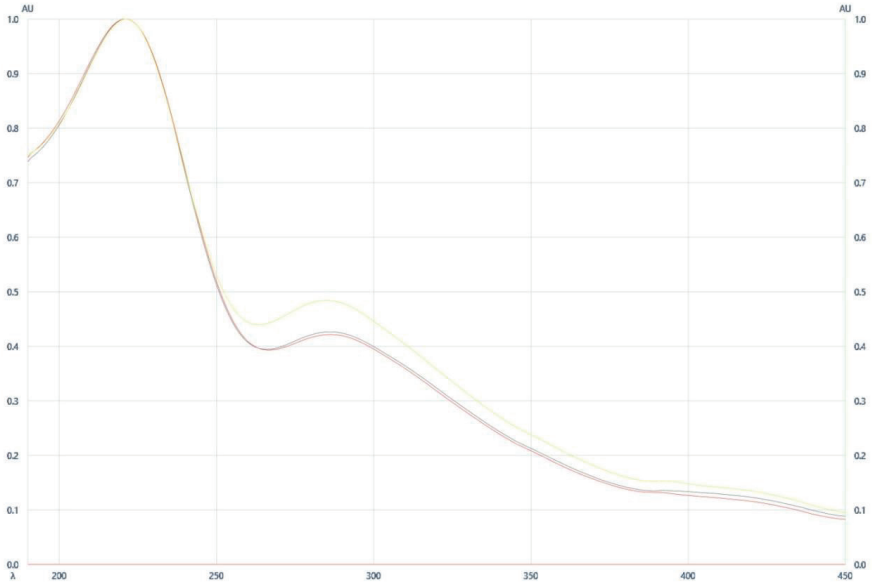
(b)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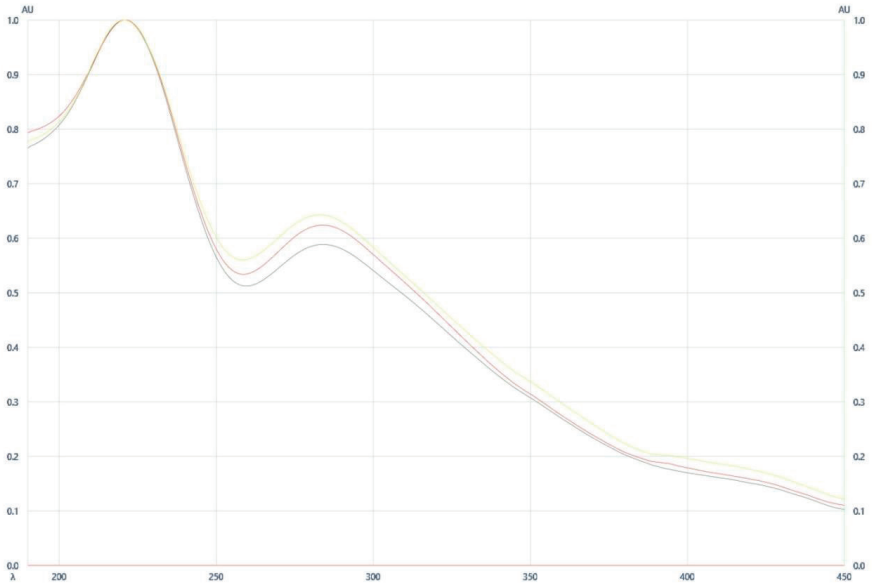
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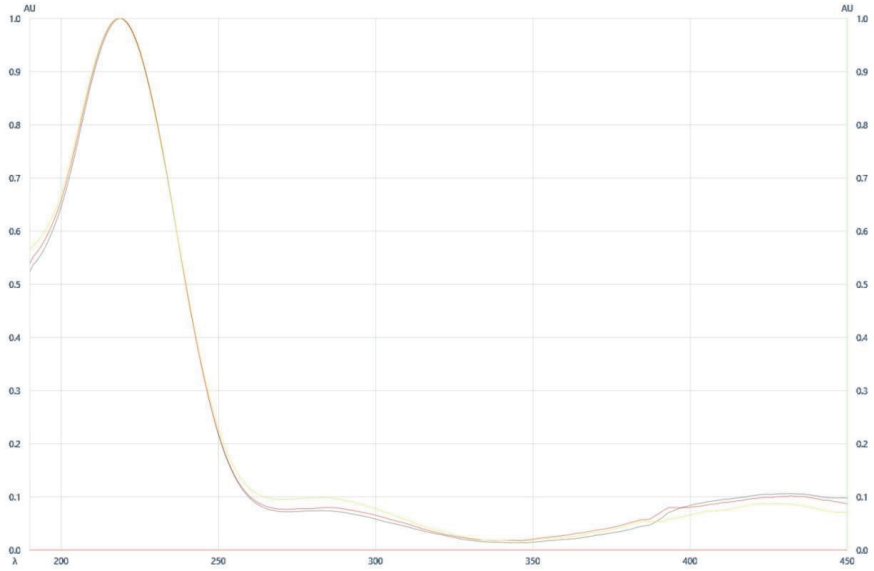
(d)



(e)

Figure 7. Overlay absorption spectra of digitoxin showing purity of up and down slopes; Max. signal @ 219 nm (a) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* in-house sample A CRI (H); r (s;m) = 0.992,r (e;m) = 0.995; (b) Peak purity of standard digitoxin; r (s;m) = 0.999, r (e;m) = 0.998; (c) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* market sample B; r (s;m) = 0.999, r (e;m) = 0.993; (d) Peak purity of test digitoxin eluted from chloroform extract of D. purpurea market sample C;r (s;m) = 0.999, r (e;m) = 0.998; (e) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* market sample D; r(s;m) = 0.999, r (e;m) = 0.998.

Precision/Reproducibility

The precision was evaluated in terms of Intra-day precision and Inter-day precision. The Intra-day and Inter-day precision was used to study the variability of the method. The average coefficient for variance (CV) value observed for Intra-day and Inter-day precision were 1.77% and 1.32%. The observed coefficient for variance CV value is less than acceptable limit of coefficient for variance (CV) 2.4% (acceptable limit of CV as per ICH guidelines is CV≤ 2.4%). Hence the developed HPTLC method for quantification of digitoxin from in-house sample CRI (H) and commercial market sample B, C and D were more precise and reproducible (Table 5).

Accuracy (Recovery)

The accuracy of the developed analytical method expresses the closeness of agreement between the value either as conventional true value or an accepta-

ble reference value and the value found sometimes termed trueness as per ICH Q2 R1 guidelines[21]. To check accuracy of the method, recovery studies were carried out by addition of standard digitoxin solution to pre-analyzed in-house sample solution at three different levels 80%, 100% and 120%. The resultant solution was reanalyzed three times and the best mean recovery % observed 88.90% (Table 5).

Table 5. Summary of validation parameters:

S.No.	Validation Parameters	Results
1.	Linearity (R)	$Y= 1.472\times10^{-9}x +6.818\times 10^{-4}(R) = 0.995$
2.	LOQ	1000 ng
3.	LOD	300 ng/mL
4.	Specificity	Specific
5.	Precision (% CV)	
	Intra-day precision	1.77% i.e. CV≤ 2.4%
	Inter-day precision	1.32% i.e. CV≤ 2.4%
6.	Accuracy (mean recovery %)	88.90%

The result of HPTLC fingerprinting profile study confirms the presence of cardiac glycosides digitoxin in in-house mother tincture (A) as well as in commercial market samples B, C and D at R_f 0.63. However, by analyzing quantitatively in-house mother tincture A has highest concentration of digitoxin i.e. 426.2 µg/mL as compare to the commercial market samples B (262.8 µg/mL), C (224.8 µg/mL) and D has lowest concentration i.e. (0.404 µg/mL) (Table 4).HPTLC Results indicate screening of extraction power of *D. purpurea* in-house CRI (H) sample and commercial market sample towards digitoxin shows maximum amount of digitoxin concentration in in-house mother tincture (A) as compare to market samples B, C and D. Therefore, in-house mother tincture (A) prepared from HPI process is best choice for future development of analytical as well as industrial plant-based developments.

A validated new HPTLC method has been developed for the identification and quantification of Digitoxin from dried chloroform extract i.e. mother tincture of the leaves of *D. purpurea* in-house sample and market samples. Developed HPTLC method was more precise, accurate and specific based on validation parameters. Present study reveals that as part of pre-formulation study, the dried chloroform extract i.e. mother tincture of the leaves of *D. purpurea*

showed promising physicochemical characteristics. The study suggests that the chloroform Leaf extract of *D. purpurea* of cardiac glycosides (digitoxin) medicinal importance that justifies its medicinal usage in Homeopathy. This is the reason for cure and healing property of leaves of *D. purpurea*. For homeopathy medicine it is absolutely necessary to laid down the standards and to check the authenticity of the plant in their extract to ascertain the quality before it enters into the market and utilized for their efficacy. There is essential need for the detailed physicochemical, Phytochemical and HPTLC finger printing study to start work on quality standard of homeopathic medicines. The present study helped in further research development work which will increase the usefulness of the plant *D. purpurea* in complementary and alternative system of medicine. Quantitative estimation of rest of the compounds present in this plant responsible for its other pharmacological activity will be evaluated in future studies.

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Formulation development of oral disintegrating film of fexofenadine hydrochloride

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ABSTRACT

The present study aimed to develop an oral disintegrating film of fexofenadine hydrochloride for the immediate management of allergies and related symptoms. The formulation of fast dissolving oral film was carried out by solvent casting method using two different grades of hydroxy propyl methyl cellulose, polyvinyl pyrrolidone, polyvinyl acetate, and pullulan as polymers and different plasticizers. Six such films were produced. The films were evaluated for their physicochemical and mechanical properties. Compatibility and thermal studies were carried out. The surface morphology of the films was studied. All six formulations were evaluated for surface pH, disintegration, and drug release study in simulated saliva. The best films were taken for stability studies. Formulations prepared with all the polymers in different proportions of plasticizers could produce a non-sticky stable film. The thermal studies revealed the uniform dispersion of the drug in the polymer matrix. Films prepared with pullulan showed better flexibility and dissolution behavior. Stability studies of the pullulan films highlighted that the films were stable and could retain their physical and mechanical properties. Hence, it can be concluded that pullulan can be successfully employed to prepare fast dissolving oral films of fexofenadine hydrochloride.

Keywords: Fexofenadine hydrochloride, Oral disintegrating film, Plasticizers, Mechanical properties, pullulan.

INTRODUCTION

Oral disintegrating films are a new type of dosage form that is particularly well

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suited to juvenile and geriatric patients. These are the thin strips that are largely transparent, biodegradable, and contain hydrophilic polymers when simply placed on the patient's tongue they disintegrate and dissolve quickly in the oral cavity when exposed to saliva, and no additional water is required for drug administration and release of the drug[1]. This type of delivery has the potential to increase patient compliance, especially for the pediatric population and for people suffering from mental illnesses, dysphagia, or emesis. Absorption of the drug by oral mucosa into the systemic circulation is an interesting approach, the drug absorbs directly into the systemic circulation, bypassing the first-pass metabolism in the liver[2].

Fexofenadine hydrochloride (FXD), an H1 antagonist is used to ease seasonal allergic rhinitis and hives. Conventional oral dosage form of FXD includes tablets and liquid oral which suffer from the drawback of slow onset of action and variable absorption on food intake. Approximately 80% of the ingested drug is eliminated primarily by biliary and renal secretion[3].

FXD belongs to class III of the Biopharmaceutical Classification System and shows variable absorption with oral bioavailability of 35%[4]. The adult daily dose of FXD is limited to 180mg, whereas the child dose is limited to 30 mg per day[5].

The present research work proposes a formulation of oral disintegrating films of FXD to provide a rapid onset of action and improvement of bioavailability. They can be administered without water, and are ideal for children. They are flexible and easy to carry[6].

The ability of the oral films primarily depends on the water solubility and film-forming ability of the polymer used. The critical parameter is the polymer to plasticizer ratio that provides the mechanical strength of the films.

Garsuch V. et al. reported the film-forming ability of hydroxypropyl methylcellulose[7]. Jantrawut P. et al. investigated and reported the effect of different plasticizers like PEG 400, glycerin, and propylene glycol on the mechanical properties of the film[8]. In another study, Pezik E. et al. commented-on pullulan as a good film-forming agent for rapid action of drugs from an orally disintegrating film[9]. The use of a combination of polymer of polyvinyl pyrrolidone and polyvinyl acetate in the formulation of fast dissolving films was reported by Chaklan N. et al[10].

Hence the present study explores the combination of different types of polymers and plasticizers to formulate oral disintegrating films of FXD and observed the effect of the variation in polymer and plasticizer content on the physicochemical and mechanical properties of the film.

METHODOLOGY

FXD was purchased from Yarrow chemicals, Mumbai, Maharashtra. Pullulan was procured from Gangwal chemicals, Mumbai, Maharashtra. Hydroxypropyl methylcellulose (HPMC) 15cp and 5 cp, polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), sodium starch glycollate (SSG), polyethylene glycol 400 (PEG 400), glycerine, propylene glycol, ethanol, and mannitol were procured from SD fine chemicals, Bangalore, India.

Fourier transform infrared (FTIR) spectroscopy study

The compatibility study of the FXD with different polymers in a physical mixture of 1:2 was carried out by Fourier transform infrared (FTIR) spectroscopy Shimadzu, Tokyo, Japan. The IR spectra of the samples were obtained using the KBr pellet method in the range between 400 to 4,000cm^{-1} at ambient temperature [11].

Preparation of oral film

The solvent casting approach was used to make the oral film. Six such FXD-loaded orally disintegrating films (F1-F6) were prepared using different polymers, and plasticizers as mentioned in table 1.

Table 1. Composition of the oral films of FXD

Ingredients	Formulation code					
	F1	F2	F3	F4	F5	F6
Drug (mg)	30	30	30	30	30	30
HPMC 15 cp (%w/v)	20	--	--	--	--	--
HPMC 5 cp (%w/v)	--	20	--	--	--	--
PVP (%w/v)	--	--	10	--	--	--
PVA (%w/v)	--	--	10	--	--	--
Pullulan (%w/v)	--	--	--	20	20	20
PEG400 (%w/v)	3	2	3	2	--	2
Propylene glycol (%w/v)	--	--	--	1	3	--
Glycerine (%w/v)	--	1	--	--	--	1
SSG (%w/v)	3	3	3	3	3	3
Mannitol (%w/v)	1.5	1.5	1.5	1.5	1.5	1.5
DM Water (q.s) (ml)	5	5	5	5	5	5

The drug was dissolved in ethanol. The polymers as per table 1 were dispersed in distilled water under magnetic stirring to which the drug solution was added. To this homogenous solution of polymer and drug, the required quantity of plasticizer, disintegrants, and sweeteners was added. The entire dispersion was continued to stir using a magnetic stirrer at 800rpm for 30min[12,13]. The prepared mixture was set aside for 15min to remove any bubbles before being poured into the fabricated 8cm² glass mold. The casted films were dried for 45–50 minutes in a laminar hot-air oven at 60°C. The film was carefully peeled off after drying, packaged in plastic zip pouches (polythene), and stored in a desiccator for further characterization.

Evaluation of the prepared films

Differential scanning calorimetric (DSC) study

Samples weighing approximately 5mg were placed in an Aluminum crucible and heated at a rate of 10/min on a Perkin Elmer STA 8000 series instrument from room temperature to 500°C. The thermograms were recorded for the pure drug and the physical mixture of drug with different polymers[14].

Drug content

Each film of the sample (1cm²) was dissolved in methanol to extract the drug. The sample was suitably diluted with pH 6.8 buffer and analyzed at 277nm using a UV spectrophotometer (UV-1900i, Shimadzu, Tokyo) against a suitable blank prepared from the non-medicated film. This experimentation was done with three trials for all the formulations (F1-F6) [15,16].

Film Thickness

The thickness of the film (2cm(L) X 8cm (W)) was measured with a digital vernier caliper (Mitutoyo), and the average value was calculated. The thickness was measured at 5 different points throughout the film to ensure uniformity. The estimation was carried out in triplicate for all the prepared films[17].

Moisture loss

To determine the moisture content in the films, the freshly prepared film (4cm²) of the formulations (F1-F6) was weighed and placed in desiccators containing anhydrous calcium chloride under airtight conditions. The film was reweighed after 3 days to determine the percentage of moisture loss [18].

$$Percentage\ moisure\ loss\ = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

Film pH

The film to be tested was moistened with distilled water and stored in a Petri dish for 1h, and the pH of the solution was recorded using the pH meter Digisun electronic system, Model No:2001. The experimentation was carried out thrice for each film [17].

Swelling index

The studies on the swelling index of the films (F1-F6) (2cm(L) X 2cm(W)) were carried out in pH 6.8 phosphate buffer solution. Each film was weighed and placed in a pre-weighed stainless-steel wire sieve. The film was submerged in phosphate buffer pH 6.8 on a Petri dish. At a specified interval (1min), the rise in film weight was measured until a constant weight was recorded. In this test, three films of each formula were employed [19]. The degree of swelling was determined by using the following formula:

$$Swelling\ index = \frac{W_t - W_0}{W_0}$$

Where, is the weight of the film at the time t; is the Weight of film at t = 0

Mechanical properties of the film

Folding endurance

Randomly selected films (2cm(L) X 2cm (W)) from each formulation were folded at the same place until any signs of fracture were visible. Folding endurance was determined for all the films (F1-F6) to observe how many times the films could be folded without breaking or showing noticeable fissures[20]. Each experiment was carried out thrice.

Tensile strength, percentage elongation, and young's modulus

The tensile strength of films, F1 to F6 was measured to estimate the resistance of the film against the applied forces from breaking down. The tensile strength was measured using a tensiometer by holding a film (an area approximately 2cm × 2cm) longitudinally in it and drawn against an increasing load at a rate of 10mm/min. Tensile strength is calculated mathematically by the equation illustrated below[21,22].

$$\sigma_{TS} = \frac{F_{max}}{A}$$

where, is the tensile strength of the film, is the maximum load applied on the film till it breaks, and A is the initial cross-sectional area of the sample.

During the process of estimating tensile strength, the length elongation was measured for each film.

The increase in the length of the film was used to determine percentage elongation (%E) using the formula [23].

$$\%\varepsilon = \frac{\Delta L}{L_0} \times 100$$

Where percent ε is elongation at break, ΔL is the increase in length, and L_0 is the sample's initial length.

The hardness of the oral disintegrating film was measured by calculating the young modulus as per the following formula.

$$E = \frac{F}{A} \times \frac{1}{\varepsilon}$$

Where, E is elastic modulus, which is Young's modulus, F is the force at the corresponding strain, *A* is the sample's initial cross-sectional area, and ε is the corresponding strain[24].

Disintegration time

The disintegration time of each film (8cm^2) was measured by placing a unit dose of the formulated film in a petri-dish containing distilled water and the time taken for the film to complete breakdown was noted[25,26].

In vitro dissolution study

The *in vitro* dissolution study was carried out by using USP dissolution apparatus type-II with 900ml of simulated salivary fluid of pH 6.8 at 37±0.5°C at 100rpm. A film size of 8cm^2 was used as dose size. In the dissolution apparatus, the film was attached with adhesive tape on a glass disk and placed at the center of the vessel[27]. At specified intervals, specific volumes of samples were collected for 60min and replaced with an equivalent volume of the blank dissolution media. The blank of each formulation was subjected to dissolution at the same condition and used as a blank to negate the effect of excipients in the determination of drug spectrophotometrically. The filtered samples were analyzed at a wavelength of 277nm for drug concentration, and the percentage (%) of drug dissolved or released was calculated. The drug release study of all the films (F1-F6) was compared with a popular commercial tablet available in India.

Scanning Electron Microscope study (SEM)

SEM study was carried out using Zeiss, ultra 55 (GEMINI® technology), to study the surface morphology of the films. The film was adhered to the stubs with adhesive carbon and then sputter-coated with a thin gold-palladium layer. The coated samples were scanned at 5kV[18].

Statistical analysis

All the six formulations were subjected to Dunnett's multiple comparison test using graph pad prism software V5, considering the parameters of disintegration time, drug release at the earliest, and the mechanical strength of the films. The ANOVA study was carried out at a significance level of $p<0.05$.

Stability testing

Stability testing was carried out for the best formulations (F4, F5, and F6). The films were stored in the aluminum package at 40 ±5°C and 75±5%RH for 28 days. The films were tested for physicochemical and mechanical properties at a specific interval of time[9].

RESULTS and DISCUSSION

FTIR study

The FTIR spectra showed the characteristics peak of the pure drug at 1704. 1470. 1278 and 1167cm^{-1} for the major functional groups like carbonyl stretching, aromatic stretching, and C-O stretching of tertiary alcohol respectively, the same peaks were preserved in the combinations of drug and different polymers used in the oral films. Major functional groups were retained in all the combinations as shown in figure 1, which indicated the absence of any interactions between the drug and polymers[28].

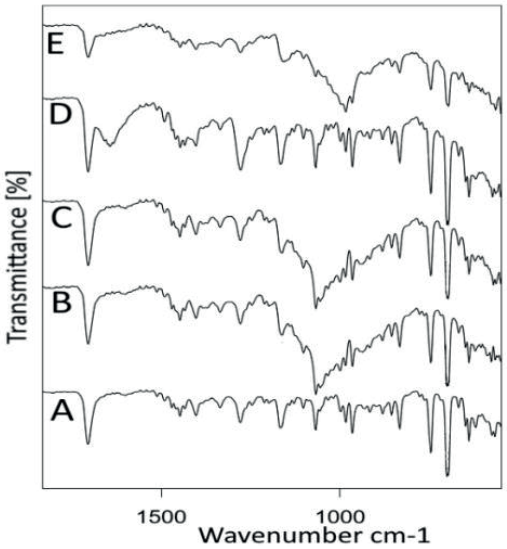

Figure 1. FTIR spectra of Pure drug FXD (A), FXD and HPMC 15cp(B), FXD and HPMC 5cp(C), FXD and PVP+PVA(D), FXD and Pullulan(E).

DSC Study

The DSC thermograms showed a sharp endothermic peak of the pure drug at 192°C[29]. The drug dispersion in the various polymer mixture showed a reduction of the peak intensities to a great extent. The reduced intensity of the endothermic peaks of the pure drug represented the conversion of crystalline to the amorphous state of the drug as shown in figure 2.

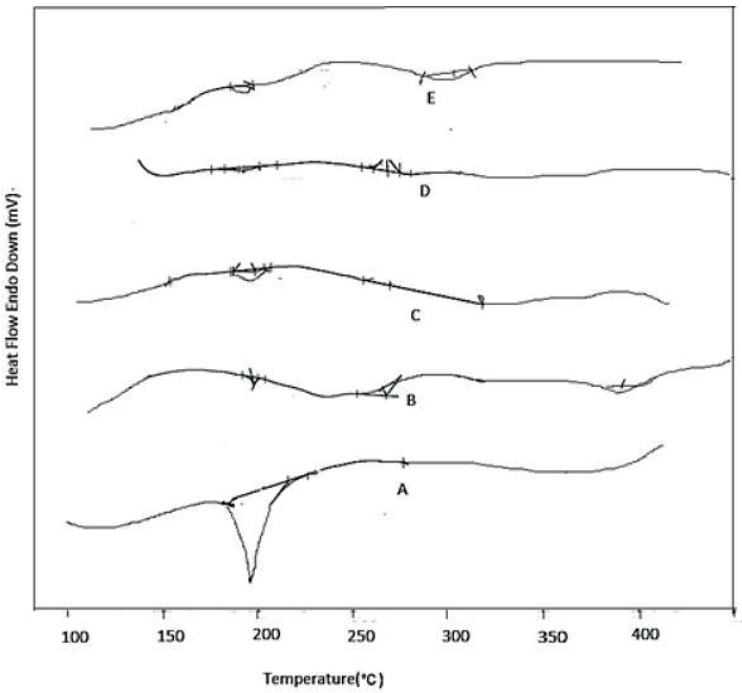

Figure 2. DSC thermograms of Pure drug FXD(A) and films of different polymers (B-HPMC 15 cps, C- HPMC 5 cps, D-PVP and PVA, E-Pullulan)

This is an indication of the dispersion of the drug in different polymer matrices. Hence it can be concluded that the drug was molecularly dispersed in the polymer matrices. Miscibility of the drug with the polymers confirms the formation of miscible dispersion. Therefore, all the selected polymers were found to be suitable for the formulation of the oral disintegrating film of FXD. The glass transition temperature of the prepared films was found to be greater than the temperature of the buccal cavity and environment, which proves the stability of the film during manufacturing, and storage.

Physicochemical evaluation of the prepared films

The prepared six films were found to be smooth and opaque with a uniform dispersion of drug in the films. The physical characteristics of the films are summarized in table 2.

Table 2. Evaluation of the oral disintegrating films of fexofenadine hydrochloride

Formulation	Drug Content (%)	Thickness (mm)	Weight (mg)	pH	Moisture content(%)	Swelling index
F1	98.78±0.01	0.032±0.01	66.3±0.03	6.8	0.85±0.85	1.76±4.70
F2	99.12±0.023	0.035±0.02	64.3±0.02	6.4	0.87±0.69	2.79±1.01
F3	99.05±0.04	0.027±0.02	57.6±0.05	6.6	0.45±0.09	6.12±2.90
F4	99.44±0.02	0.025±0.03	56.6±0.01	5.5	0.88±0.21	1.73±1.04
F5	99.61±0.12	0.021±0.02	60.5±0.04	5.6	0.83±0.68	1.77±1.94
F6	99.75±0.05	0.023±0.01	58.5±0.04	5.5	0.84±0.74	1.33±1.93

The drug content of the films was found to be more than 98%, which indicated that the drug was distributed homogeneously in the polymer matrix. The drug content of all the films was found to be in the acceptable pharmacopeial range for standard oral solids.

The thickness of the films was measured at 5 different points of the prepared films and found that the standard deviation was less than 0.05, which indicated the thickness of all the films was uniform. It also revealed that the prepared drug-polymer dispersion had optimum viscosity to be spread over the fabricated dish uniformly by solvent casting method. For an oral film, the thickness should be 12 to 100μm[4]. The thickness of the film was found to be high for the films prepared by HPMC polymers (F1 and F2). Rest all the films prepared had a thickness varied from 0.021 to 0.027mm. This variation in thickness might be due to the difference in composition of the films which resulted in various viscosity and the spreadability of the dispersion over the petri dish. The average weight of the films was slightly high for the films made with HPMC. The pH of all the films was found to be in the range of 5.5-6.8. The surface pH of the films indicated the non-irritability of the films for oral mucosa. The mechanical properties of the films are greatly affected by the moisture content. This quality control parameter enables to detect the protection of the films against drying out during storage. All the formulations showed a moderate moisture content to retain the plasticizing effect of the films. Formulation F3 containing PVP and PVA was found to be brittle on storage as indicated by the moisture content and folding endurance. The swelling index for the films varied widely because of the composition of the films. A high swelling index is an indication of high viscosity on the absorption of moisture and thereby a slow release. Formulation F3 showed a very high swelling index compare to the other formulations. Folding endurance was found to be the best for films containing

HPMC (F1 and F2), it is due to the comparatively high thickness and weight, and moisture content of the films. Films prepared with pullulan (F4, F5, and F6) were also found to be good for arresting the breakage during folding[30]. But the films prepared with PVP and PVA (F3) are not found to be satisfactory in durability as indicated by the low value of folding endurance.

Mechanical Properties

The deformation of a film under applied stress determines the mechanical strength of the film. Mechanical strength is an ideal property of an orally disintegrating film. The mechanical strength of oral disintegrating films is estimated by the determination of folding endurance, tensile strength, percentage elongation, and young modulus. The folding endurance of all the films was found to be more than 110 except for formulation F3. Higher folding endurance was indicative of high mechanical strength of the films. The elongation of the film increases as the plasticizer content rises. The use of a mixture of plasticizers was found to be good in formulations F4, F5, and F6. An optimum elongation was obtained from each formulation. Young's modulus, also known as elastic modulus, is a measurement of the film's stiffness and a correlation between applied stress to strain. An ideal film is characterized by higher folding endurance and tensile strength, and low elastic modulus[31]. The films produced by HPMC (F1 and F2) and Pullulan (F4, F5, and F6) were quite strong compared to films produced by PVP and PVA (F3) as shown in table 3.

Table 3. Mechanical properties of the prepared oral films of fexofenadine hydrochloride.

Formulation code	Folding endurance	Tensile strength (dyne/cm^2)	% Elongation	Young modulus (Pascal)
F1	120±2.13	5	22.33	0.224
F2	110±1.16	4.5	22.12	0.203
F3	89±1.67	3	20.21	0.148
F4	115±1.78	4.5	21.03	0.214
F5	114±2.34	3.75	20.59	0.182
F6	118±2.50	4.5	21.76	0.207

Disintegration time

The oral films should disintegrate quickly in the mouth and should be able to release the drug immediately for instant therapeutic action. The composition and the physicochemical property of the polymer play an important role. All the polymers used in the present study showed good film-forming properties.

But the disintegration time of the films becomes critical to identify the best polymer for the proposed hypothesis. Hence in the present study, the formulation with different polymers was prepared to identify the best polymer for the oral disintegrating films of FXD, the disintegration time was found to be the least for films prepared with pullulan (F4, F5, and F6) as shown in table 4. According to European pharmacopeia, the oral disintegrating tablets should disintegrate within 3min, and in the present study all the films were disintegrated below 1min and that satisfies the limit prescribed by the pharmacopeia[32].

Table 4. Disintegration time of the films

Formulation code	Disintegration time (sec)
F1	35±0.05
F2	43±0.01
F3	65±0.02
F4	29±0.65
F5	29±0.05
F6	26±0.03

In vitro Dissolution profile

The dissolution of all the oral films of FXD was carried out in simulated salivary fluid at pH 6.8 for 60min. The dissolution profile of all the films is expressed in figure 3. The maximum release was exhibited by formulation F6 in 30min. The marketed formulation exhibited 75% release in 30min.

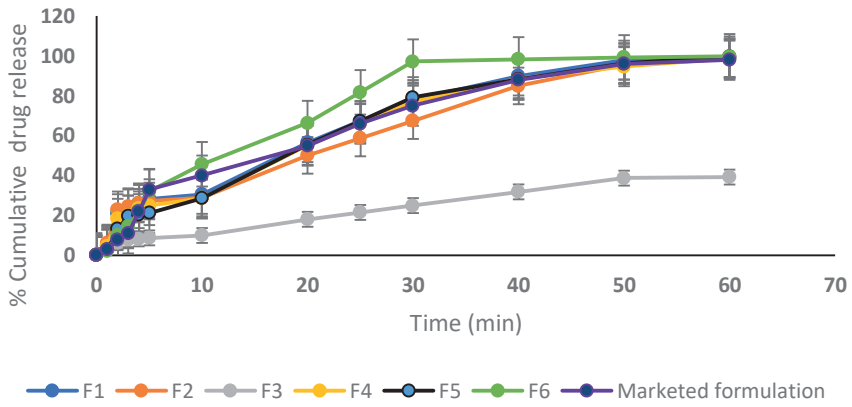

Figure 3. *In vitro* dissolution of the prepared oral films of fexofenadine hydrochloride.

The *in vitro* release data was analysed for release kinetics and found that all the formulations except F3 followed first-order kinetics. The release of the drug from formulation F3 was slow it might be due to the high swelling of the film resulting in high viscosity, hence retarded the drug release. The regression analysis of rate kinetics of dissolution is shown in table

Table 5. Regression analysis for release data

Formulation code	R_1	R_0	R_{HG}	R_{KM}
F1	0.968	0.960	0.956	0.922
F2	0.959	0.962	0.949	0.910
F3	0.983	0.985	0.977	0.950
F4	0.975	0.963	0.959	0.938
F5	0.984	0.966	0.969	0.928
F6	0.986	0.899	0.968	0.967

R_1- Correlation coefficient for first order kinetics, R_0- Correlation coefficient for zero order kinetics, R_{HG}- Correlation coefficient for Higuchi model, R_{KM}- Correlation coefficient for Korsemeyer Peppas model.

SEM Study

The SEM photographs (figure 4) for all the formulations revealed the uniform texture of the prepared film upon dispersion of the drug in the polymer matrix. The films F4, F5, and F6 showed a smooth surface with lesser cracks indicating good mechanical strength of the films prepared by pullulan and different plasticizers. Films prepared by HPMC 15cps (F1) and 5CPs (F2) had comparatively lesser cracks than films prepared by PVP and PVA(F3). Hence the surface morphology also indicated films prepared with pullulans were the best in mechanical strength[14].

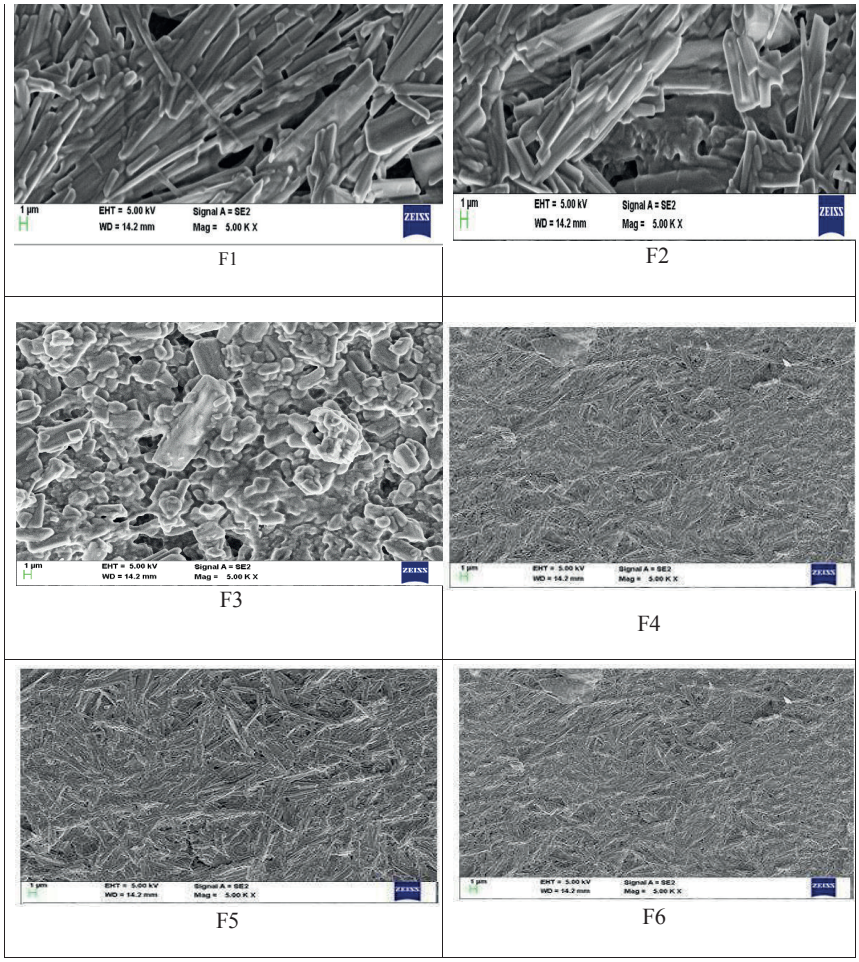

Figure 4. SEM of the different oral films(F1-F6) of Fexofenadine hydrochloride.

Statistical analysis

Dunnett's multiple comparison tests for all the formulations were carried out considering the main characteristics of an oral film like fast disintegration, fast release, and high mechanical strength of the films. A random scaling of 1-5 was assigned for the evaluation of the important characteristics, 5 being the highest, and 1 being the lowest. The low disintegration, rapid dissolution at the earliest, and high mechanical properties were assigned with the highest score. An ANOVA study at a significance level, $p<0.05$ was conducted and it revealed that the characteristics of the formulation F6 were significantly better than F1, F2, and F3 statistically. The formulations F4 and F5 were almost equivalent to F6 and did not show any significant statistical differences. The statistical analysis is listed in Table 6.

Table 6. Dunnett's Multiple Comparison Test

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
F6 vs F2	1.500	3.464	Yes	*
F6 vs F3	3.250	7.506	Yes	***
F6 vs F4	0.5000	1.155	No	ns
F6 vs F5	0.0000	0.0000	No	ns
F6 vs F1	1.000	2.325	Yes	*

Hence, it can be concluded that the formulations F4, F5, and F6 have the desired properties of an orally disintegrating film. All the films made up of polymer Pullulan have good characteristics to deliver drug immediately from an oral film, irrespective of the type of plasticizer in the film. Therefore, these formulations were taken for stability studies.

Stability study

The selected films were packed in an aluminium package and stored at 40 ±5°C and 75±5% RH for 28 days. The short-term stability data are presented in table 7.

Table 7. Short-term stability study

Properties evaluated	F4		F5		F6	
	14th Day	28th Day	14th Day	28th Day	14th Day	28th Day
Drug content (%)	99.14±0.01	98.64±0.03	99.6±0.02	99.54±0.05	99.69±0.02	99.64±0.03
Thickness (mm)	0.026±0.01	0.0256±0.09	0.023±0.03	0.022±0.09	0.025±0.06	0.0248±0.01
Moisture content (%)	0.91±0.11	0.89±0.19	0.84±0.21	0.82±0.90	0.845±0.44	0.840±0.12
Folding endurance	110±1.08	108±0.23	116±1.30	114±0.92	113±1.05	113±0.07
Young modulus (Pascal)	0.210	0.207	0.178	0.176	0.206	0.205
Disintegration time (sec)	30±0.15	32±0.55	28±0.15	31±0.0.70	26±0.07	27±0.05

Short-term stability studies revealed that the films prepared with pullulans (F4, F5, and F6) were stable, there were no significant changes in the properties of the films ($P>0.05$). Under accelerated conditions, the change in moisture content in the films was found to be minimum. Hence it can be concluded that oral disintegrating films of FXD made with polymer pullulan and different plasticizers were found to be stable and retained their mechanical and pharmaceutical properties.

The current research concentrated on the formulation development of oral disintegrating film of FXD for immediate control of the symptoms of allergy. The study screened a variety of polymers and plasticizers to evaluate their effect on the mechanical property and dissolution behaviour of the drug. Among all the polymers used the polymer pullulan was found to be the best to achieve the desired characteristics of the fast-dissolving film of FXD. The films disintegrated in less than 30 seconds and showed a high drug release at 30 min. The formulations F4, F5, and F6 were found to be stable. The formulation showed good mechanical properties and drug release and could retain flexibility, and strength even after a short-term stability study for 4 weeks. Hence it can be concluded that a fast-dissolving film of FXD with pullulans could be a promising combination to achieve the objective of the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Grewia asiatica Linn. root extract loaded suspension, microparticulate and nanosuspension dosage form: Fabrication, characterization and anthelmintic evaluation

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ABSTRACT

The current study was planned to formulate the *Grewia asiatica* Linn. root extract loaded herbal formulation and evaluated its potential for *in vitro* anthelmintic activity. The root extract of *Grewia asiatica* Linn. was standardized by HPTLC using quercetin and naringenin, as a marker compound. The extract was formulated into a novel microparticulate and nanosuspension dosage form based on the Eudragit S100. The prepared dosage forms were characterized by particle size, morphology, and *in vitro* release behavior. Further, the comparative evaluation of dosage form revealed the presence of higher *in vitro* anthelmintic activity in nanosuspension as compared to conventional suspension and microparticulate dosage form.

Keywords: *Grewia asiatica*; nanosuspension; microparticles; suspension; anthelmintic

INTRODUCTION

Helminths i.e. parasitic worms of the gastrointestinal tract are pathogens, present as a constraints in livestock all over the world. Helminths not only tend to increase other bacterial infections in the body but also reduce growth rate and food production (e.g. milk and meat). Approximately, ten billion people are

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affected in remote areas [rural (68%), & urban (50%)] from soil-transmitted helminthiasis diseases like *Ascariasis*, hookworm, *Trichuriasis,* etc. To treat the infections, several synthetic anthelmintic drugs are available in the market with high cost, toxicity, and drug resistance[1,2]. So on reviewing the literature, herbal drugs were chosen to formulate the required anthelmintic formulation to avoid any side effects and to improve the efficacy with synergistic effects.

Herbal drugs, a part of the traditional system of medicine, are widely present all around. The safer pharmacological profile, economical value, and easy availability make herbal tonics a choice of interest for the development of pharmaceutical formulation[3]. *Grewia asiatica* Linn. (belonging to the family – *Tiliaceae*) is commonly known as "Phalsa" and is found in Asian countries. It is comprised of fruits, shrubs, and trees and has a height of up to 6-8 m. Of 150 species of this genus, nearly 40 species are used in the folklore medicine[4,5]. The term "*Grewia*" comes from the 'Nehemiah Grew' i.e. name of plant physiology founder and "*asiatica*" which reflects the Asian origin of species[6]. This seasonal summer plant contains phenols, tannins, saponins, and flavonoid compounds. Fruits of *Grewia asiatica* contain vitamins, proteins, minerals, fibers, and ash. It has also many reported therapeutic properties such as antimicrobial; analgesic; antipyretic; antioxidant; anthelmintic; antimalarial; anti-hyperlipidemic; rheumatism, radioprotective and anti-diarrhoeal etc[7]. It has already been reported that leaves and extract from *Grewia asiatica* Linn. have high anthelmintic activity but due to the lack of scientific data, and its clinical effectiveness, it needs to be explored more efficiently for further evaluation.

Henceforth, the present study was designed to formulate three different pharmaceutical dosage forms such as suspension, microparticulate, and nanosuspension of *Grewia asiatica* Linn. root extract. However, a standardized extract of *Grewia asiatica* Linn. was used to formulate dosage forms which were characterized by using FT-IR, SEM, and TEM and further evaluated for *in-vitro* anthelmintic activity.

METHODOLOGY

Chemicals and reagents

Grewia asiatica Linn. roots were taken and authenticated in CSIR-NISCAIR, New Delhi, India under voucher specimen no. NISCAIR/RHMD/Consult/2013/2631-141-1 dated 07-01-2014. Albendazole was collected from Coax Bioremedies (Hisar, India). Quercetin, toluene, and ethyl acetate were purchased from Hi-Media Laboratories Pvt. Ltd., (Mumbai, India). Naringenin, formic acid, and methanol were procured from Sisco Research Laboratories

Pvt. Ltd., (Mumbai, India). Silica gel F_{254} HPTLC plates were obtained from Machery-Nagel (Germany). Silica gel UV254, Sodium carboxymethyl cellulose, Tween 80, methyl and propylparaben, lemon oil, Eudragit S100, and Poloxamer 188 were collected from S.D. Fine-Chem Ltd. (Mumbai, India). Other analytical grade chemicals and reagents were used as received.

Preparation of *Grewia asiatica* extract

Grewia asiatica Linn. roots were dried under shade and powdered coarsely which was extracted with distilled water and ethanol. Briefly, alcoholic extracts were prepared with 95% ethanol by a continuous soxhlation process and aqueous extracts were prepared by a cold maceration process. Both extracts were concentrated to a semi-solid residue. Aqueous and alcoholic extracts were suspended in saline phosphate buffer for further usage[8].

Evaluation of anthelmintic activity

Adult Indian earthworms of size 9-12 cm long and 0.1-0.2 cm wide (*Pheretima posthuma*) were collected from the moist soil of the farm field (Hisar, India). The anthelmintic activity of the aqueous and ethanolic extracts of *Grewia asiatica* Linn. roots against *Pheretima posthuma* were evaluated comparatively by employing albendazole as a standard drug. The earthworms were divided into 12 groups of six worms each.

Group 1 : Control (treated with 25 ml of phosphate buffer saline)

Group 2 : Standard (treated with 500 mg of albendazole)

Group 3-7 : Treated with ethanolic extracts of *Grewia asiatica* Linn. (GAEE) roots at five levels of concentration i.e. 12.5, 25, 50, 75 and 100 mg/ml respectively

Group 8-12 : Treated with aqueous extracts of *Grewia asiatica* Linn. (GAAE) roots at five levels of concentration i.e. 12.5, 25, 50, 75 and 100 mg/ml respectively

The worms in group 1 (Control) were treated with phosphate buffer saline, while the group 2 (Standard) worms were treated with albendazole suspension (20 mg/ml) in phosphate buffer saline. Further, GAAE and GAEE were screened at five levels of concentration i.e. 12.5, 25, 50, 75, and 100 mg/ml. The earthworms were placed in petridishes (diameter 9cm) containing 25 ml of the aqueous vehicle (control) or albendazole (standard) or extract as per their treatment protocol. The earthworms were observed for paralysis and death over a period of time and the time taken for paralysis and death of worms was noted and the mean was calculated. The paralysis of worms was established

when the worms become immobile even in warm saline phosphate buffer, while the death was confirmed when the worms showed loss of motility followed by fading of body color [9, 10, 11].

HPTLC Analysis

HPTLC analysis was carried out using aluminum-backed plates pre-coated with silica gel F_{254} (20 x 20 cm, 200 µm layer thickness). The samples were spotted with CAMAG 100 µl syringe using a CAMAG Linomat V automatic sample spotter, starting at the point x = 15 mm and y = 10 mm, in the form of distinct bands of width 4 mm and 10 mm apart. The optimized equipment parameters used were:- application rate 160 nl/sec, slit dimension:- 3 x 0.45 mm and scanning speed of 20 mm/sec. The plates were developed in a linear ascending mode in a twin trough (20 x 10 cm) glass chamber which was previously saturated with the mobile phase for 15 min at a temperature of (25±2)°C and RH of (60±5)%. The chromatogram was developed with a run height of 80 mm and a mobile phase volume of 15 ml (a TLC study was carried out to select the suitable mobile phase for the separation of compounds). The densitometric analysis of plates was performed at 254 nm in absorbance mode employing CAMAG TLC scanner-III having a tungsten lamp as a radiation source and win CATS software (version 1.2.0)[12,13,14].

GAEE-loaded suspension dosage form

The suspension dosage form of GAEE (5%) was formulated using sodium carboxymethyl cellulose as suspending agent (0.5%, 1%, 1.5% and 2%), Tween 80 as wetting agent (0.1%), lemon oil as flavouring agent (0.1%), and methylparaben (0.08%) and propylparaben (0.03%) as preservative.

Evaluation

The prepared GAEE conventional suspensions were evaluated organoleptically for color, odor and taste; pH and viscosity were determined at 25°C; sedimentation volume and ease of redispersibility were measured after 1 day and 1 week respectively; followed by an accelerated stability study at 25°C and 40°C for 3 months.

GAEE-loaded Eudragit S100 microparticles

GAEE-loaded Eudragit S100 microparticles were prepared by spray drying technique. Briefly, an ethanolic solution (250 ml) of Eudragit S100 (1%, w/v) containing GAEE (10%-50%, w/w of Eudragit S100) was sprayed through the spray dryer (LSD-48, JISL, Mumbai, India) under the following conditions:- nozzle: ultrasonic nozzle; inlet air temperature: 85°C; outlet air temperature:

43°C; aspirator: 41%; feed rate: 1%; a vacuum in the system:- 80 mmWC and atomization pressure: 2 kg/cm^2 [15, 16].

Characterization of microparticles

GAEE-loaded Eudragit S100 microparticles were characterized for size by optical microscopy and morphology by scanning electron microscopy.

Microscopical size

Micromeritic analysis of microparticles was carried out using optical microscopy. A pinch of powdered GAEE-loaded Eudragit S100 microparticles was taken on a glass slide and observed under an optical microscope. The eyepiece of the microscope was fitted with an ocular micrometer previously calibrated with a stage micrometer. The photomicrographs were taken using Zeiss Primostar trinocular microscope with a Canon photomicrograph unit.

Morphology

Scanning electron microscope (JEOL, JSM-6100, Tokyo, Japan) was used to observe morphological features and surface topography of microparticles. The gold-coated sample was mounted on a sample holder for capturing the photomicrographs at an accelerating voltage at 10kV at different magnification.

Evaluation of microparticles

Drug content

GAEE-loaded Eudragit S100 microparticles were assayed for the contents of quercetin and naringenin. An accurately weighed 50 mg of the microparticles were dissolved in 10 ml methanol. The contents of quercetin and naringenin were determined by the HPTLC method of analysis as reported above.

In vitro drug release

The *in vitro* release behavior of GAEE-loaded Eudragit S100 microparticles was determined by employing USP type-II dissolution rate test apparatus (TDL-08L, Electrolab, Mumbai, India). GAEE-loaded Eudragit S100 microparticles equivalent to GAEE (50 mg) were placed in muslin cloth, which was tied to the paddle. The paddle was dipped in 500 ml phosphate buffer saline (pH 7.4), maintaining the temperature at (37±0.1)°C, and rotated at speed of 50 rpm. Aliquots of 0.1 ml sample were withdrawn at a specific time interval and substituted with an equivalent volume of release media[17,18]. The withdrawn specimens were investigated for the contents of quercetin and naringenin using the HPTLC method of analysis as mentioned earlier.

GAEE-loaded Eudragit S100 nanosuspension

GAEE-loaded Eudragit S100 nanosuspension was synthesized using the nanoprecipitation method [19]. Briefly, 250 ml solution of GAEE (0.5 %, w/v), Poloxamer 188 (1%, w/v), and Eudragit S100 (1%, w/v) in acetone was prepared. This solution was introduced steadily using a hypodermic glass syringe into distilled water (500 ml), with continuous stirring. A rotary vacuum evaporator (Strike 102, Steroglass, Italy) was used to evaporate the acetone in obtained dispersion. The resulting nanosuspension was dried employing a lyophilizer and spray dryer. For lyophilizer, mannitol (5%, w/v) as cryoprotectant was added in suspension and kept in a deep freezer at -80°C for overnight, followed by lyophilization in a laboratory model freeze dryer (Alpha 2-4 LD Plus, Martin Christ, Germany) at -90°C for 24 h, at 0.0010 mbar. The other batch of nanosuspension was spray-dried using a lab model spray drier (LSD-48, JISL, Mumbai, India) under the following conditions:- nozzle: ultrasonic nozzle; inlet air temperature: 150°C; outlet air temperature: 61°C; aspirator: 42%; feed rate: 4%; a vacuum in the system:- 80 mm WC and atomization pressure: 2 kg/cm^2. The lyophilized powder of GAEE-loaded Eudragit S100 nanosuspension containing equivalent to 1250 mg of GAEE was dispersed in 25 ml Sorenson's phosphate buffer (0.0667M, pH 7.4) to prepare the nanosuspension containing GAEE equivalent of 50 mg/ml [20].

Characterization of nanosuspension

Particle size and zeta potential measurement

The mean particle size and zeta potential of the GAEE-loaded Eudragit S100 nanosuspension were evaluated using Zetasizer Nano ZS90 (Malvern Instruments, UK). The equilibration time and temperature were kept at 120s and 25°C respectively[21].

Transmission electron microscopy (TEM)

A transmission electron microscope (FEI Tecnai G^2 F20 S-Twin, Bellaterra, Spain) was used to determine the morphology of the prepared nanosuspension at 200 kV[22].

Evaluation of nanosuspension

Drug content

An accurately weighed lyophilized powder equivalent to 50 mg of GAEE was dissolved in methanol with the help of sonication. The contents of quercetin and naringenin were determined by the HPTLC method of analysis.

In vitro release behavior

The *in vitro* drug release of GAEE nanosuspension was studied in USP type-II dissolution rate test apparatus (TDL-08L, Electrolab, Mumbai, India) using the dialysis sac method. The specific amount of nanosuspension (10 ml), placed in a dialysis membrane (cut off: 10,000 Da) was immersed into phosphate buffer solution (500 ml, pH 7.4) at 37°C and stirred at 50 rpm with the help of a sinker. At respective time intervals, aliquots of 0.1 ml sample were removed and replaced with an equal volume of release media[19]. The withdrawn samples were analyzed for the contents of quercetin and naringenin by HPTLC.

Evaluation of GAEE formulations for *in vitro* anthelmintic activity

The conventional suspension, GAEE-loaded Eudragit S100 microparticles, and GAEE-loaded Eudragit S100 nanosuspension, all equivalent to GAEE 50 mg/ml each, were screened for *in vitro* anthelmintic activity using adult Indian earthworm (*Pheretima posthuma*) as mentioned earlier [23]. To rule out the anthelmintic action due to the presence of excipients, the anthelmintic action of excipients was also carried out *i.e.* vehicle control (for suspension), Eudragit S100 (for microparticles and nanosuspension), and Poloxamer-188 (for nanosuspension). In addition, the effect of quercetin, naringenin, and their combination was also comparatively evaluated.

Statistical analysis

All the results are expressed as mean ± standard error of the mean. Analysis of the statistics was determined using one-way ANOVA and Dunnett's *t*-test. A value of $P>0.05$ was considered significant at $P>0.05$.

RESULTS and DISCUSSION

Synthetic anthelmintic drugs have been the mainstay of treatment for the eradication of helminths even though the resistance to them is developing. Their toxicity, increased cost, and inaccessibility in remote areas have added barriers to achieving the goal of deworming. A large proportion of populations in the developing world rely on the traditional system of medicine which use herbs and herbal derivatives to treat several diseases. Several plants have been documented to possess anthelmintic activity in ancient literature and some of them have been tested but still, a large number of plants have not been used clinically[24,25]. Among these plants, *Grewia asiatica* Linn. is one of the active plants which is used traditionally for the treatment of helminthiasis but there is no scientific report to validate their use as anthelmintic. Thus, the roots of *Grewia asiatica* Linn. were used to prepare the aqueous and alcoholic extract.

From both prepared extracts, the alcoholic extract was chosen based on its extractive value (38.6±0.54%,w/w)[8]. Further, the extract was formulated into different pharmaceutical dosage forms and comparatively evaluated for *in vitro* anthelmintic activity.

Selection of extract and its anthelmintic activity

The effect of different concentrations of GAEE and GAAE roots with albendazole on the paralysis and death time of worms were compared in figure 1. It can be observed that the worms treated with vehicle control (saline phosphate buffer) did not show any paralysis or death during the 2 h period of observation. The worms treated with albendazole suspension (20 mg/ml) get paralyzed and died in 29 min and 58 min respectively. It was estimated that GAEE was more efficacious than aqueous extracts. The result of the ANOVA analysis revealed that there was no significant difference in the paralysis and death time of worms treated with albendazole (20 mg/ml) and ethanolic extract (GAEE, 50 mg/ml). However, ethanolic extracts at a concentration of 75 and 100 mg/ml showed better efficacy than albendazole for paralyzing and killing the earthworm. In the previous literature, the anthelmintic activity of methanolic extract of *Grewia asiatica* leaves is already reported with *Ferula assafoetida* Linn. resin, *Ipomoea hederacea* Jacq. seeds, *Lepidium sativum* Linn. seeds, and *Terminalia chebula* Retz. fruits[26].

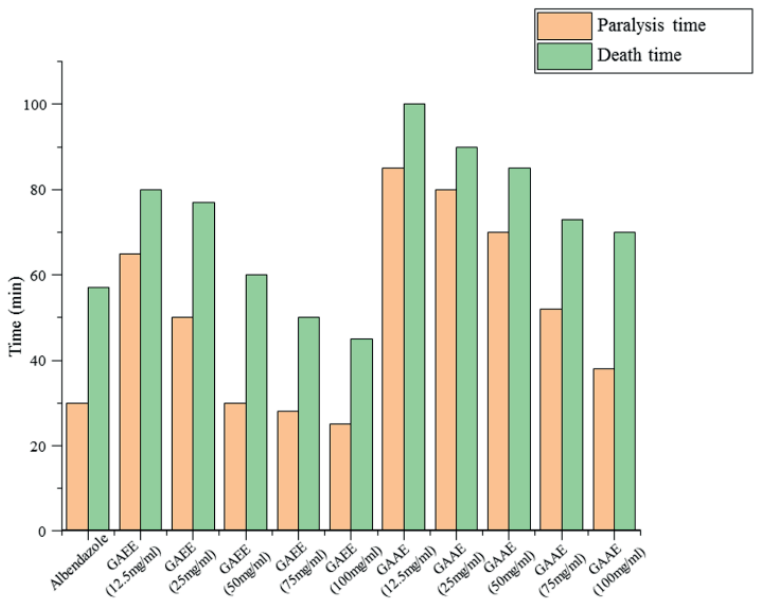


Figure 1. Anthelmintic activity of GAEE and GAAE

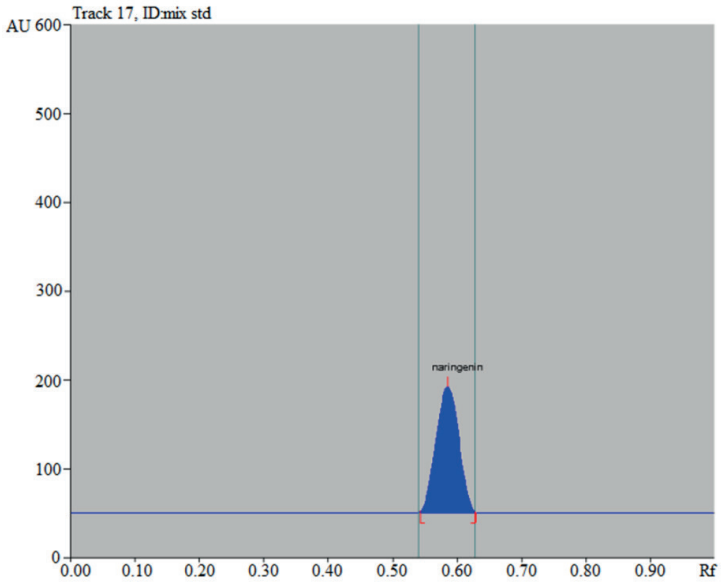
The results of anthelmintic screening of selected plant extracts reveal that the anthelmintic action of extracts followed the order GAEE > GAAE. Thus, GAEE which showed more anthelmintic activity (at a concentration of 50 mg/ml) comparable to the albendazole (at a concentration of 20 mg/ml) was selected for further development of the herbal formulation.

HPTLC analysis

The selection of the mobile phase for running the HPTLC chromatogram was done by a preliminary TLC study. Various composition of mobile phase such as ethyl acetate: methanol: water (60:30:10, 90:20:10, 80:15:05, v/v/v), toluene: ethyl acetate: formic acid (60:40:02, 60:40:04, 60:40:06, 60:40:08, v/v/v) were tried to optimize the resolution of bands. Among these, toluene: ethyl acetate: formic acid (60:40:08, v/v/v) was observed to give the best resolution of quercetin (R_f = 0.44) and naringenin (R_f = 0.62), which act as marker components [27]. Thus, this mobile phase composition was employed further to develop a suitable HPTLC densitometric method of analysis in the GAEE. The identity of bands of quercetin and naringenin in the plant matrix was confirmed by comparing their spectra with the spectra of respective standards.

The HPTLC chromatogram of quercetin, naringenin, a combination of quercetin and naringenin dosage form, and GAEE dosage form is shown in figure 2. The plot between the concentration of naringenin (200-3000 ng/band) and the peak area of naringenin was found to be linear with the equation of line being Y = -159.295 + 5.188X and correlation coefficient (R^2) of 0.99403 [28]. However, the plot between the concentration of quercetin (100-3000 ng/band) and the peak area of quercetin was found to be linear with the equation of line being Y = 99.297 + 1.417X and correlation coefficient (R^2) of 0.99787. The content of quercetin and naringenin in the GAEE were calculated and found to be 2.24 mg and 21.117 mg/gram of the extract respectively. The limit of detection (LOD) & limit of quality (LOQ) was found to be 50 ng & 100 ng respectively for naringenin and 100 ng & 200 ng for quercetin. Also, the 3D densitometric TLC profile of the GAEE suspension dosage form is shown in figure 2[29].

Thus, the developed HPTLC method is suitable for quantification of quercetin and naringenin in *Grewia asiatica* extract and would be useful for standardization of *Grewia asiatica* extract and its formulation.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naringenin
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0.40
0.50
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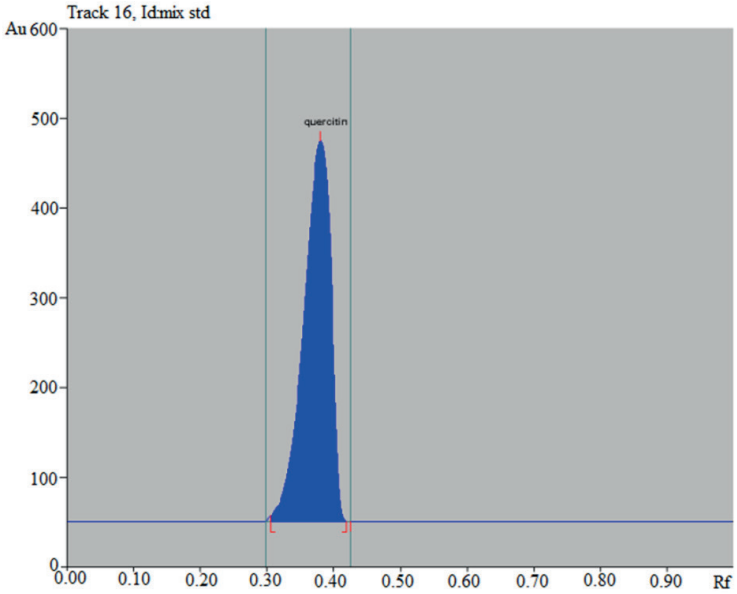
0.80
0.90
Rf

(a)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Au 600
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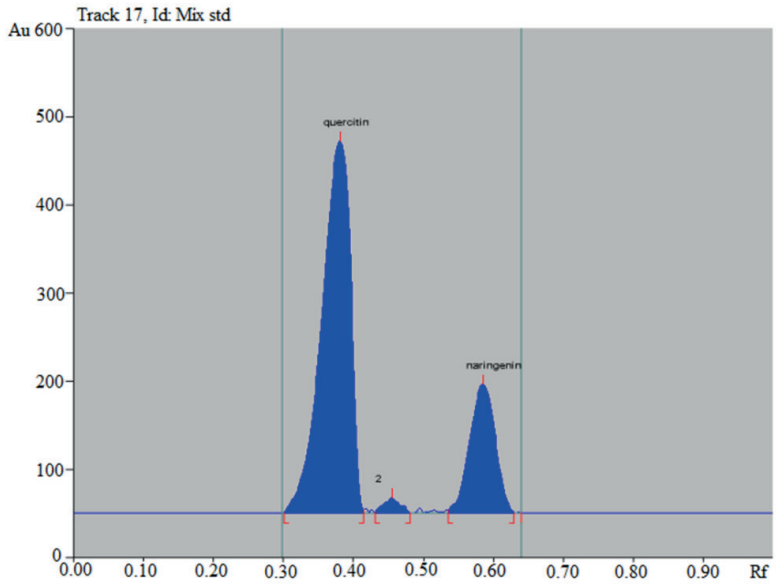
(b)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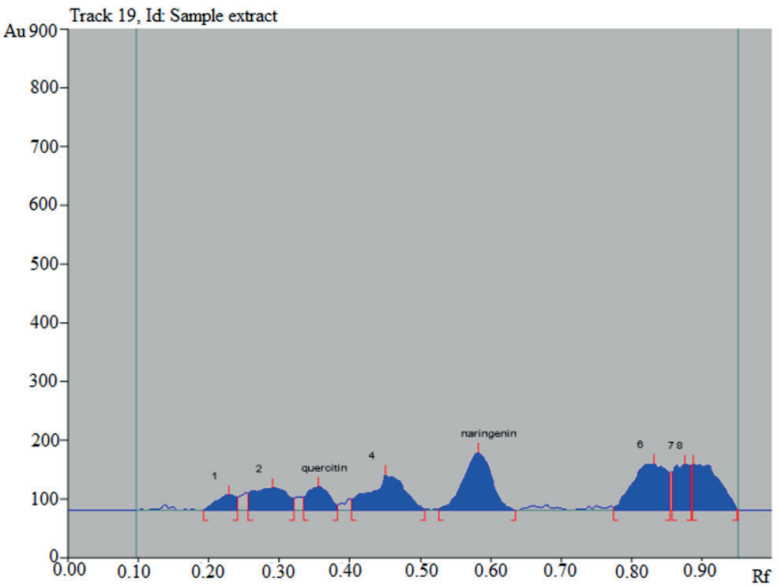
(c)


Track 19, Id: Sample extract
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1
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naringenin
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0.20
0.30
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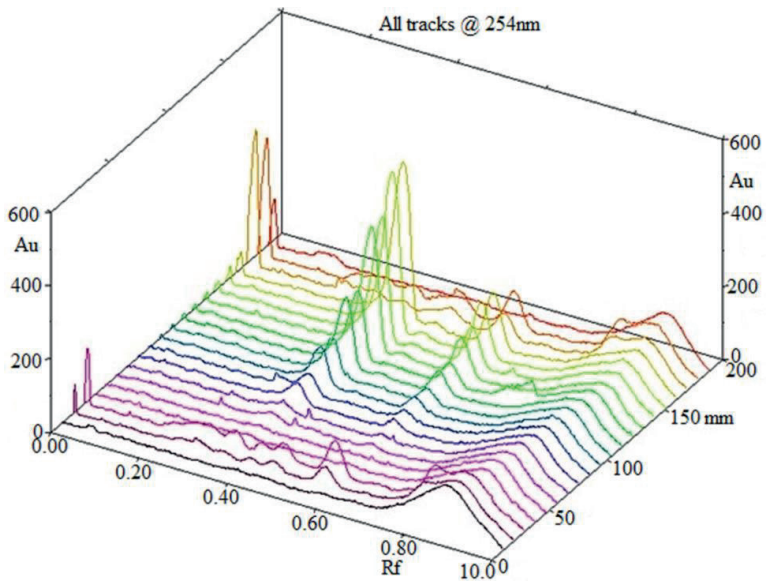
Rf

(d)



(e)

Figure 2. HPTLC chromatogram of standards naringenin (a), quercetin (b), a combination of quercetin and naringenin (c), and GAEE (d), and densitometric TLC profile showing GAEE (e)

Development of herbal formulation

After confirmation of anthelmintic activity present in standardized *Grewia asiatica* extract, different herbal formulations were developed by using the ethanolic extract for further pharmaceutical applications.

GAEE-loaded Eudragit S100 suspension dosage form

Solution dosage forms, if palatable, are usually chosen, as, these dosage forms are absorbed rapidly with better bioavailability in the human body. Since GAEE was partially soluble in water; so its suspension was selected for developing the dosage form. Sodium carboxymethyl cellulose in concentration of 0.5% (GES_1 and GES_2), 1% (GES_3), 1.5% (GES_4) and 2% (GES_5) was used as suspending agent. As, the suspension without a wetting agent (GES_1) was not dispersible, so Tween-80 was added as a wetting agent at a concentration of 1% (v/v) in formulation GES_2 which was found to adequately disperse the extract. A combination of methyl and propyl paraben was employed as a preservative. To impart a pleasant flavor, lemon oil was incorporated.

Table 1. Physicochemical parameters of GAEE suspension formulations

Physicochemical Parameters	Formulations				
	GES_1	GES_2	GES_3	GES_4	GES_5
Color	Brownish Yellow	Brownish Yellow	Brownish yellow	Brownish yellow	Brownish yellow
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.51	6.54	6.91	7.12	7.18
Nature	Liquid	Liquid	Liquid	Liquid	Liquid
Texture	Pourable	Pourable	Pourable	Poorly pourable	Poorly pourable
Sedimentation Volume	16.76	16.66	7.69	5.55	5.26
Redispersibility	—	100%	95%	70%	50%
Viscosity (cps)*	300	310	330	370	430

** Viscosity at 100 rpm determined using spindle 3*

Table 1 shows the results of the characterization of various batches of suspension. The pH of the suspension was found to vary from 6.51-7.18, with an increase in the pH of suspension with an increase in the content of sodium CMC. The batch of suspension without Tween-80 was not dispersible while batches prepared with Tween-80 were homogeneously dispersed. Further, it can be observed that the rate of sedimentation decreased with an increase in the concentration of sodium CMC. However, the increase in sodium CMC concentration also affects their redispersibility, which can be attributed to the increase in viscosity. Moreover, suspension prepared using sodium CMC (1.5 %, w/v and 2.0 %, w/v) were poorly pourable. Similar results have been reported in the study evaluating the *Grewia ferruginea* mucilage as a suspending agent in metronidazole suspension[30]. Considering the settling rate, ease of redispersion, and pourability, suspension of batch GES_3 was selected for conducting further studies.

The results (Table 2) of accelerated stability studies done on batch GES_3 of suspension revealed that there was no significant difference in the physicochemical properties of the suspension over the study period. Thus, this batch of *Grewia asiatica* extract suspension (GES_3) appears as the optimal formulation, for further *in-vitro* anthelmintic evaluation.

Table 2. Accelerated stability studies of GES_3 suspension

Physicochemical Parameters	Time Period									
	Initial		15 Days		30 Days		45 Days		90 Days	
	25°C	40°C	25°C	40°C	25°C	40°C	25°C	40°C	25°C	40°C
Color	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow
Odor	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.
pH	6.91	6.91	6.85	6.95	6.80	6.85	6.78	6.89	6.80	6.85
Nature	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Texture	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable
Sedimentation Volume	7.69	7.69	7.60	7.72	7.62	7.65	7.55	7.60	7.52	7.55
Redispersibility (%)	95	95	95	95	95	90	95	90	95	95
Viscosity (cps)*	330	330	330	330	330	330	330	320	330	320

** Viscosity at 100 rpm determined using spindle 3, Char. = Characteristics*

GAEE-loaded Eudragit S100 microparticles

Three batches of microparticles containing GAEE [10% (GEM1), 20% (GEM2), and 50% (GEM3), w/w of the Eudragit S100] were prepared as shown in Table 3. The average yield of microparticles for the three batches varied from 56% to 67%. The dry product was found to be brownish-yellow in color. The average length–number diameter (*dln*) of three batches was found to be 9.89, 12.31, and 15.99 **µ**m for GEM1, GEM2, and GEM3 respectively, while the respective average volume-surface diameter (*dvs*) was determined to be 15.44, 19.43 and 21.32 **µ**m respectively. Thus, increasing the quantity of *Grewia asiatica* extract from 10%-50% (w/w of Eudragit S100), lead to an increase in the size of microparticles.

Table 3. Characteristics of *GAEE*-loaded Eudragit S100 microparticles

Sr. No.	Batch	Eudragit S100 (mg)	GAEE (%, w/w)	Yield (%, w/w)	Color	dln (µm)	dvs (µm)	Assay
1	GEM1	500	10	56	Creamish-white	9.89	15.44	98%
2	GEM2	500	20	61	Yellowish-white	12.31	19.43	97%
3	GEM3	500	50	67	Brownish-yellow	15.91	21.32	101%

**GAEE = Grewia asiatica ethanolic extract; dln = Average length-number diameter; dvs = Average volume-surface diameter*

Figure 3 (a), (c), and (e) display the particle size distribution curves of three batches of microparticles. The microparticles of GEM1 show a normal distribution curve with a narrow size distribution, while the GEM2 batch shows a bimodal distribution with a broad particle size distribution with two peaks. Microparticles of batch GEM3 also show a very broad distribution of microparticles. The photomicrographs of GAEE-loaded Eudragit S100 spray-dried microparticles of three different batches were observed using an optical microscope. The microparticles appear disc shape to ovoid shape in an optical microscope. However, the exact morphological feature and surface topography can be seen in scanning electron micrographs [figure 3 (b), (d), and (f)]. It can be seen in the micrographs that Eudragit S100 microparticles are disc-shaped with grooved surfaces. It is not unusual to obtain microparticles of irregular shapes, in some earlier studies on microparticles[30,31], microparticles of different shapes such as ribbon, biocone, elongated hexagonal disks, porous, wrinkled, bullets, barrels, pills, and biconvex lens-shaped, etc. were obtained by different methods such as self-assembly, microfluidics, photolithography, and spray drying. The morphology of spray-dried microparticles depends upon a no of factors such as the type of polymer, polymer concentration, solvent composition, feed pump rate, the temperature of drying air, rate of air aspiration, etc. Esposito, et al. 2000 observed that during the spray drying of Eudragit RS 100 microparticles from ethanol/water mixtures, the crust formed during initial drying is impermeable to the solvent which leads to fracture of the crust. The microparticles formed under such conditions are shriveled. Further, it was reported that the collapsed, irregular-shaped microparticles were obtained by spray drying polymer solutions of Eudragit E, Eudragit R, Eudragit S, and Eudragit RL[32]. The results obtained in our study are consistent with the results obtained earlier. In this study, it can be observed that increasing the amount of extract in the microparticles increases the particle size as well as the width of particle size distribution.

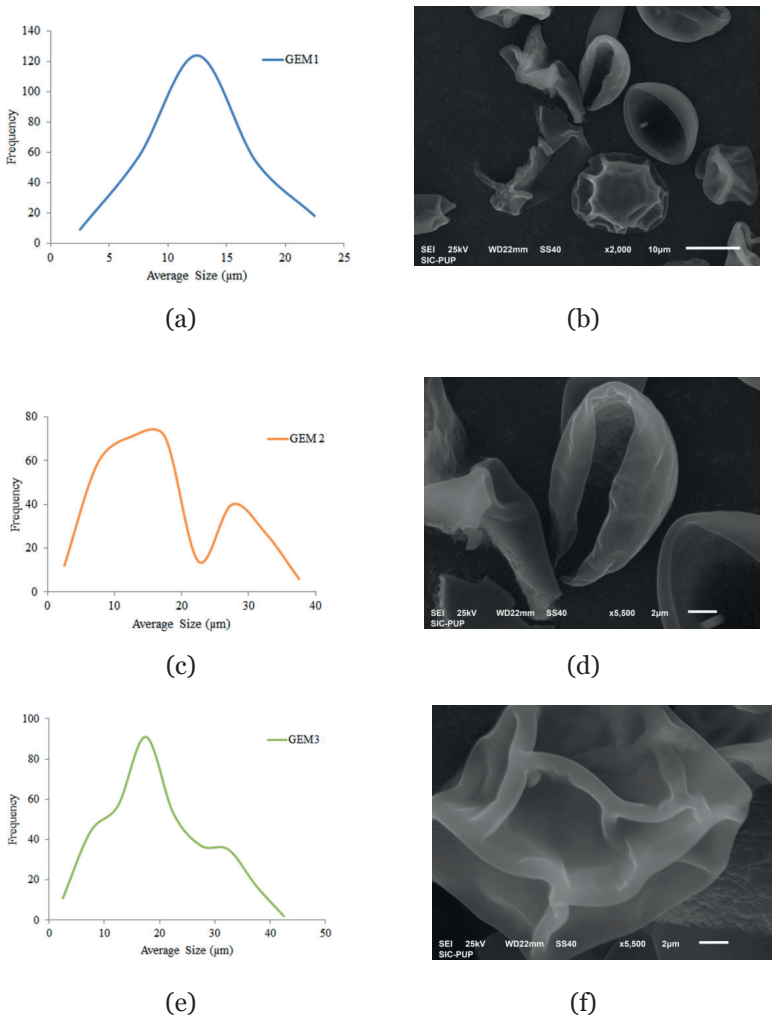
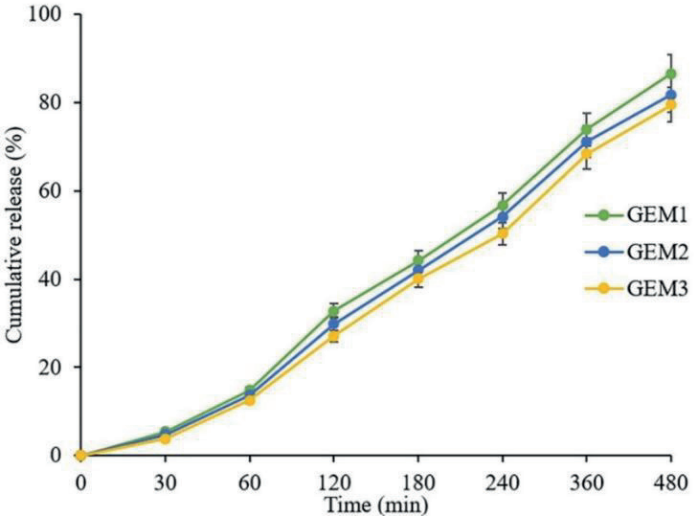
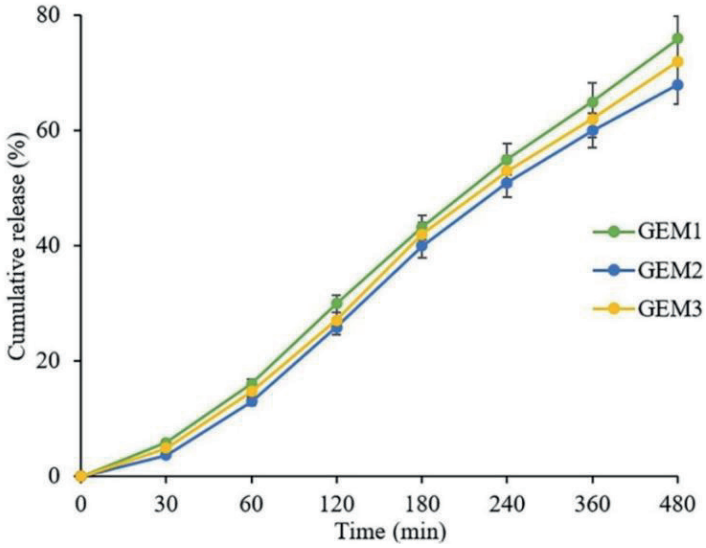


Figure 3. Size distribution frequency curve (a, c, e) and scanning electron micrograph (b, d, f) of GAEE-loaded microparticles of batch GEM1, GEM2, and GEM3

Figure 4 shows the *in vitro* release behavior of three batches of microparticles. The microparticles were observed to release the drug over 8 h with 73%, 68%, and 70% of quercetin release from GEM1, GEM2, and GEM3 respectively. In terms of naringenin, the percentage release was found to be 85%, 82%, and 79% from GEM1, GEM2, and GEM3 respectively. Since there is no significant difference in the release rate of microparticles, the microparticles of batch GEM3 owing to their higher drug loading were selected for further evaluation of *in vitro* anthelmintic activity.



(a)



(b)

Figure 4. *In-vitro* release profile of quercetin (a) and naringenin (b) from microparticles batches of GEM1, GEM2 and GEM3

GAEE-loaded Eudragit S100 nanosuspension

GAEE-loaded Eudragit S100 nanosuspension (GEN) dried using a spray dryer was found to lose redispersibility after drying while the nanosuspension dried using a lyophilizer was found to be redispersible. Thus, the spray drying method was rejected and lyophilization was adopted. The lyophilized powder of GAEE-loaded Eudragit S100 nanosuspension after redispersion was analyzed for particle size and zeta potential. The Z-average particle size of 224.3 (dnm) and polydispersity index (PDI) of 0.153 indicate that the nanoprecipitation method provides suspension of nanometric size particles while the zeta potential was found to be -13.7 which represents the stability of nanosuspension. Further, the lower PDI values indicate monodisperse nanosuspension.

Figure 5 displays the transmission electron micrographs of the GAEE-loaded Eudragit S100 nanosuspension. The nanoparticles are spherical in shape having size consistent with the results of particle size analysis.

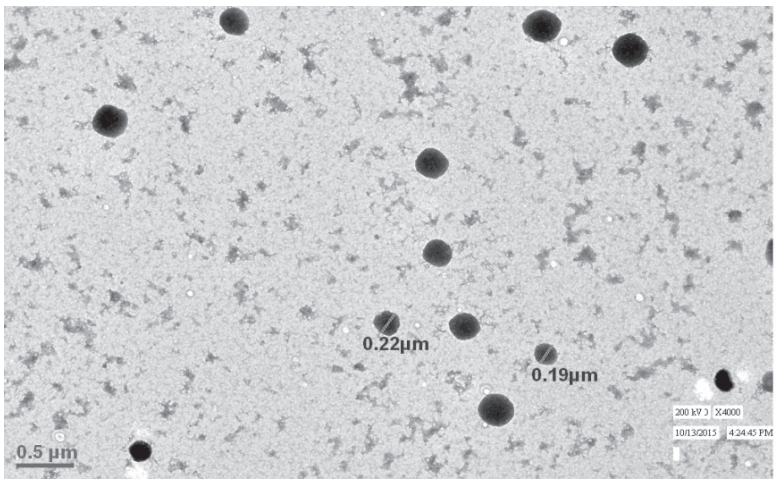
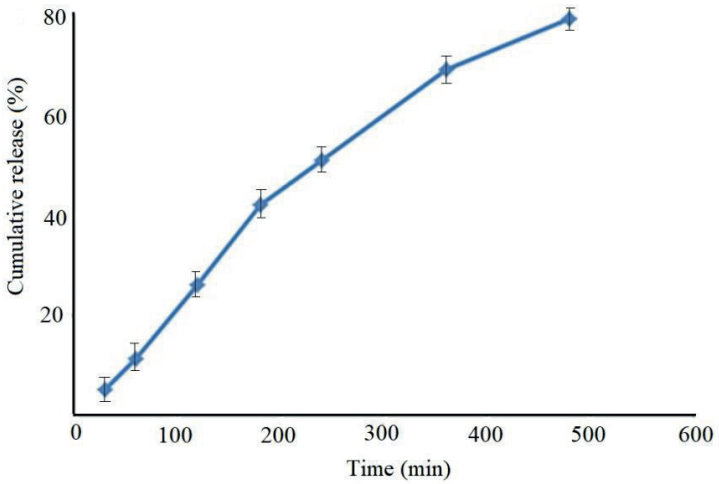


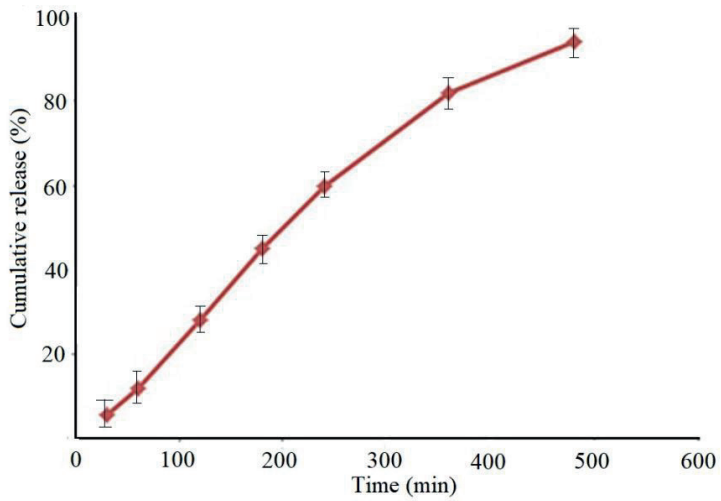
Figure 5. Transmission electron micrographs of GAEE nanosuspension dosage form

Figure 6 shows the *in-vitro* release profile of *Grewia asiatica* extract nanoparticles in terms of quercetin (a) and naringenin (b) respectively. It can be observed that nanosuspension provided 76% release of quercetin and 92% release of naringenin over 8 h period. Quercetin and naringenin have poor oral bioavailability which is attributed to their poor aqueous solubility[33,34]. Nanosuspensions of hydrophobic bioactive have been prepared to improve their solubility and bioavailability. During earlier studies, the nanosuspensions of quercitin[33] and naringenin[34] were observed to show a sustained release profile.

In the present study, microparticulate and nanosuspension dosage forms of GAEE have been prepared, it can be observed that the release of quercetin and naringenin (biomarker components) is relatively faster from the nanosuspension dosage forms as compared to the microparticulate dosage form.



(a)



(b)

Figure 6. In vitro release profile of quercetin (a) and naringenin (b) from the nanosuspension dosage form

Comparative anthelmintic evaluation of suspension, microparticles, and nanosuspension

The results of *in vitro* anthelmintic screening of conventional suspension of GAEE (GES_3), GAEE-loaded Eudragit S100 microparticles (GEM), and GAEE-loaded Eudragit S100 nanosuspension (GEN) with albendazole drug as a standard is shown in figure 7. The formulations of GAEE were tested at a concentration equivalent to 50 mg/ml of GAEE as a drug while the preliminary screening of GAEE at 50 mg/ml was found to be comparable with albendazole (20 mg/ml). It can be observed that the conventional suspension paralyzed and killed the earthworms at a comparable time to that of GAEE. Further, there was no effect of vehicle control (GES) on earthworms. The results of HPTLC studies showed the presence of eight phytoconstituents in GAEE. Among these, quercetin and naringenin were identified to contain 2.24 mg and 21.117 mg/gm of the extract respectively. So, quercetin and naringenin equivalent to that present in GAEE at 50 mg/ml were also tested for *in vitro* anthelmintic activity. The results show that the effect of quercetin is more than naringenin while the combined effect of quercetin and naringenin indicates synergistic action. However, the combined effect of quercetin and naringenin is still less than the effect of GAEE, indicating that other ingredients present in the GAEE are also responsible for its anthelmintic activity.

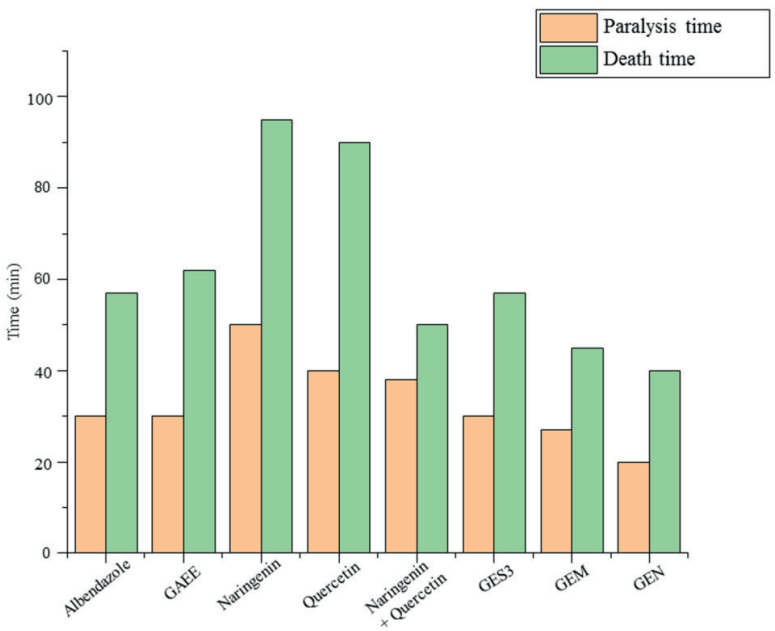


Figure 7. Anthelmintic activity of suspension, microparticulate, and nanosuspension

On comparing the effect of microparticles and nanosuspension dosage forms, it can be observed that both microparticulate and nanoparticulate dosage forms of GAEE showed significantly higher anthelmintic activity than the standard albendazole. Further, the effect of nanosuspension is more pronounced than the microparticulate dosage form. Also, there was no effect of the blank microparticles and nanosuspension which confirms that the effect is due to GAEE.

Herbal drugs i.e. *Grewia asiatica* Linn roots have numerous therapeutic and curative properties. So, the extract of the plant was employed to develop a new and low-cost herbal formulation with better activity and efficacy. For qualitative fingerprinting, the HPTLC study was carried out by using quercetin and naringenin as marker compounds. Then the conventional suspension dosage form of ethanolic extract of *Grewia asiatica* was prepared and evaluated. Microparticles and nanosuspension dosage forms were also prepared simultaneously with the help of spray drying and freeze-drying process respectively. Further, the comparative anthelmintic evaluation of all the selected dosage forms was done against the Indian earthworm *Pheretima posthuma*. The results of the study revealed that in nanosuspension dosage form, significant anthelmintic activity is present as compared to conventional suspension and microparticulate dosage form. However, the excipients exert no anthelmintic action. So, it can be concluded that a stable and non-toxic herbal anthelmintic formulation can be prepared with improved stability, convenience, and compliance.

STATEMENT OF ETHICS

All the necessary ethical rules were followed while performing research.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Conceptualization	:	Tarun Kumar
Methodology	:	Tarun Kumar
Validation	:	Tarun Kumar, Rimpy
Formal Analysis	:	Rimpy, Munish Ahuja
Investigation	:	Tarun Kumar
Resources	:	Tarun Kumar, Munish Ahuja
Writing - Original draft	:	Tarun Kumar
Writing - Review and editing	:	Rimpy, Munish Ahuja
Supervision and Project administration	:	Munish Ahuja
Funding acquisition	:	Munish Ahuja

FUNDING

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Formulation and evaluation of transdermal ultradeformable vesicles of aspirin

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ABSTRACT

Transfersomes are incredibly elastic and deformable vesicles composed of phospholipids and edge activators. This study proposed aspirin-loaded transfersomes for transdermal administration to prevent gastrointestinal side effects, boost drug permeation rate, and extend drug action. The formulations were prepared via the thin-film hydration method using soy lecithin as a vesicle forming agent and tween 80 as an edge activator. The formulation trials were optimized by 'Custom design' JMP SW13. The optimum formulation yielded a vesicle size of 74.4 nm, a zeta potential of -27.4 mV, and a % entrapment efficiency of 90.5%±0.25 with a drug release of 88.65 %±0.34. A 1% carbopol gel incorporated the optimum formula. The homogeneous gel had a drug content of 95.8±1.5 %, a viscosity of 1762cP, a pH of 5.74±0.78, and % a drug release of 85.5%±0.85. The study concluded that transdermal transfersomes would be a promising approach to treating angina.

Keywords: Transfersomes, Ultra-deformable, Vesicles, Phospholipids, Optimization

INTRODUCTION

Classically, angina (or angina pectoris) refers to a pressure-like substernal chest discomfort brought on by physical or emotional stress. It is the most acute symptom of ischemic heart disease. Angina can be acute or chronic. A restriction in coronary blood flow due to coronary artery spasm, obstructive

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atherosclerotic plaque, a non-coronary problem such as acute anemia, coronary microvascular dysfunction, or hypotension might limit myocardial oxygen supply. The cause may be a significant rise in myocardial oxygen demand. A rise in heart rate (HR), supraventricular tachycardia (atrial fibrillation or flutter), hyperthyroidism, or other factors are some of the incidences that lead to oxygen demand[1].

Medications used to treat angina are calcium channel blockers, nitrates, and beta-blockers. These agents decrease myocardial ischemia through heart rate regulation and vasodilatory processes[2]. Ranolazine and ivabradine are unconventional antianginal drugs used to treat symptomatic angina in chronic, stable ischemic heart disease patients[3]. Most -adrenoceptor antagonists are effectively absorbed after oral treatment. However, many are subjected to first-pass hepatic degradation, limiting their oral bioavailability to different degrees[4].

Aspirin's mechanism of action involves irreversible inhibition of platelet-dependent enzyme cyclooxygenase, [COX], identified as isoenzymes as COX -1 and Cox-2. Platelet aggregation happens from the production of thromboxane A2, a powerful promotor produced by COX -1, thus preventing prostaglandin synthesis. This irreversible inactivation of COX -1blocks thromboxane A2 and produces the antiplatelet effect. Platelet activation and aggregation with subsequent activation of the clotting cascade play critical roles in the onset of acute occlusive vascular events, such as MI and occlusive cerebrovascular accident (CVA). Because platelets do not have a nucleus and thus cannot regenerate COX, they become an excellent target for antithrombotic therapy, while aspirin shows both immediate and long-term effects on platelets. Aspirin belongs to the biopharmaceutics classification system (BCS) II drug with high solubility and permeability profile[5]. The reported water solubility of aspirin is 2-4 mg/ml.[5]

The transdermal route has several advantages over other traditional drug delivery routes. The benefits include reducing unfavorable side effects, preventing hepatocytes' metabolism, and extending predictable drug action. Also, it avoids fluctuation in drug blood levels, improves the physiological and pharmacological response, and, last but not least, the ability to deliver drugs with a short half-life. Specific features such as higher encapsulation capacity for hydrophobic and hydrophilic drugs, biodegradability, non-toxicity, and drug encapsulation in the vesicular structure make them preferential to other vesicular carriers. The prolonged drug presence in the circulation, the potential to target different organs and tissues, and enhanced bioavailability are also additional advantages[6]. Transferosomes are lipid-based-vesicular carriers comprised of four elements: phosphatidylcholine., dipalmitoyl phosphatidyl-

choline, edge activator such as a surfactant or a bile salt, low concentration of alcohol, and water. Transferosomes mimic the behaviour of a cell engaged in exocytosis, making them ideal for regulated and targeted drug administration. When added to aqueous systems, the carrier aggregate comprises at least one amphipathic molecule (such as phospholipids), which self-assembles into a bilayer of lipid that finally shuts into a lipid vesicle in the presence of a bilayer softening agent (a biocompatible surfactant).[7,8]

Transdermal anti-anginal drugs are helpful for the treatment of moderate to severe chronic angina. The advantages of transdermal transferosomes of the anti-anginal drug include ease of administration and therapeutic efficacy. These factors, coupled with preventing the drugs from hepatic metabolism, and lowering the untoward side effects, are likely to contribute to targeted delivery and better therapeutic efficacy. Therefore, the current work concentrated on developing transferosomes via a thin-film hydration technique to optimize the formulation via a custom approach and evaluate the formulation for its percentage of drug loading, vesicle size, stability, and *in vitro* drug release profile to substantiate its ability to pass via skin.

METHODOLOGY

Materials

Aspirin from Central Drug House (P) Ltd, soya lecithin, and tween 80 from Sigma Aldrich Ltd. All of the other chemicals and reagents utilized in this experiment were of an analytical grade.

Methods

Preparation of Transfersomes

Screening of surfactants and preparation of blank formulation

Two surfactants were screened (tween 80 and span 80) with different concentrations (10-25%). The blank formulation was dissolving the lipid (Soya lecithin) in a volatile organic solvent (chloroform: methanol) (1:1) mixture, and the organic solvent was allowed to evaporate in a rota evaporator; once the organic solvent evaporates, a thin film formed. (Phase transition temperature of soy lecithin is 570C, and cholesterol is 370C) This thin film was kept for 12-24 hr, ensuring the complete evaporation of the organic solvent, thin-film hydrated with the phosphate buffer solution 7.4 pH with gentle shaking above its phase transition temperature[9].

Preparation of drug loaded transfersomes

Based on the blank formulations, tween 80 as the surfactant (10-25% w/w of phospholipids) and soya lecithin as the phospholipid/vesicle forming agent (300-600mg) was selected (Figure1.)

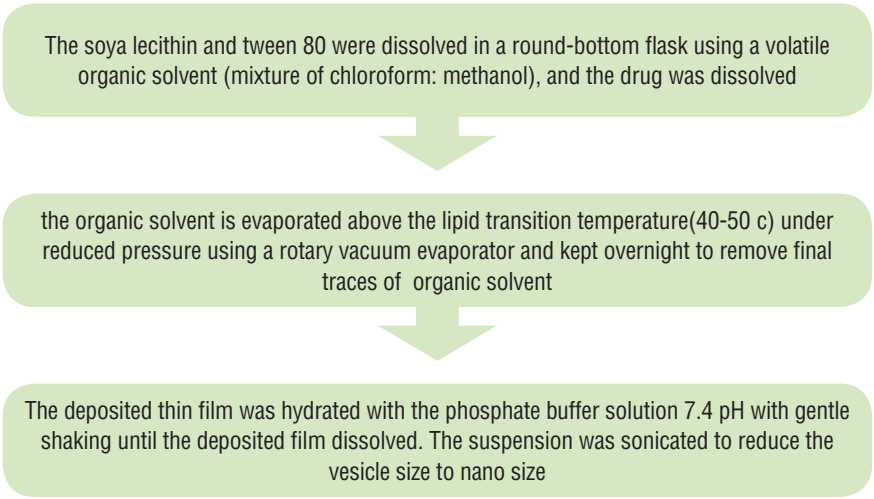

Figure 1. Schematic representation of preparation of transfersomes

Design of experiment

Using custom design via JMP version 13, the effect of formulation variables on quality attributes was studied. The formulation variables such as the number of phospholipids (300-600 mg), the concentration of surfactant (10-25%), and hydration volume (10-15ml) as factors on responses entrapment efficiency (%), particle size (nm), zeta potential (mV) were studied is given in Table 1. The design generated 12 runs given in Table 2[10]

Table 1. Experimental design

Factors	Low		High
X1= Weight of phospholipids	300mg		600mg
X2= Concentration of surfactants	10% w/w of phospholipid		25% w/w of phospholipid
X3= Hydration volume	10ml		40ml
Responses	Goal	Low limit	High limit
Y1=%Entrapment efficiency	Maximise	80%	95%
Y2= Vesicle size	Minimise	50nm	200nm
Y3= Zeta potential	Minimise	-20mV	-35mV

Table 2. Formulation table

Formulation code	Drug (mg)	Weight of phospholipids (mg)	Concentration of surfactant (%)	Hydration volume (ml)
F1.	100	600	25	10
F2.	100	600	15	40
F3.	100	300	15	40
F4.	100	300	25	40
F5.	100	600	15	40
F6.	100	300	15	10
F7.	100	300	25	10
F8.	100	600	25	10
F9.	100	300	25	40
F10.	100	300	15	10
F11.	100	450	20	25
F12.	100	450	20	25

Characterization of transfersomal suspension

Entrapment efficiency (%EE)

To separate the entrapped drug from the unentrapped drug, 2ml of transfersomal suspension was centrifuged at 11000 rpm for one h. The supernatant diluted in methanol and drug content was quantified spectrophotometrically as the free drug. The percentage of drug entrapment is calculated by the below-mentioned formula[10].

$$\%\ Entrapment\ efficiency = \frac{Total\ drug - free\ drug}{Total\ drug} * 100$$

Vesicle size analysis (Horiba SZ-100 particle size analyser)

The dynamic light scattering (DLS) technique measured the vesicle size of all drug-loaded transfersomes. Transfersomal suspension was diluted with double distilled water before subjecting for measurements, and each sample was measured in triplicate at a scattering angle of 90° at 25.2°C[11].

Zeta potential

The zeta potential measurement was carried out by SZ-100 HORIBA scientific using the principle of electrostatic light scattering. Zeta potential was determined after suitable dilution of the trasfersomes samples with distilled water. The diluted samples were placed into were placed in the zeta measurement cell. The electrophoretic mobility of the nanodispersion was measured by tracking the movement of trasfersomes in an electrical field, and the electrical charges were determined.7 Electrophoretic mobility (μ) measurements were used for determining Z. The Smoluchowski equation converted the mobility to ZZ = μη/ε Where η is the viscosity and ε is the permittivity of the solution[11].

***In vitro* drug release of formulations**

Using Franz diffusion cell assembly, in vitro drug release studies were performed on trasfersomes suspension. The study used a dialysis membrane (70; Hi media) with a diffusional area of 70 cm2. The donor compartment contained (50 mg equivalent) formulation, and the receiver compartment had a volume of 50 ml of phosphate buffer solution (PBS) pH 7.4. The dissolution medium in the receptor compartment was magnetically stirred (at 100 rpm) and kept at 37 ± 0.5 ⁰C for 24 h. The sample aliquots (1 ml) collected at predetermined intervals were replaced with the same buffer volume. The aliquots were filtered through a 0.45 mm membrane filter. After appropriate dilution, the samples were analyzed using UV–a visible spectrophotometer at a λmax 266.4nm against PBS as blank. The measurements were done in triplicate[12].

Evaluation of experimental design

The results of the responses from the experimental study were substituted into the experimental design and evaluated for model fit. The model identified the design space and determined the desirability function and surface response curves. The transfersome gel was prepared from the optimized formulation.

Fourier transform infrared spectroscopy (FTIR)

Spectra of optimized formulation were recorded using the Perkin Elmer FTIR spectrophotometer (RXIFT-IR system, USA). Samples were mixed with dry potassium bromide in a 1:1 ratio and then compressed into a transparent disc by applying 10kg/cm2 pressure in a hydraulic press at a scanning range from 4000-400 cm-1. The spectra obtained were compared and interpreted for the functional group peaks[13].

Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Perkin Elmer) was used to measure the thermal nature of drugs and additives. The pure drug and a physical mixture of aspirin, soya lecithin, and tween 80 were used in the study. A sealed aluminum pan was availed to place the sample, which was flushed with nitrogen (50 ml/min). The sample was scanned at a ten °C/min rate from 20 °C to 250 °C. Thermograms were recorded. An empty aluminum pan was used as a reference[13].

Morphology using Transmission electron microscopy (TEM)

The morphology of transferosomes containing the drug was examined by the 200-transmission electron microscope (TEM) (FEI type FP5018/40 Tecnai G2 Spirit Bio TWIN). The transferosomal suspensions containing aspirin in Milli-Q water were dropped on a standard carbon-coated copper grid (mesh) and air-dried for five h, and the surface pictures were observed[13].

Preparation of aspirin loaded transfersomal gel

Preparation of blank gel

The gel was prepared with different concentrations (0.1, 0.5, 0.75, and 1%) of the gelling agent carbopol 934. The accurate weight of carbopol 934 was sprinkled into a beaker containing 100 mL boiling distilled water and soaked overnight. A homogenous gel prepared under magnetic stirring, followed by the neutralizing agent triethanolamine, was added. The gels were examined for consistency, pH, and viscosity[14].

Preparation of drug loaded transfersomal gel

Drug-free gel (1%w/w) was prepared using the procedure mentioned in the blank gel formulations. The optimized aspirin transfersome was introduced into the gel base with continuous stirring on a magnetic stirrer. Preservatives such as methylparaben and propylparaben were added, followed by triethanolamine dropwise, and adjusted the prepared gel's pH to pH 5.5[14].

Preparation of drug solution gel

A gelling agent of 1% carbopol 934 was used to form a drug solution gel. Carbopol 934 was accurately weighed and dusted into a beaker containing 100 mL boiling distilled water, where it soaked overnight. The drug solution was introduced with continuous stirring on a magnetic stirrer to ensure homogeneous dispersion inside the gel base. Preservatives such as methylparaben and propylparaben were used. The solution was neutralized by adding triethanolamine as a neutralizing agent drop by drop, constantly mixing until a homogeneous gel was produced. Then the amount of added neutralizing agent was controlled to adjust the pH of the prepared gel to pH 5.5 using a pH meter[14].

Evaluation of the transfersomal gel

Rheology of the gel

The prepared gels were evaluated for the viscosity using Brook-field Viscometer (Brookfield Engineering Laboratories, Inc. Middleboro, MA, USA) with an S94 spindle; at speeds of 10, 12, 20, 30, 50, 60, and 100 rpm at 37^{0}C. After a predetermined time of 5 minutes, constant viscosity readings were obtained and recorded in centipoises[15].

pH measurement, Homogeneity and spreadability

A digital instrument16 measured the pH of the prepared gel.

A small amount of gel was pressed between the thumb and index finger and checked for the presence of a lumpy feeling[16].

Spreadability was checked by pressing 0.5 g of transfersomal gel between two transparent circular glass slides by measuring the diameter of the produced circle[13].

Drug content

The drug content of the transfersomal gel was determined by diluting 0.5g of gel (10 mg equivalent trasfersomal suspension) with methanol and stirred for 2hr. The solution was filtered and was analyzed spectrophotometrically at 276 nm[15,16].

In vitro release study

Using Franz diffusion cell assembly, in vitro drug release studies were performed on trasfersomes suspension. The study used a dialysis membrane (70; Hi media) with a diffusional area of 70 cm 2. The donor compartment contained (50 mg equivalent) formulation, and the receiver compartment had a volume of 50 ml of phosphate buffer solution (PBS) pH 7.4. The dissolution

medium in the receptor compartment was magnetically stirred (at 100 rpm) and kept at 37 ± 0.5 ºC for 24 h. The sample aliquots (1 ml) collected at pre-determined intervals were replaced with the same buffer volume. The aliquots were filtered through a 0.45 mm membrane filter. After appropriate dilution, the samples were analyzed using UV–a visible spectrophotometer at a λmax 266.4nm against PBS as blank. The measurements were done in triplicate[17-20].

Stability testing

The stability of transfersomes and transfersomal gel was studied at 250±20 C/60 % RH ±5% and 5º±3ºC. In this study, the samples were stored for three months and evaluated for % EE, vesicle size of transfersomes in transfersomal suspension, and drug content in transfersomal gel[18].

RESULTS and DISCUSSION

Preparation of transfersomes

Screening of surfactants and preparation of blank formulation

Surfactants were evaluated for their vesicle size, physical observation, and microscopic structure. The microscopic image showed that the vesicles were distributed widely, and the lamellae were visible, whereas they were not much prominent in the formulation with a span of 80, as shown in Figure 2.

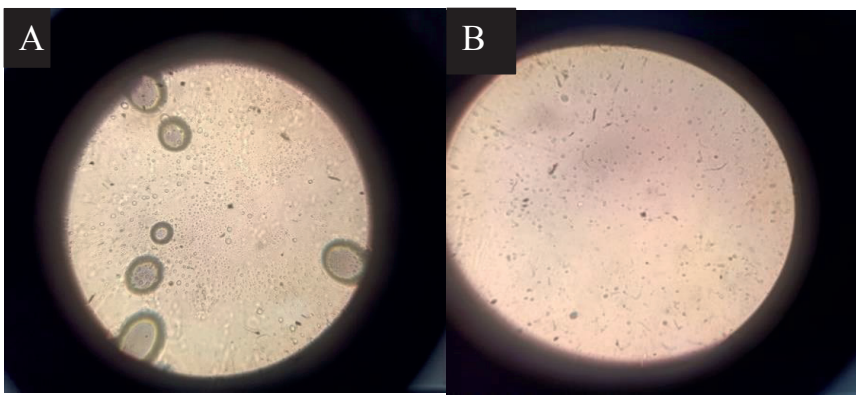

Figure 2. Microscopic view of transfersomes formulated with (A) tween 80(15%) (B) Span 80 (15%)

Formulation with Tween 80 and span 80 resulted in a transparent film, which on subsequent hydration and sonication, resulted in a vesicle size of 1025.5 nm and 1436 nm, respectively, as given in Figure 3. With this evaluation, tween 80 was used further in formulation trials.

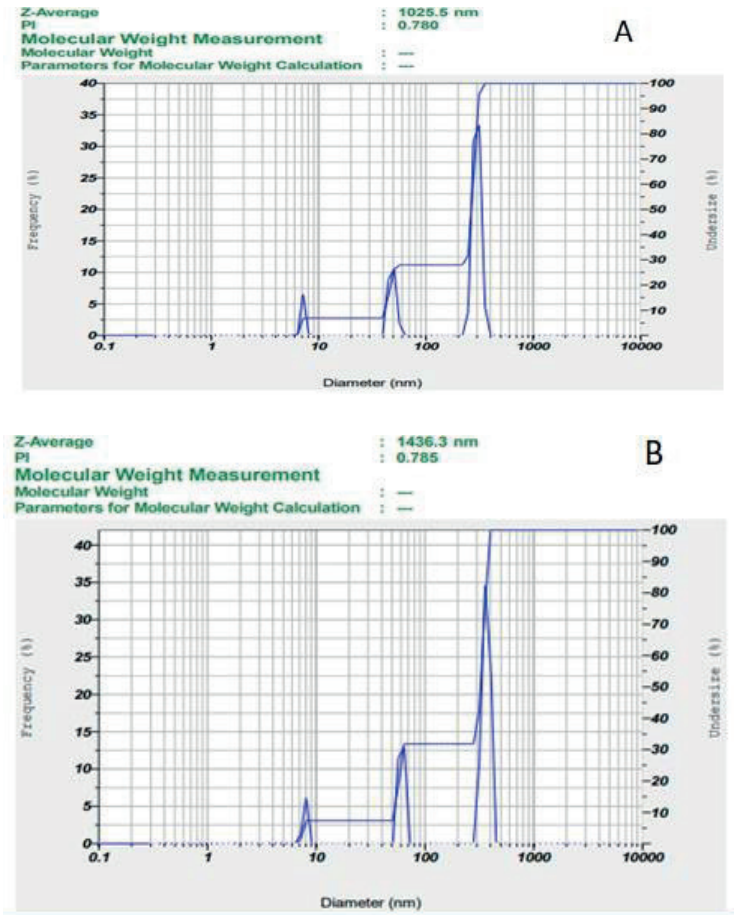

Figure 3. Vesicle size of formulation A) with tween 80 surfactant, B) span 80 surfactant

Vesicle size, zeta potential and entrapment efficiency

Formulations were optimized via a custom design approach using JMP 13 SW. Weight of phospholipid, the concentration of surfactant, and hydration volume was selected as factors for the responses % EE, vesicle size, and zeta potential. The experimental design generated 12 runs with two center points. A vesicle size of 50.4- 100.5 nm, the zeta potential of -32.6 to -21.5mV, and % EE in the 82.3-92.5% range were observed. The results are reported in Table 3.

Table 3. Physical observation of blank formulation

Surfactant	Vesicle size (nm)	% EE	Physical observation
Tween 80	1025.5	89.4%	Clear thin film
Span 80	1436.3	81.5%	Clear Thin film

In vitro drug release of drug loaded transfersomal suspension

In vitro drug release of drug-loaded transfersomal suspension of all formulations was carried out for 24 h and the percentage release was in the range of 49.7-89.4 %, as given in Figure 4. The full release was found in the F6 formulation; this data can support the most miniature particle size and highest entrapment of the drug in the vesicle.

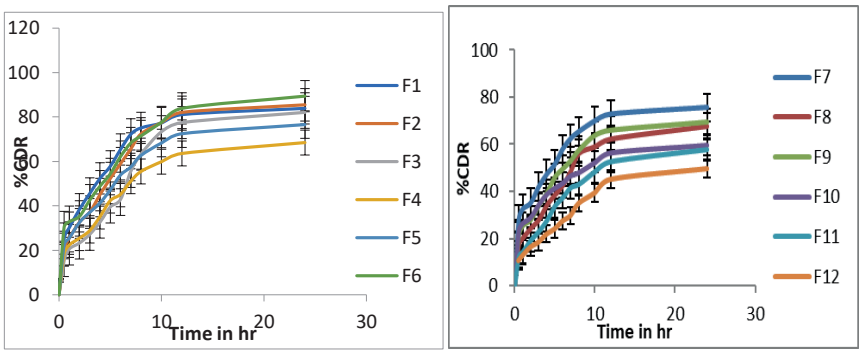

Figure 4. *In vitro* drug release profile of transfersomal suspension (F1-F6) and (F7-F12)

Optimization of experimental design

The actual vs. predicted plot for the responses % EE, vesicle size, and zeta potential showed the R2 value of 0.80, 0.85, 0.86, and the P-value of 0.0039, 0.0012, and 0.001 as given in Figure 5. This data indicated that the factors and selected factors' levels were statistically significant ($p \leq 0.05$) for all the responses. The optimized formula was obtained from the desirability plot with desirability of 0.703 and found maximum desirability of 0.80, as shown in Figure 6. The optimized formulation contains 381mg of a phospholipid, 15% w/w concentration of surfactant, and 10ml hydration volume, all factors in lower levels. The surface profiler indicates that the variables selected had a linear influence on the responses and no curvature effect, as seen in Figure 7. The prediction variance profile demonstrates that about 50 % of the fraction of de-

sign space had a variance less than 0.175, a desirable parameter in optimizing the formulations. Both prediction variance profiler and the fraction of design space are given in Figure 8 and figure 9, respectively.

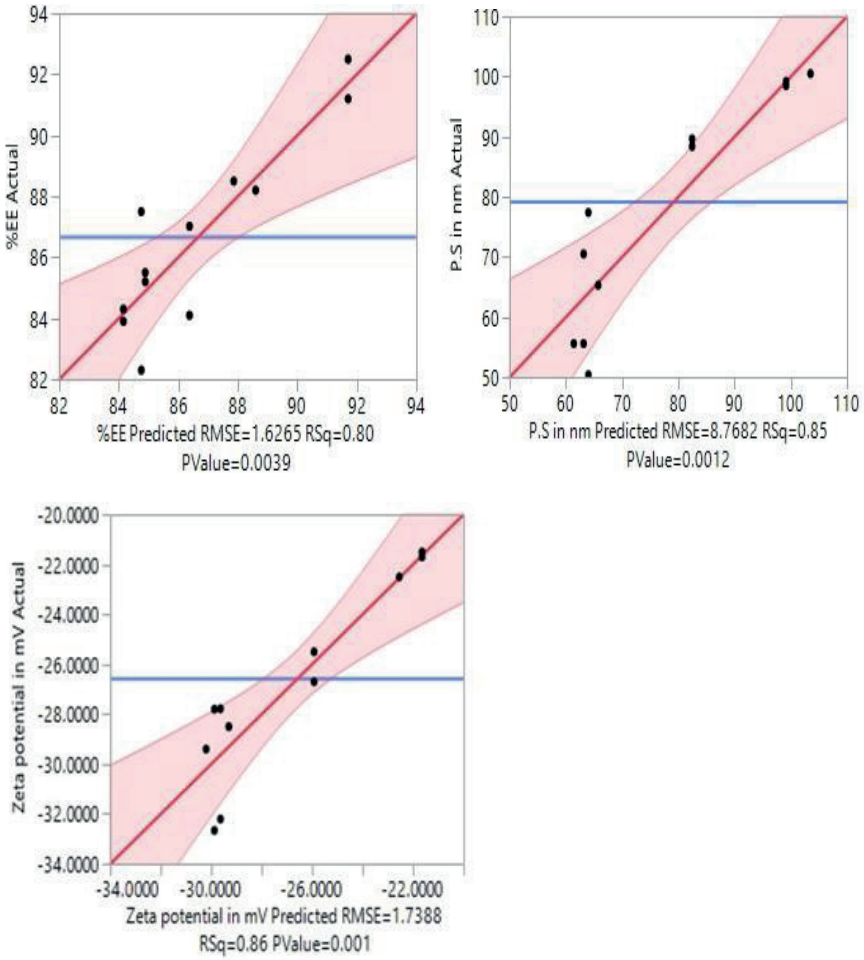

Figure 5. The graph of % EE, Vesicle size and Zeta potential

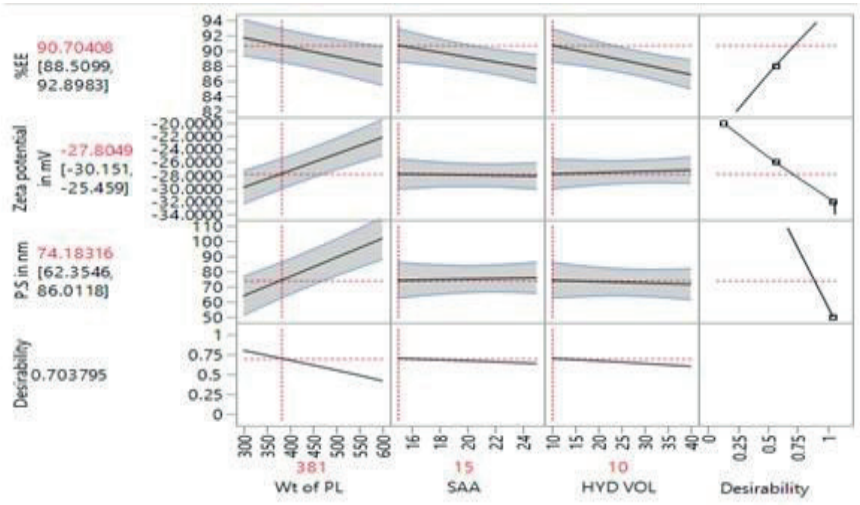

Figure 6. Prediction profiler and desirability for DOE

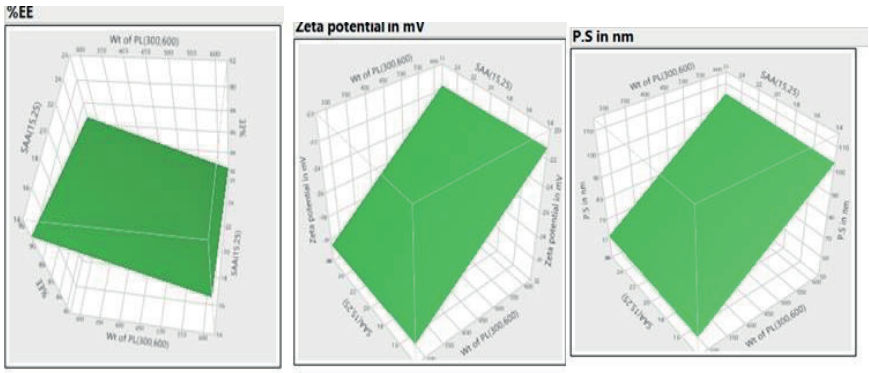

Figure 7. Surface profiler for % EE, zeta potential, vesicle size

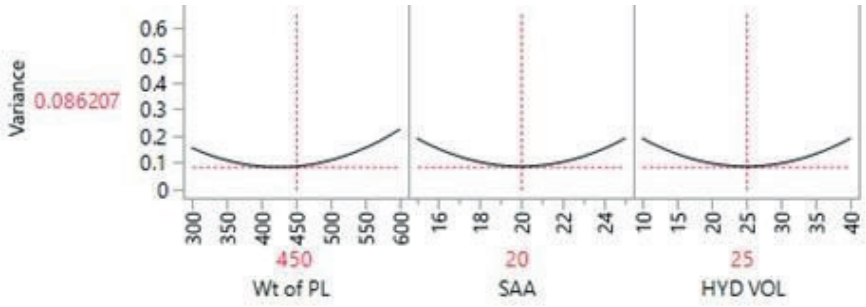

Figure 8. Prediction variance profile

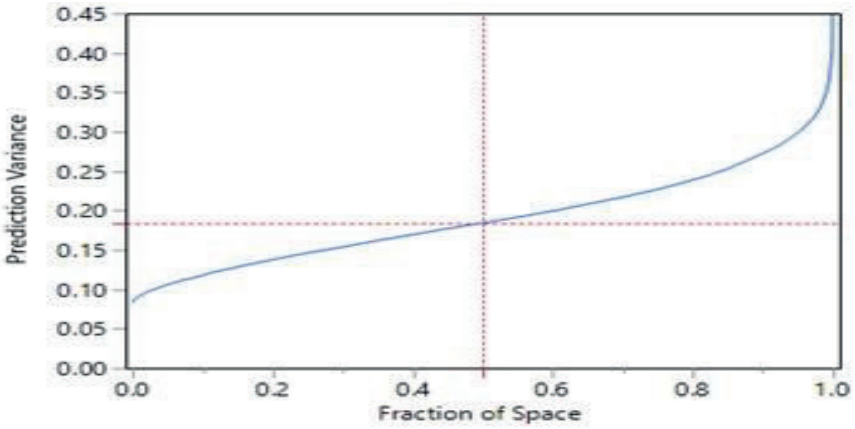

Figure 9. Fraction of design space plot

Evaluation of optimised formulation

The optimized formulation was prepared per the predicted formula based on the desirability approach. The formulations evaluated for the vesicle size, zeta potential, % EE and % CDR were found through diffusion studies. The vesicle size was 74.4 nm, zeta potential to be -27.4mV as shown in Figures 10 and 11, and % EE to be 90.5%±0.25. As reported in the literature, the optimum ratio of phospholipid: surfactants can affect the size, zeta potential, and drug entrapment efficiency. It is seen that the surfactant at higher concentration has, on the contrary, an effect on entrapment efficiency as the changes in membrane permeability can expel the drug from the core. However, higher surfactant concertation results in smaller vesicle size up to an optimum level. After that, it promotes micellar formation rather than vesicles.

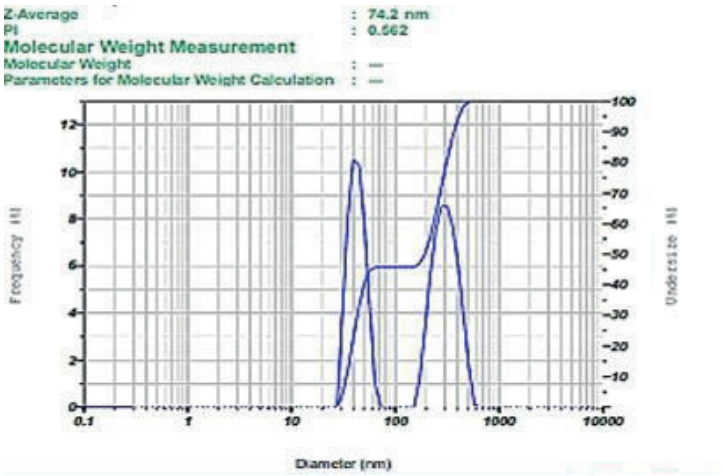

Figure 10. Particle size analysis of optimised formulation

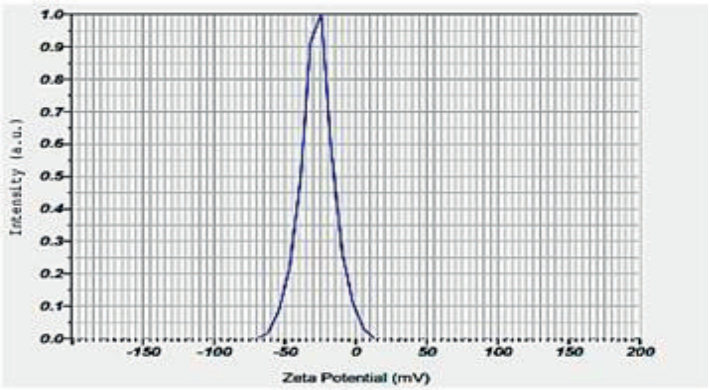

Figure 11. Zeta potential of Optimised formulation

Moreover, the poydispesibility index is found to be low, which may be due to reduced interfacial tension. The results showed that the phospholipid has a prominent effect on the responses; a low amount of phospholipid resulted in low zeta potential value, vesicle size, and higher % EE. The hydration volume had an almost negligible effect on the responses. The lesser the hydration volume is, the more secondary is zeta potential, and with more hydration volume, a slight increase in the vesicle size and % EE was observed18-20. The actual values are as per the practical observation, as shown in Table 4, and the observation of optimized formulation is given in Table 5.

Table 4. Results showing %Entrapment efficiency, Zeta potential and Vesicle size

Formulation code	% Entrapment Efficiency	Zeta potential (mV)	Vesicle size (nm)
F1	85.5	-22.5	100.5
F2.	84.3	-21.5	98.5
F3.	88.5	-28.5	55.6
F4.	82.3	-32.2	70.5
F5.	83.9	-21.7	99.2
F6.	92.5	-32.6	50.4
F7.	88.2	-29.40	65.3
F8.	85.2	-22.5	100.5
F9.	87.5	-27.78	55.6
F10.	91.2	-27.8	77.4
F11.	87.02	-26.7	88.4
F12.	84.1	-25.50	89.65

Table 5. Evaluation of optimised formulation

	Vesicle size predicted	Vesicle size actual	Zeta potential predicted	Zeta potential actual	% EE predicted	% EE actual	% CDR
Optimised formulation	74.1nm	74.4nm	-27.9mV	-27.4mV	90.70%	90.5%±0.25	88.65%±0.34

Morphology of transfersomes

The TEM image shows the transfersomes are present in a spherical shape, and a unilamellar structure was observed, which is given in Figure 12.

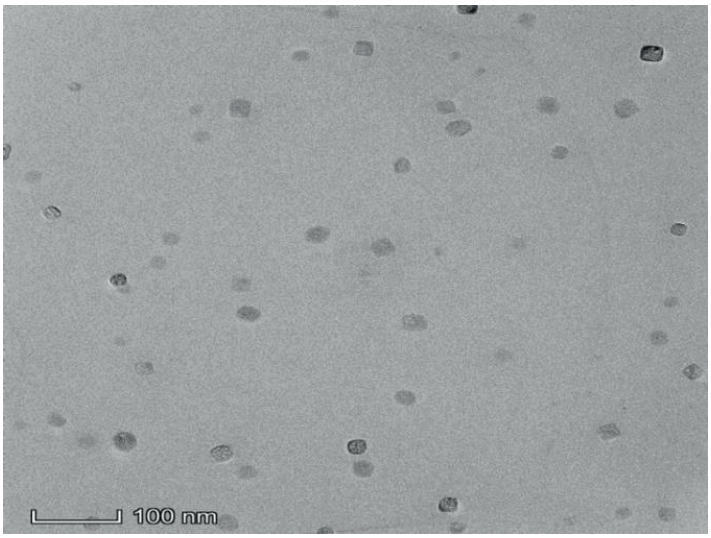

Figure 12. TEM image depicting the structure and morphology of transfersomes

FTIR Study of optimised formulation.

The FTIR graph of the optimized formulation showed a peak at 1634.5 cm-1, establishing the presence of the carboxylic acid group, which was found to agree with the standard range 1500-1700cm-1. C-O stretching for carboxylic acid and ester can be observed at 1239.46cm-1 and 1197.8cm-1, which follow the standard range 1200-1250cm-1 and 1100-1200cm-1, respectively represented in Figures 13 and 14. And the peaks of O-H stretching meta substitution and C-H bending signify the drug's presence in the formulation, and no interaction was observed.

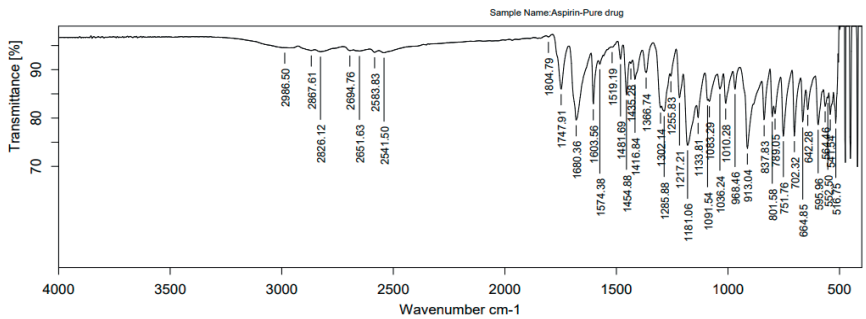

Figure 13. FTIR spectrum of pure drug aspirin

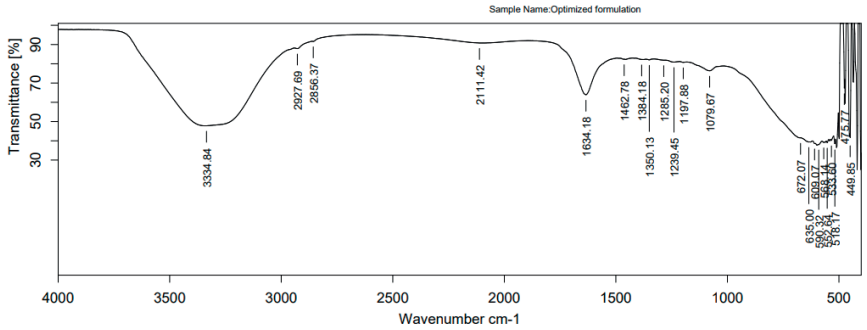

Figure 14. FTIR spectrum of optimised formulation

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is one of the most extensively used calorimetry techniques for characterizing the solubility and physical state of drugs in lipid vesicles is differential scanning calorimetry (DSC). Figure 15 shows the DSC analysis of the pure drug and the optimized formulation. The DSC study of the pure drug shows a significant endothermic peak at 144.9°C, which corresponds to aspirin's melting point. This peak intensity was reduced in the DSC thermogram of optimized transfersomes produced with tween 80. The absence of aspirin's melting endotherm suggested that the drug was in a more soluble amorphous state. (Figure 16)

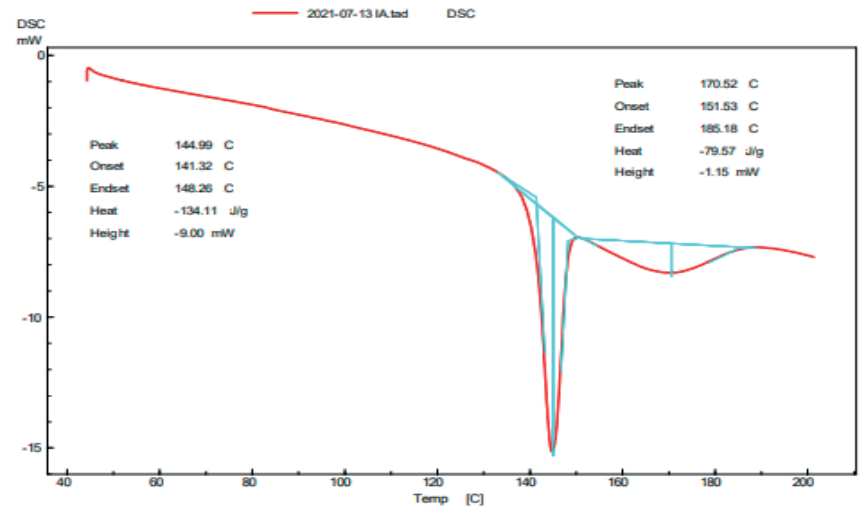

Figure 15. DSC thermogram of pure drug

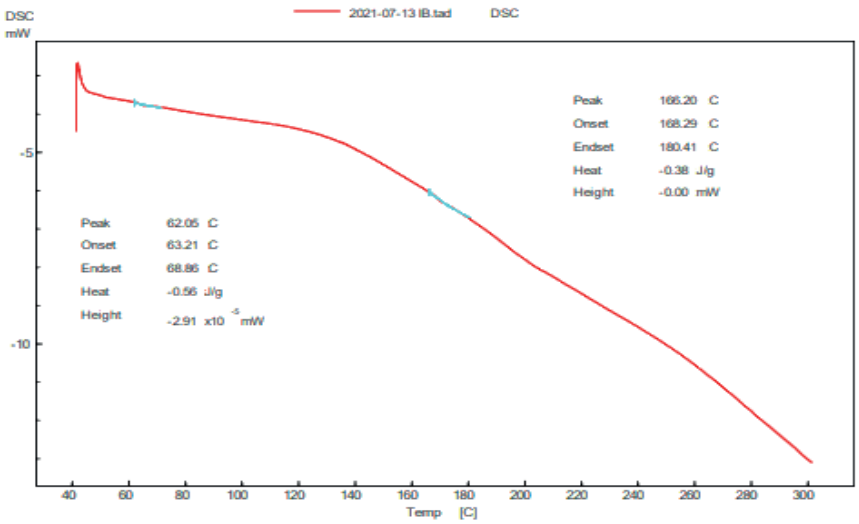

Figure 16. DSC thermogram of optimised formulation

The inhibition of aspirin crystallization and solubilization in transferosomes could explain the shift in melting behavior. This shows that the aspirin in the prepared transfersomes was amorphous. The conversion of a drug's physical state to an amorphous or partially amorphous state results in a high-energy state with the high disorder, resulting in increased solubility.

Evaluation of transfersomal gel

The viscosity of drug solution gel and transfersomal gel was evaluated at 100 pm. The gel formed with the drug solution was thick compared to the transfersomal gel, represented in Figure 17.

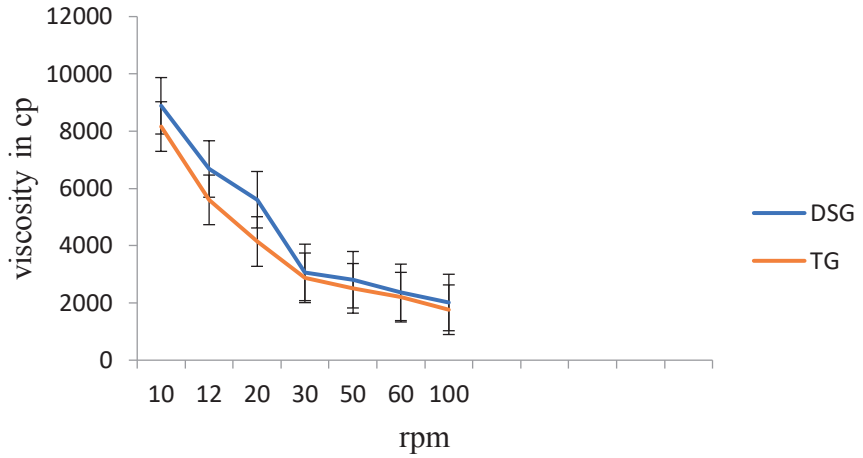

Figure 17. Viscosity in cP of drug solution Gel (DSG) and transfersomal Gel (TG)

The viscosity and consistency of the plane gel are represented in Table 6, and the pH, homogeneity, spreadability, and drug content are given in Table 7.

Table 6. Evaluation of drug solution gel and transfersomal gel

Evaluation parameters	Drug solution gel	Transfersomal gel
Viscosity(cP) at 100 rpm	2014.5	1762
pH	5.04±0.38	5.74±0.78
Homogeneity	Good	Good
Spreadability (cm)	10.6 ± 0.73	7.2 ± 0.85
Drug content (%)	85.54±2.2	95.8±1.5

Table 7. Effect of carbopol concentration on viscosity and consistency

Carbopol 934 concentration	Viscosity(cP) at 100 rpm	Consistency
0.1%	57	**Liquid**
0.5%	455	**Slightly gel consistency**
0.75%	501	**Gel consistency**
1%	600	**Gel consistency**

In vitro drug release study

Drug release from the drug solution, drug solution gel, and transfersomal gel was compared to see the effectiveness of the drug release when presented as the solution and solution gel. The drug solution showed a % CDR of 45.6 ±0.48% after 24 hrs; release is less due to the partial solubility of the drug in water and permeation of the drug through the membrane. Drug solution gel and transfersomal gel % CDR were 70.9%±0.45 and 85.5±0.52%, respectively. The graph of % CDR v/s time was plotted and shown in Figure 18.

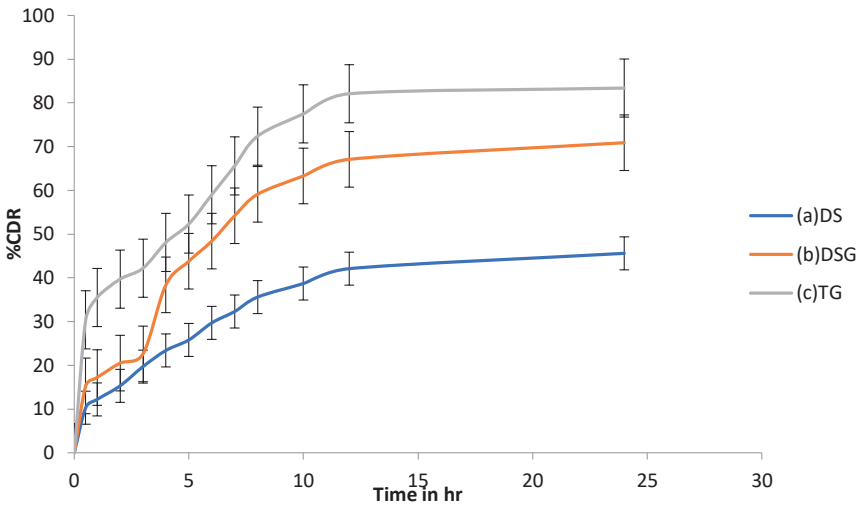

Figure18. *In vitro* drug release profile of (a) (DS) Drug solution (b) (DSG) Drug solution gel (c)(TG) Transfersomal gel

Stability studies

The optimal transfersomal suspension and gel were studied at 250±20 C/60 % RH ±5% and 50±30C. The samples were evaluated for % EE, vesicle size, and drug content. After 90 days of the study period, the % EE and drug content remain the same without any changes indicating a stable formulation, as shown in Table 8.

Table 8. Accelerated stability testing

Sl no	Days	% Entrapment efficiency Transfersomal suspension		Vesicle size transfersomes in suspension		Drug content of gel	
		Optimal formulation	25°±2° C/60 % RH ±5%	Optimal formulation	25°±2° C/60 % RH ±5%	Optimal formulation gel	25°±2° C/60 % RH ±5%
1	0	90.5%±0.25	90.5%±0.25	74.4nm	74 nm	95.8±0.38	95.8±0.38
2	90	90.5%±0.25	89.15%±0.15	74.4nm	73.4nm	94.1±0.08	95.12±0.09

Transferosomes are flexible and biocompatible non-invasive carriers utilized in drug delivery systems to achieve adequate drug concentration. The present work was an attempt to formulate ultra-deformable vesicles of aspirin to improve the therapeutic efficacy of the drug via a non-invasive drug delivery system. The vesicles prepared by the thin-film hydration method preserved their properties, such as zeta potential and particle size. The formulation trials were optimized via a custom design approach. The optimized vesicle incorporated gel formulation exhibited appreciable viscosity. The drug release from trasferosomal gel formulation exhibited a 3-fold increment in drug release compared to the solution and a two-fold to solution gel. The study can be extrapolated to *ex vivo* permeation using biological tissue samples, and also pharmacokinetic studies can be carried out to support *in vitro* data. Hence, the study proved the feasibility of transferosomes as a stable carrier to aspirin in treating angina pectoris.

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The effect of breadfruit (*Artocarpus altilis* (Parkinson) Fosberg) leaf extract on blood glucose, lipid profiles, and weight loss in alloxan-induced diabetic rats

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ABSTRACT

Diabetes mellitus is associated with abnormalities in lipid metabolism and weight loss. This study aimed to examine breadfruit (*Artocarpus altilis* (Parkinson) Fosberg) leaf extract's effects on lipid profiles and weight loss in alloxan-induced diabetic rats. Forty-five male Wistar rats were injected with alloxan and divided into treatment groups: placebo, *Artocarpus altilis* leaf extract (100, 200, or 400 mg/kg) or insulin (6U/200 g). Five additional rats were included as normal controls. Following 14 days of treatments, *Artocarpus altilis* extract lowered the blood glucose (BG) level, but only significant at 400 mg/kg dose. Eighty percent of rats in the placebo group had a significant weight loss compared to 40% of rats in the 400 mg/kg group. The placebo group had significantly higher total cholesterol (TC) compared to controls ($p<0.05$) and the *Artocarpus altilis* extract treatment significantly reduced the TC level ($p<0.05$). In conclusion, *Artocarpus altilis* extract treatment improves BG, lipid metabolism, and weight loss in alloxan-induced diabetic rats.

Keywords: *Artocarpus altilis*, breadfruit, alloxan, dyslipidemia, weight loss

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INTRODUCTION

Diabetes mellitus (DM) is a condition characterized by hyperglycemia due to insulin resistance. It is estimated that people with diabetes mellitus have reached 463 million worldwide and are predicted to rise to 578 million by 2030[1]. In addition to hyperglycemia, diabetes mellitus is associated with dyslipidemia due to insulin modulation on lipid metabolism, which subsequently alters the activities or the transport of lipid metabolism enzymes. The impact of diabetic-induced dyslipidemia includes vascular complications, which lead to increased comorbidity in DM patients[2].

Weight loss is one of the clinical symptoms of non-insulin or insulin-dependent DM. In the absence of insulin, the transport of blood glucose into the cells is averted. As the cells fail to receive glucose as the primary energy source, the body stimulates excessive mobilization of fat from the adipose tissues[3], leading to diabetic weight loss. There is evidence that diabetic patients who lost weight without having a lifestyle change had increased risks of mortality compared to those who did not lose weight[4].

The use of herbal products and supplements has increased rapidly over the past three decades, with no less than 80% of people relying on herbal products[5]. It has been reported that more than 1200 traditional plants may have been used as the folklore of antidiabetic treatments[6]. *Artocarpus altilis* (Parkinson) Fosberg or breadfruit is one of the plants that have been empirically used for DM treatments and lipid disorders. In animal studies, *Artocarpus altilis* leaf extract was found to increase pancreatic beta cell number in streptozotocin-induced diabetic rats[7]. In line with this, *Artocarpus altilis* leaf extract also improves Langerhans islands and exocrine tissue structures in the pancreas of alloxan-induced diabetic rats[8, 9]. It is believed that *Artocarpus altilis* roles are not limited to glucose control since *Artocarpus altilis* leaf extract was also beneficial to reduce free fatty acid levels in obese rats[10]. Therefore, this study aimed to examine the effect of *Artocarpus altilis* leaf extract administration on lipid profiles and weight loss in diabetic rats induced by alloxan injection. The lipid profiles examined include cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels.

METHODOLOGY

Preparation and extraction of *Artocarpus altilis*

Artocarpus altilis leaves were harvested in Gowa, South Sulawesi. Only leaves with yellowish color were handpicked from the trees based on the empirical use of *Artocarpus altilis* leaves as a diabetes treatment in the area. The *Artocarpus*

altilis leaves were authenticated by Dr. A. Mu'Nisa from the Department of Biology, State University of Makassar, Indonesia, with an authentication number of 096/SKAP/LAB.BIOLOGI/VII/2019. The herbarium specimen was collected and deposited in the Laboratory of Pharmacognosy, Hasanuddin University, Indonesia. The leaves were cleaned with tap water, sorted, dried, and finely chopped. *Artocarpus altilis* leaves (150 g) were macerated using 70% ethanol (2.5 L) for three days with an occasional stirring. The result of maceration was evaporated using a rotary vacuum evaporator at 175 mbar coupled with a water bath at 40°C until thick extract was obtained. The remaining solvent was further reduced using a desiccator.

Chemical preparation

Alloxan monohydrate was purchased from Sigma Aldrich. Diagnostic kits for cholesterol, triglycerides, LDL, and HDL were purchased from Human Diagnostics Worldwide (Germany).

Animal preparation

Fifty male Wistar rats were purchased from an animal laboratory breeding facility (UD. Wistar, Yogyakarta, Indonesia). Animals were accustomed to the laboratory environment for 14 days before starting the experiment. Animals received standard food and water ad libitum.

Experimental procedures

Forty-five rats were intraperitoneally injected with alloxan at a dose of 155 mg/kg. The alloxan dose was chosen based on a previous study[11] and adjusted in our preliminary study. Ten minutes after injection, rats were given 5% glucose (2 ml) through oral gavage to prevent hypoglycemia in rats. The blood glucose levels were checked daily with a glucometer. After three days post-alloxan injection, only rats with blood glucose levels> 200 mg/dl were defined as diabetic and received treatments according to their groups.

Group I was given a placebo (sodium carboxyl methylcellulose, Na CMC 1%, n=5); group II was given *Artocarpus altilis* leaf extract at the dose of 100 mg/kg; group III was given *Artocarpus altilis* leaf extract at the dose of 200 mg/kg; group IV was given *Artocarpus altilis* leaf extract at the dose of 400 mg/kg; group V received insulin injection at the dose 6 IU/200 g. An additional group of rats (n=5) that did not receive alloxan injection was also involved as normal controls.

Rats were weighed every day before receiving treatments to adjust the dose accordingly. The *Artocarpus altilis* extract treatments, as well as insulin injections, were done once daily for 14 consecutive days. Blood samples were withdrawn at

the end of experiments to measure cholesterol, triglyceride, LDL, and HDL levels. All lipid fractions were measured using Humalyzer 3500 according to the kits' instructions.

Statistical analysis

All data are presented as mean ± SEM. Kolmogorov-Smirnov analysis is used to test data distribution, while Levine's test is used to determine the data's homogeneity. Normally distributed data were analyzed using one-way ANOVA followed by a Least Significance Difference (LSD) post hoc test. Meanwhile, not normally distributed data were analyzed with a Kruskal-Wallis analysis, followed by a Mann-Whitney test. A significant difference was considered achieved when $P<0.05$.

RESULTS and DISCUSSION

Dyslipidemia in diabetic patients is common since insulin-dependent pathways of lipid metabolisms were considerably altered[12]. Effective treatment of diabetic-induced dyslipidemia can significantly reduce the risk of cardiovascular disorders. In an effort to find an effective treatment for diabetic-induced dyslipidemia, this study examined the effectiveness of *Artocarpus altilis* leaf extract in improving lipid metabolism in diabetic rats.

In this study, 25 out of 45 rats experienced hyperglycemia or increased blood glucose level after 72 hours from alloxan (155 mg/kg BW) injection. A previous review has pointed out that alloxan's diabetogenic effect could be unpredictable, as alloxan may induce diabetes in 33% to 60% of rats injected with 150 mg/kg to 170 mg/kg of alloxan[13]. Some factors may influence alloxan diabetogenic effects, including the route and rate of injection, animal age, and type of diet[14]. Two different pathogenesis were associated with alloxan-induced diabetes: 1) through a selective inhibition of insulin secretion by specific inhibition on glucokinase; 2) induction of the formation of ROS, causing selective necrosis of beta-pancreatic cells.

The increase in blood glucose levels varied among rats after alloxan injection, but most rat BG levels reached >300 mg/dl, and the average BG levels were not significantly different among groups. Figure 1 shows the blood glucose level after 14 days of treatment administration. It is found that only the highest dose of *Artocarpus altilis* extract (400 mg/kg) significantly reduced the blood glucose level, which was similar to that seen with insulin treatment ($p<0.05$).

The body weights of alloxan-induced diabetic rats are depicted in Table 1. Eighty percent of the placebo group, while only 40% of rats treated with extract 400 mg/kg or insulin, experienced weight loss.

Table 1. The profile of rat body weight before alloxan injection (baseline), after treatment, and overall changes in body weight of rats

Groups	Baseline weight (a)	Post-treatment (b)	Weight change (b-a)	Percentage of an animal with weight loss (%)
Normal (n=5)	243.4±20.0 g	259.2±18.3 g	+15.8±6.0 g	0
Placebo (n=5)	210.4±15.3 g	188.8±13.3 g	-21.6±13.1 g	80
Ext 100 (n=5)	239.0±17.8 g	224.6±35.5 g	-14.4±19.4 g	40
Ext 200 (n=5)	232.4±17.8 g	216.0±17.8 g	-16.4±9.3 g	80
Ext 400 (n=5)	206.0±3.3 g	205.0±16.2 g	-1.0±14.3 g	40
Insulin (n=5)	226.0±14.4 g	225.2±7.3 g	-0.8±11.3 g	40

+ weight change=weight gain; -weight change=weight loss

Weight loss could happen as the body has to compensate for the lack of energy production from glucose by switching the source of ATP to non-carbohydrate molecules, such as fat and protein from muscle tissues[15]. The body weight changes varied in each treatment group. While the normal group gained weight after 14 days of the experiment (15.8±6.0 g), a significant weight loss was shown in the placebo, extract 100 and extract 200 groups, with an average of weight loss of 21.6±13.1 grams, 14.4±19.4 grams, and 16.4±9.3 grams, respectively. Meanwhile, the insulin group and the 400 mg/kg *Artocarpus altilis* extract group did not experience a marked decrease in their body weights. This result could suggest a potential role of *Artocarpus altilis* leaf extract to improve glucose metabolism in diabetic rats.

Figure 2 shows the levels of total cholesterol, triglycerides, LDL, and HDL in alloxan-induced diabetic rats following 14 days of treatment. It is revealed that the normal groups had total cholesterol (TC) levels of 41 to 116 mg/dl, with an average of 71 ±12.3 mg/dl. Meanwhile, the diabetic rats that only received a placebo had increased TC level (175 ±22.8 mg/dl), which was significantly elevated compared to normal control. The *Artocarpus altilis* extract of 100 mg/kg, 200 mg/kg, and 400 mg/kg were found effective to lower the TC levels of alloxan-induced diabetic rats ($P<0.05$), which was similar to that achieved with the insulin treatment ($P<0.05$).

As seen in Figure 1, the placebo group had a slightly increased triglyceride level compared to the normal group (65.6±2.7 mg/dl vs. 78.3±6.3 mg/dl); however, it did not reach statistical significance. Out of the treatments given, only 100 mg/kg of *Artocarpus altilis* extract administration resulted in a reduction of

triglyceride level compared to the placebo group. Unlike the TC and triglyceride levels, the HDL levels of diabetic rats were not significantly altered compared to the normal group. Interestingly, there was an increase in LDL level with 100 mg/kg of *Artocarpus altilis* extract and a reduced LDL level with insulin treatment; yet, these changes did not reach statistical significance compared to the normal group.

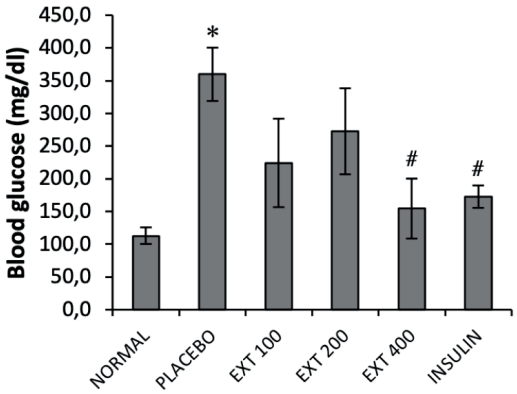

Figure 1. The blood glucose levels in non-diabetic (normal) and diabetic rats after receiving a placebo, Artocarpus leaf extract 100, 200, 400 mg/kg, or insulin injection. *P<0.05 between the placebo group and normal controls. #P<0.05 between treatment groups and placebo.

The common characteristics of diabetic dyslipidemia include hypertriglyceridemia, low HDL cholesterol, and elevated LDL, with hypertriglyceridemia being more dominant[16]. Interestingly, alloxan injection in this study only slightly induced hypertriglyceridemia in rats. The discrepancy may originate from the fact that alloxan-induced diabetes had different mechanisms from that develop in humans.

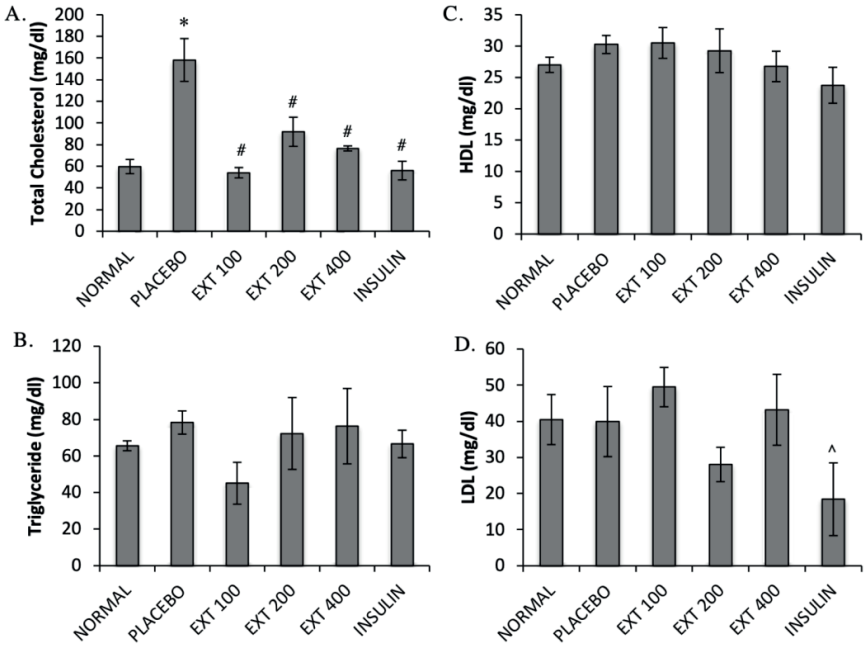

Figure 2. The lipid profiles of non-diabetic (normal) and diabetic rats after receiving placebo, Artocarpus leaf extract 100 mg/kg, 200 mg/kg, 400 mg/kg, or insulin injection. A. Total cholesterol; B. Triglyceride; C. High-density lipoprotein; D. Blood glucose. *P<0.05 between the placebo group and normal controls. #P<0.05 between the treatment and placebo groups. ^P<0.05 between the insulin and EXT 100 groups.

Alloxan shares a similar molecular structure with glucose, hence. its cellular uptake is also facilitated by GLTU2 in beta-pancreatic cells. Alloxan diabetogenicity comes from the selective inhibition of glucose-stimulated insulin release through glucokinase inactivation and ROS production[17]. Meanwhile, the development of diabetes mellitus in humans is far more complex, involving a range of factors, including genetics, nutritional state, and environment[18]. Dyslipidemia found in diabetes mellitus type 2 patients is progressively developed due to insulin resistance. In contrast, alloxan injection acutely damages the Langerhans islands of the pancreatic tissues, leading to a degeneration of beta-pancreatic cells and a massive reduction in insulin production and release[19]. This form of diabetes is repeatedly associated with persistent ketoacidosis and hypercholesterolemia[20]; yet, a significant increase in LDL and triglyceride levels or a reduction in HDL levels may inconsistently be observed in alloxan-induced diabetic rats[21, 22].

At the dose of 400 mg/kg, *Artocarpus altilis* extract improved blood glucose levels and prevented weight loss in alloxan-induced diabetic rats. In addition to its hypoglycaemic effect, the administration of *Artocarpus altilis* leaf ethanol extract significantly improved total cholesterol level compared to placebo ($p<0.05$) and triglycerides, to a lesser extent, with the low dose. The effect of *Artocarpus altilis* leaf extract treatments was similar to insulin in reducing total cholesterol levels in alloxan-induced diabetic rats. This result may indicate the potential roles of *Artocarpus altilis* leaf extract as an alternative treatment for diabetic hypercholesterolemia. Another study also reported the antidiabetic effect of *Artocarpus altilis* extract occurred along with an improvement in histological structures of the islets of Langerhans[23]. Indeed, the protective effect of *Artocarpus altilis* extract has also been demonstrated in the liver and kidneys of alloxan-treated animals[24]. The antidiabetic, antihypercholesterolemic, and organ protective effects of the *Artocarpus altilis* leaf extract may be related to the presence of high content of a range of phytochemicals content, including polyphenol compounds and flavonoids. It has been shown that flavonoids and flavonoid-rich extracts, such as luteolin, curcumin, wild berry extract, hawk tea, and anthocyanin extract, may inhibit cholesterol uptake via inactivation of a cholesterol transporter, the Niemann–Pick C1-like 1 (NPC1L1) protein, resulting in a decrease of blood cholesterol levels[25]. Further study is still warranted to elucidate the specific bioactive compounds and the putative mechanisms underlying the therapeutic potential of *Artocarpus altilis* extract.

STATEMENT OF ETHICS

All animal procedures have been approved by the institutional ethics committee at the Faculty of Medicine, Hasanuddin University, with an ethical clearance number of 544/UN4.6.4.5.31/PP36/2019.

CONFLICT OF INTEREST

The authors declare to have no conflict of interest

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Does eugenol have potential as an anti-campylobacter and antioxidant compound in the food industry and clinical settings?

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ABSTRACT

Though there exist high food safety standards and legislations in developed countries, *Campylobacter* infections have remained a major public health problem. *Campylobacter jejuni* and *Campylobacter coli* are the principal causes of bacterial foodborne gastroenteritis in humans. *Campylobacter* infections are closely related to the consumption of contaminated chicken. *Campylobacter* species develop resistance to existing antibiotics thanks to the genomic plasticity. There is therefore a higher need to develop effective approaches for bacterial control in modern food industry and clinical settings. Presented study has evaluated the anti-campylobacter activity and antioxidant capacity of eugenol, the primary phenolic component of clove oil. Two *C. jejuni* strains (ATCC 33560 and one chicken isolate) and two *C. coli* strains (NCTC 12525 and one chicken isolate) were used in the study. The anti-campylobacter activity of eugenol was analyzed by microbroth dilution method. Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) were in the range of 0.64-1.28 mg mL^{-1}. Besides, our MTT assay findings showed that eugenol has strong scavenging ability. These outcomes have supported both the anti-campylobacter and antioxidant activity of eugenol. Eugenol is generally recognized as safe and is a promising antimicrobial compound against the genus *Campylobacter*.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, Eugenol, Clove oil, Antibacterial Activity

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INTRODUCTION

Campylobacter species, specially *C. jejuni* and *C. coli*, are the primary cause of bacterial food-borne gastroenteritis in humans [1]. The World Health Organisation (WHO) has suggested that food-borne *Campylobacter* infections affected more than 95 million people around the earth in 2010 [2]. Human Campylobacteriosis has currently been recorded as the mostly reported infection in the European Union. It is notable that *Campylobacter* infections represent 50% of the total cases of gastrointestinal infections [3]. Apart from acute morbidity, chronic several complications such as reactive arthritis (RA), inflammatory bowel disease (IBD) and Guillain Barre Syndrome (GBS) have also been reported following *Campylobacter* infection in humans [4]. Moreover, *Campylobacter* infections are responsible for 31% of GBS cases globally [5].

Campylobacter species are common in the digestive tract of livestock and domestic animals. Essentially the thermophilic *Campylobacter* species are a commensal microorganism in broiler flocks with up to 10^{10} colony forming units (CFU) per gram of faeces [1]. Modern poultry slaughter relies on automated equipment posing a challenge to processors to avoid bacterial contamination in the slaughter line. As a result, contamination of poultry carcasses during slaughter is often inevitable [6]. The most *Campylobacter* infections in humans are caused by the consumption of contaminated foods of animal origin, such as under-cooked poultry and un-pasteurized milk [7,8]. However, it has been reported that the main cause of human campylobacteriosis is eating contaminated chicken in developed countries [3]. Chicken consumption is responsible for 29.2%, 65-69% and 56.5% of human campylobacteriosis in USA, Canada and UK, respectively [9]. Thus, ensuring microbiological control at all stages of the food chain will reduce the risk of human *Campylobacter* infections.

Campylobacter infections are typically self-limiting and not require medical treatment. The focus of medical interventions in the most cases are hydration and electrolyte repletion. However, antibiotics are used in high-risk patients such as the immunocompromised and the elderly. The genus *Campylobacter* creates a variety of mechanisms for resistance to clinically important antibiotics thanks to the genomic plasticity [8]. Antibiotic-resistant *Campylobacter* is listed as one of the underlying pathogens on the WHO list for creating new treatment strategies [10]. Concerns about antibiotic-resistant pathogens and food safety have increased the interest in developing alternative approaches to combat pathogens. Natural compounds from roots, leaves and flower buds of medicinal plants may have a prominent medicinal potential without developing resistance in several pathogens [11]. Besides, the inclusion of natural compounds in combination therapies for medical treatment is also of great interest.

Clove has been used by civilizations for many years due to its fragrance and flavor. This desirable characteristics make it worth for culinary and therapeutic uses. Eugenol is the major phenolic component of clove essential oil, derived from the flower buds and leaves of *Syzygium aromaticum* (L.) Merr. et L.M.Perry (syn. *Eugenia caryophyllus* (Spreng.) Bullock et S.G.Harrison). It is a yellow colored liquid with the molecular weight of 164.2 g mol^{-1}. The antioxidant property of eugenol, which has been proven by various studies, has recently became the focus of attention of researchers [12,13]. Current studies have also suggested the analgesic, antiproliferative and anti-inflammatory properties of eugenol [14,15]. Further, there is extensive literature on the antimicrobial activity of eugenol against wide variety of human pathogens such as *Salmonella* Typhmurium, *Candida albicans* and *Escherichia coli* [16-18]. However, there are limited studies reporting the *in-vitro* antimicrobial activity of eugenol against *Campylobacter* species. The present study therefore assessed the *in-vitro* antimicrobial activity of eugenol against *C. jejuni* and *C. coli* strains. We have also aimed to present the *in-vitro* antioxidant activity of eugenol as ascorbic acid equivalent.

METHODOLOGY

Chemicals

Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Chemie GmbH, Germany), Mueller Hinton Broth (MHB; Sharlau Microbiology, Belgium), Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA; CM0739, OXOID), Mueller Hinton Agar (MHA; M1084-500G, HIMEDIA), Eugenol (Sigma-Aldrich, Chemie GmbH, Germany), Peptone Water (PW; LAB104, LABM) and CampyGen 3.5 L sachet (CN0035A, OXOID) were used in the present study.

Bacterial strains and preparation of bacterial suspension

In the present study, *C. jejuni* (one chicken isolate and ATCC 33560) and *C. coli* (one chicken isolate and NCTC 12525) strains were used. Isolated and reference strains were obtained from chicken meat purchased from a local market and Refik Saydam National Type Culture Collection Laboratory (Ankara, Turkey), respectively. All bacterial strains were preserved in a growth medium containing 20% glycerol at -80 °C. Initially, each *Campylobacter* strain was cultivated on separate mCCDA plate and incubated at 37 °C for 48 hours under the microaerobic condition created with CampyGen 3.5 L sachet. The cells were then adjusted to 0.5-McFarland opacity (1-2 x 10^8 CFU mL^{-1}) with 10 mL of PW [19]. Each prepared bacterial suspension was homogenized by vortexing for 1 minute prior to the antibacterial activity test.

Preparation of eugenol solution

Eugenol (99% reagent plus) was purchased from Sigma-Aldrich (Chemie GmbH, Germany). The stock solution was prepared by dissolving 0.96 mL of eugenol in sterilised distilled water (50 mL) containing 1% DMSO. The stock solution (20.480 mg mL^{-1}) is then 2-fold-diluted with sterile MHB to yield 10.240 mg, 5.120 mg, 2.560 mg, 1.280 mg, 0.64 mg, 0.32 mg, 0.16 mg, 0.08 mg and 0.04 mg mL^{-1} solutions.

Analyze of bacterial growth inhibiton and determination of minimum inhibitory concentration

The MIC was determined using the microbroth dilution method recommended by Clinical and Laboratory Standards Institute [20]. Briefly, 100 µL of the serial dilution of eugenol (0.04-10.240 mg mL^{-1}) was transferred to 96-well microplates. Subsequently, bacterial suspension (1-2 x 10^8 CFU mL^{-1}) were prepared for each *Campylobacter* strain. 10 µL of each bacterial suspension was inoculated to the wells of separate microplates in six replicates. The control wells were prepared with broth media (sterilized product testing), eugenol (for negative control), and bacterial suspension (for positive control). Optical density (OD) was determined for each well by a spectrophotometer (Byonoy GmbH Absorbance 96, Germany) at an absorbance of 600 nm prior to incubation, T_0. Then, microplates were incubated in the jar for 24 hours at 37 °C under microaerobic condition created with CampyGen. The OD of the wells was measured again by the same spectrophotometer at an absorbance of 600 nm after the incubation, T_{24}. The OD for each well at T_0 was subtracted from the OD for each well at T_{24} and the average of the differences for six replicates was ultimately calculated.

The average OD of the positive control well was assigned a value of 100% bacterial growth. Afterwards, a standard curve was generated using the known percentage of bacterial growth by linear regression analysis. Accordingly, the percentage of bacterial growth was determined for each dilution of eugenol in the test wells. The growth inhibition data were obtained using the following equation. Percentage of inhibition = 100 – Percentage of growth. The MIC value was considered as the minimum eugenol concentration achieved growth inhibition above 95% at the absorbance of 600 nm.

Determination of minimum bactericidal concentration

The MBC values were determined after obtaining the MIC values. Briefly, the dilutions corresponding to ½MIC, MIC and 2MIC were cultured on MHA and incubated under microaerobic conditions created with CampyGen at 37°C for

48 hours. The MBC was regarded as the lowest eugenol concentration that inactivated more than 99.99% of bacteria present. Six replicates were performed for each *Campylobacter* strain and eugenol dilution.

Determination of antioxidant activity

The antioxidant activity of eugenol was determined by the MTT [3- (4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] method previously defined by Liu & Nair (2010) [21]. The MTT (1 mg mL^{-1}) was dissolved in sterilised distilled water. A stock solution of vitamin C (L-ascorbic acid, 600 µmol, 0.105 mg mL^{-1}) and eugenol (6.5 µmol, 1.067 g/mL) was two-fold diluted with distilled water. 190 µL of MTT solution and 10 µL of each eugenol dilution were mixed in an eppendorf tube and incubated at 37 °C for 4 hours. After incubation, 200 µL of DMSO was added to each tube and vortexed again to dissolve the blue formazan salt. Then 100 µL of each mixture was transferred to 96-well microplates in four replicates. The results were evaluated by spectrophotometer (Byonoy GmbH Absorbance 96, Germany) at an absorbance of 570 nm. A standard curve was generated with the OD values of two fold dilution of Vitamin C (y=bx+a) by linear regression analysis. Vitamin C equivalence of eugenol was determined according to the standard curve.

Statistical analysis

The results are stated as mean and standard deviation (X ± SD). Antimicrobial and antioxidant activity tests were performed in 6 replicates and 4 replicates, respectively. Data were analyzed using standard curves created with Microsoft Excel graphical charts.

RESULTS and DISCUSSION

Anti-campylobacter activity of eugenol

The antimicrobial activity of essential oils has long been described by Martindale (1910) [22]. In the literature to date, significant results on the antimicrobial activity of eugenol have accumulated. Despite limited research reporting the activity of eugenol against the genus *Campylobacter*, our findings have supported that eugenol has significant antimicrobial activity as noted in the literature. The antimicrobial activity of eugenol has been demonstrated against a wide range of pathogens such as parasites, fungi, gram positive and negative bacteria [23,24]. The antimicrobial potential is generally attributed to the free –OH groups in the structure of eugenol [12]. Previous studies have reported that eugenol targets microorganism viability by increasing cell membrane permeability, inhibiting membrane-associated ATPase activity, and arresting cellular

respiration [25,26]. Recent studies have reported the effects of eugenol on targeting microorganism viability as well as modulating various aspects of virulence [27]. However, *Campylobacter* has a natural ability to develop antimicrobial resistance thanks to its genomic plasticity [28].

Research on antimicrobial activity against *Campylobacter* species has mainly focused on essential oils of thyme and oregano [29,30]. A very few studies have been conducted on the anti-campilobacter activity of eugenol obtained from clove essential oil. Friedman et al. (2002) previously reported that *C. jejuni* strains are highly sensitive to eugenol [31]. Similarly, our findings revealed the activity of eugenol against *C. jejuni* and *C. coli* strains. The limited research has showed that the MIC value of eugenol against thermophilic *Campylobacter* species ranges from 0.05 to 1.280 mg mL^{-1}. According to Kuete (2010), antimicrobial activity is classified as weak (MIC ≥ 0.625 mg mL^{-1}), moderate (0.1 < MIC < 0.625 mg mL^{-1}) and significant (MIC < 0.1 mg mL^{-1}) [32]. In our study, MIC values of eugenol against the tested *Campylobacter* strains ranged from 0.64 to 1.280 mg mL^{-1} (Table 1).

Table 1. Minimum inhibitory and minimum bactericidal concentration of eugenol against *Campylobacter* strains.

Bacterial strain	MIC[#] (mg mL^{-1})	MBC[¥] (mg mL^{-1})	MBC/MIC
C.jejuni (chicken isolate)	0.64	1.28	2
C. jejuni ATCC 33560	1.28	1.28	1
C. coli (chicken isolate)	0.64	1.28	2
C. coli NCTC 12525	0.64	0.64	1

[#] *minimum inhibitory concentration*

[¥] *minimum bactericidal concentration*

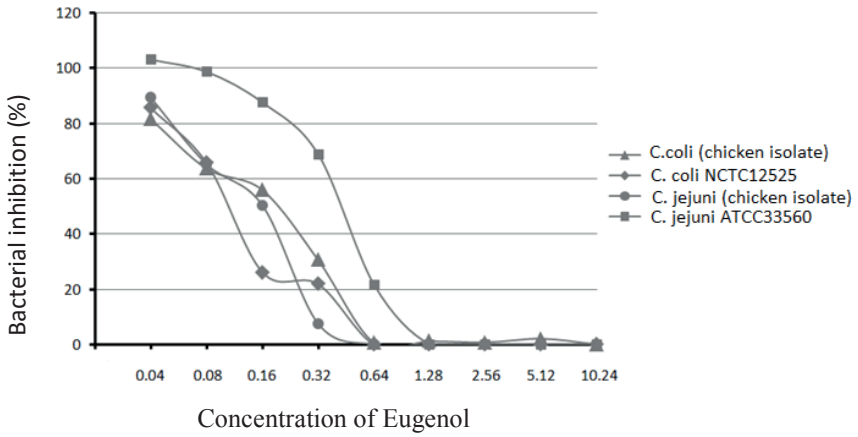

Figure 1. Assesing the efficiency of eugenol on bacterial growth inhibition at different concentrations. The studied concentrations were 0.04, 0.08, 0.16, 0.32, 0.64, 1.280, 2.560, 5.120 and 10.240 mg/mL, respectively.

The percent growth inhibition of tested eugenol concentrations against *C. jejuni* and *C. coli* strains is shown in Figure 1. Understanding the antimicrobial activity of essential oils is possible by determining the MIC value, which is the starting point of the research. However, different MIC findings reported in the literature have revealed some contradictions. These conflicts may arise from factors such as bacterial subtype, laboratory method, solvent and emulsifier used in research [33]. We determined the MIC of eugenol (1% DMSO) as 0.64 and 1.280 mg mL^{-1} against isolated and reference strains of *C. jejuni*, respectively. Similarly, Grilli et al. (2013) has reported that the MIC of eugenol (with ≤ 5% ethanol) is the 1.280 mg mL^{-1} for the both strains using the microbroth dilution method [34]. Although the solvent types were different, the MIC value in the study by Grilli et al. (2013) is in line with our findings. Also, we have observed the MIC of eugenol as 0.64 mg mL^{-1} against both isolated and reference strains of *C. coli*. Similarly, the MIC of eugenol was found to be 0.5 mg mL^{-1} against *C. coli* strains by Hassan et al. (2019) using the agar dilution method [35]. The MIC of eugenol against *Campylobacter* have mostly been determined using the microbroth dilution method. The lowest MIC values (0.05 mg mL^{-1} and 0.01 mg mL^{-1}, respectively) of eugenol against *C. jejuni* and *C. coli* strains by microbroth dilution method were recently recorded by Gahamanyi et al (2020) [36].

Some authors have reported synergistic interaction between eugenol and certain antibiotics as a possible strategy. Palaniappan et al. (2010) have suggested that eugenol (0.1 mg mL^{-1}) can reduce the MIC of penicillin and tetracycline against antibiotic-resistant *S.* Typhimurium, *E. coli* and *S. aureus* [37]. Addition-

ally, synergistic interactions between eugenol and streptomycin against *S.* Typhimurium and *L. monocytogenes* have been reported [38]. Remarkably, more than 8,000 *C. jejuni* sequence types have been reported to be registered by 2020 [39]. In the last decade, ciprofloxacin- and tetracycline- resistant *Campylobacte*r has been gradually increasing in the European population [40]. Therefore, the combination of antimicrobial compounds against *Campylobacter* strains are considered as a new alternative strategy [41]. To our knowledge, the anti-campylobacter activity of eugenol in combination with antibiotics or essential oils has not been evaluated to date. Further studies evaluating the anti-campylobacter activity of eugenol in combination with various antimicrobial agents should be warranted.

The increasing prevalence of antibiotic-resistant bacteria remains one of the major threats to public health worldwide. Knowledge of the pharmacodynamic characteristic of antimicrobial compounds can provide a reasonable approach to determine the optimal concentration against pathogens, improving public health. Therefore, it may be clinically necessary to determine the minimum bactericidal concentration (MBC) in order to understand the pharmacodynamic characteristics of antimicrobial molecules. Antimicrobial activity can be subdivided into two main categories as bacteriostatic and bactericidal activity, depending on the pharmacodynamic characteristics. In several studies, the activity of antimicrobial compounds is considered as bactericidal if the MBC/MIC ratio is ≤ 4. Some researchers have therefore suggested the bactericidal potential of eugenol against *Campylobacter* species [36]. Similarly, our findings demonstrated that the MBC/MIC ratio of eugenol against both *C. jejuni* and *C. coli* strains was < 4, resulting in bactericidal activity (Table 1). However, bactericidal activity of eugenol should be confirmed by further studies using time-kill tests.

Antioxidant activity of eugenol

Free radicals are responsible for a variety of adverse effects on health promotion and food stability. Scavenging free radicals and reducing the formation of free radicals is important for maintaining human health and food quality [42,43]. Free radical scavenging is a known reaction in which antioxidant molecules inhibit oxidation. These reactions are used in antioxidant activity studies and provide rapid detection of the scavenging activity of antioxidant compounds. The ability of antioxidant molecules to scavenge free radicals is often attributed to the bond-dissociation energy of the hydroxyl (–OH) group [44]. There is no widely used reasonable method for the determination of antioxidant activity. The 2,2, diphenyl-picrylhydrazil (DPPH) method and the trolox equiva-

lent antioxidant capacity (TEAC) method are commonly used [18]. In the present study, the scavenging ability of each antioxidant compound was determined by a simple and inexpensive MTT method [21].

As seen in Table 2, our findings showed that eugenol and vitamin C (ascorbic acid) dose-dependently reduced the MTT.

Table 2. Optical density of antioxidant compounds at 570 nm and vitamin C equivalence of eugenol

Concentration of Vitamin C (µmol/mL)	X ± SD[#] (nm)	Concentration of Eugenol (µmol/mL)	X ± SD[¥] (nm)	Vitamin C Equivalent (µmol/mL)
600	0.224±0.014	6.500	1.075±0.068	3478.222
300	0.106±0.028	3.250	0.999±0.057	3224.519
150	0.068±0.010	1.625	0.585±0.023	1844.519
75	0.060±0.014	0.812	0.253±0.019	739.333
37.5	0.051±0.012	0.406	0.105±0.003	246.000
18.75	0.039±0.004	0.203	0.063±0.002	106.370

[#] *optical density values of two fold dilution of Vitamin C*

[¥] *optical density values of two fold dilution of Eugenol*

Vitamin C is known as a broad spectrum antioxidant that can react with a range of detrimental radicals such as reactive oxygen species (ROS) and organic radicals [44]. In this study, we have demonstrated that eugenol has greater scavenging and reduced ability compared to vitamin C. Similarly, there are a wide range of *in-vivo* and *in-vitro* studies proving the potent antioxidant activity of eugenol [12,13]. Eugenol has been generally recognized as safe (GRAS) food additive by the US Food and Drug Administration. Further, it has significant potential to prevent oxidative stress-related diseases [13]. Therefore, eugenol has promising bioactivity for the prevention of chronic diseases as well as for use as a natural preservative in the food industry. However, the pro-oxidative and harmful effect of high concentrations have been highlighted. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has proven that the maximum allowable daily intake of eugenol for humans is 2.5 mg kg^{-1} body weight [45].

Campylobacter infections are the most common bacterial cause of food-borne illness around the earth. Consumption of contaminated chicken is mainly responsible for *Campylobacter* infections in developed countries. In the last decade, antibiotic-resistant *Campylobacte*r has been gradually increasing in

the European population. The researchers therefore focus on alternative treatment methods using natural antimicrobial agents. The antimicrobial activity of eugenol is one of the most popular research areas in recent years. Additionally, the antioxidant capacity of eugenol is remarkable for maintaining human health and food stability. Therefore, the goal of the presented research was to assess the anti-campylobacter as well as antioxidant action of eugenol. Our findings have highlighted the importance of eugenol for *Campylobacter* control. Eugenol may be useful to control *Campylobacter* in the modern food industry, where food preservation technologies are becoming increasingly important. However, it is necessary to comprehend the action mechanism and synergistic action of eugenol before it is used in clinical settings.

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AUTHOR CONTRIBUTIONS

Murat Gürbüz: Conceptualization, Methodology, Investigation, Software, Data curation, Writing – original draft, Writing - review & editing.

Burcu İrem Omurtag Korkmaz: Conceptualization, Methodology; Investigation, Writing - review & editing.

Serol Korkmaz: Conceptualization, Methodology, Investigation, Software, Writing - review & editing.

DECLARATION OF INTEREST

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

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Formation of an *N-oxide* metabolite following metabolism of 1-(3-chlorobenzyl)[1,2,4] triazolo[4,3-a]quinoxaline by *in vitro* rat liver microsomal preparations

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ABSTRACT

Metabolic formation of *N*-oxides has always been important because of their biological activity profiles. Many *N*-oxide derivatives today are registered on the market for their diverse clinical use. Tertiary amines and ring nitrogens are main structures in drugs and xenobiotics for metabolic production of *N*-oxides in biological systems. Recently a new class of quinoxaline derivatives were synthesized and their anti-inflammatory activity was studied. In the present study, we studied *in vitro* microsomal metabolism of 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline (substrate) selected as the most active compound out of these quinoxaline derivatives using rat liver microsomes. The preliminary results from LC-MS experiments revealed that this substrate underwent *N*-oxidation in the presence of microsomes and co-factors.

Keywords: *in vitro* metabolism, *N*-oxide, 1,2,4-triazolo[4,3-*a*]quinoxaline

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INTRODUCTION

Medicinal chemistry held diverse structures as drug molecules throughout history. Heterocyclic ring systems are one of the leading structures for drug development research. Since their biological activities have tremendous importance on pharmacological treatments, their metabolic routes could always have a probability of producing active/toxic chemicals during biotransformation. Heterocyclic compounds with nitrogen are formerly thought to be substrates of *N*-oxide metabolites and are believed to have no further use in medicine [1]. After the development of minoxidil and chlordiazepoxide, these compounds gained importance in drug research [2]. This discovery had led to the development of many active *N*-oxide compounds from antiviral to antifungal activities [3-8]. *N*-oxides today keep an important place as metabolites of tertiary nitrogens and ring nitrogens in heterocyclic structures. They can sometimes be biologically active. *N*-oxides are known to be biologically unstable molecules and most of the time they revert back to its original compound. The instability of *N*-oxides sometimes create challenge in research and development studies[9]. Recent studies presented the formation of *N*-oxide metabolites from tertiary amines. Metabolic *N*-oxide formation depends on NADPH and species differences (rats, pigs, humans, etc.) [10]. An *N*-Oxide metabolite of lignocaine was observed following *in vitro* metabolism by rat liver microsomes [11]. Phenanthridine, a fused azaheterocyclic ring was also reported to produce the corresponding *N*-oxide by rat hepatic microsomes [12]. The *in vitro N*-oxidation of isomeric aromatic diazines to the corresponding *N*-oxides were also reported [13].

Recently, Doğan and co-workers carried out an important study on anti-inflammatory activity of novel quinoxaline derivatives. Quinoxaline hydrazine and iminoester derivatives were used in a cyclo-condensation reactions. Compounds were screened for their anti-inflammatory and cytotoxic activities using MTT assay. Among the synthesized compounds, 3-chlorobenzyl derivative was found to show the best inhibition on nitrite-reducing effect with 65 % compared to the standard. Therefore, we aimed to study the compound's metabolic profile on rat liver microsomes *in vitro* [14].

METHODOLOGY

General

All the chemicals were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO). An Agilent 1260 Infinity II LC-MS spectra equipped with G7114A 1260DAD detector, G7311B 1260 Quad Pump system, G1328C 1260 manual injection unit and G6125B LC/MSD detector was used for both

HPLC and mass analysis. Retention times were recorded with ACE C18 column (particle size: 3 µm, pore size: 100Å). The column temperature was adjusted to 25°C in the column compartment. The mobile phase consisted of acetonitrile-water (60:40, v/v) mixture and was delivered at a flow rate of 0.6 ml/min. The injection volume was 20 µL. The UV detector was operated at 254 nm.

Chemistry

The substrate was synthesized previously by Doğan et al. [14] Figure 1.

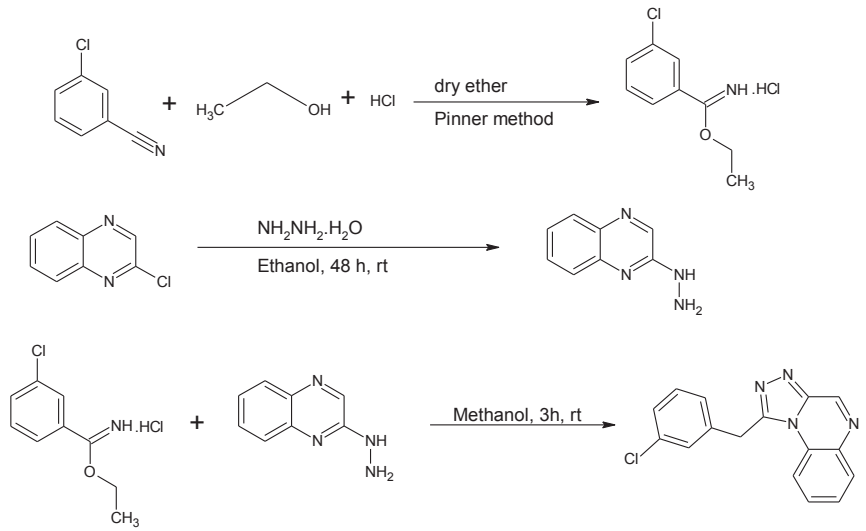


Figure 1. Synthesis method for the substrate by Doğan et al.

Preparation of rat liver microsomes and incubation/extraction procedures

Wistar albino rat liver microsomes were used in this study. ß-Nicotinamide dinucleotide phosphate (disodium salt, NADP) and glucose-6-phosphate (disodium salt, G-6-P) were purchased from Sigma. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml; G-6-PD) was obtained from Sigma Aldrich. Dichloromethane was obtained from Merck. The animals were deprived of food overnight prior to sacrifice, but were allowed water ad libitum. They were previously fed on a balanced diet. Hepatic washed rat microsomes were prepared as described by Schenkman and Cinti [15] and Ulgen [16]. Incubations were carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of NADP (2 µmole), G-6-P (10 µmole), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 µmole) in phosphate

buffer (0.2M, pH 7.4, 2 ml) at pH 7.4 (Table 1). Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmole) in methanol (50 μl). Briefly, seven test tubes for each substrate [S] were prepared (3 for test, 4 for controls) and co-factors (2ml in each tube), microsomal fraction (1 ml for each tube) and substrate (50 μl for each tube) were added respectively (Table 1). The incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in methanol (200 μl) for LC-MS. The reconstituted extracts were analyzed using the reverse-phase LC-MS system described in the text.

Table 1. Contents of the co-factor solution for each tube

NADP disodium	1.57 mg	2 μmole
G6P disodium	3.04 mg	10 μmole
G6P dehydrogenase	1.40 μl	1 unit
$MgCl_2$ (50% w/w)	8.00 μl	20 μmole

The materials above were prepared right before incubation, by dissolving in 2 mL of phosphate buffer for one incubation tube. The G-6-P dehydrogenase enzyme was added to the co-factor solution right before pre-incubation. All co-factors were "pre-incubated" at a 37°C water bath for 5 minutes, to cause NADPH creation. The amount of microsomal preparation added into each incubation tube was 1 ml, and the co-factor solution, was 2 ml. Control tubes were also prepared (Table 2).

Table 2. Incubation protocol

Test Tube	No	Substrate	Microsome	Co-factor
Test	1	+	+	+
Test	2	+	+	+
Test	3	+	+	+
Control	4	+	Denatured	+
Control	5	+	Denatured	+
Control	6	+	+	Buffer
Control	7	+	+	Buffer
		50 ml in each tube	1 ml in each tube	2 ml in each tube

Denaturation of microsomes

For control experiments, microsomes were denaturized using boiling water. The necessary amount of freshly de-frosted microsomes were taken in a test tube and it was placed in boiling water for 5 mins. After the heat-denaturation, the denaturized microsomes were used for control experiments.

Extraction of substrates and metabolites from the biological system

At the end of the incubation period, the tubes having the unchanged substrate and metabolites, were placed immediately on an ice bath. The enzymatic process was stopped with the addition of dichloromethane, extracted and evaporated under nitrogen. The extracts were analyzed by LC-MS.

LC-MS analysis

An acetonitrile/water (60:40, v/v) mobile phase mixture was used. The substrate and metabolically formed *N*-oxide were separated according to their mass/charge ratio and their molecular ion peaks were determined in the mass spectroscopy section and the retention times (*Rt*) of the substrate and metabolic standards were recorded. A DAD detector was also used to compare UV spectra of standard and metabolic products.

Autoxidation studies

The substrate (2 μM) was dissolved in methanol (50 μl). Then, phosphate buffer (0.2 M, pH 7.4) (3ml) was added in the same incubation conditions with test experiments. The incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in methanol (200 μl) for LC-MS. The reconstituted extracts were analyzed using the reverse-phase LC-MS system described in the text.

RESULTS and DISCUSSION

The aim of this present study is first to observe and prove any qualitative *in vitro* microsomal metabolite formation of the anti-inflammatory drug candidate, 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline. The only possible metabolite formation from the substrate was hydroxylation of aromatic ring, or oxidation of ring nitrogen.

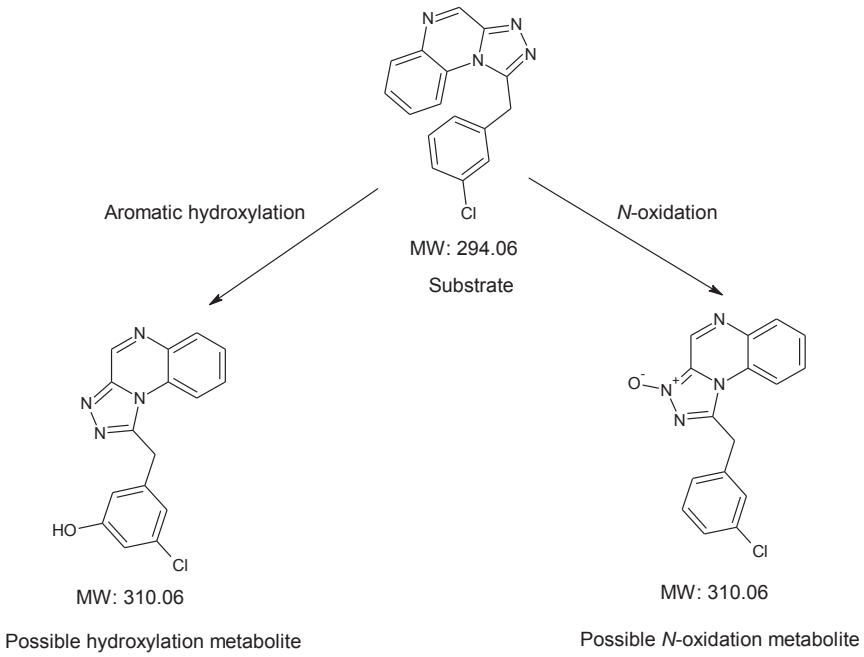


Figure 2. Potential metabolic pathways of the substrate

LC-MS

Several attempts were made in order to analyze substrate with LC-MS. Acetonitrile/water (60:40, v/v) was found to be the best mobile phase for the LC-MS analysis of substrate and therefore thought to be give the best retention time for the analysis of any possible oxidative metabolites (Figure 3).

Following the metabolic study of the substrate, the *N*-oxide formation was only observed in test experiments and not in control experiments. The metabolite was more polar than the substrate. It was clearly understood from this study that the compound undergoes *N*-oxidation in the presence of NADPH.

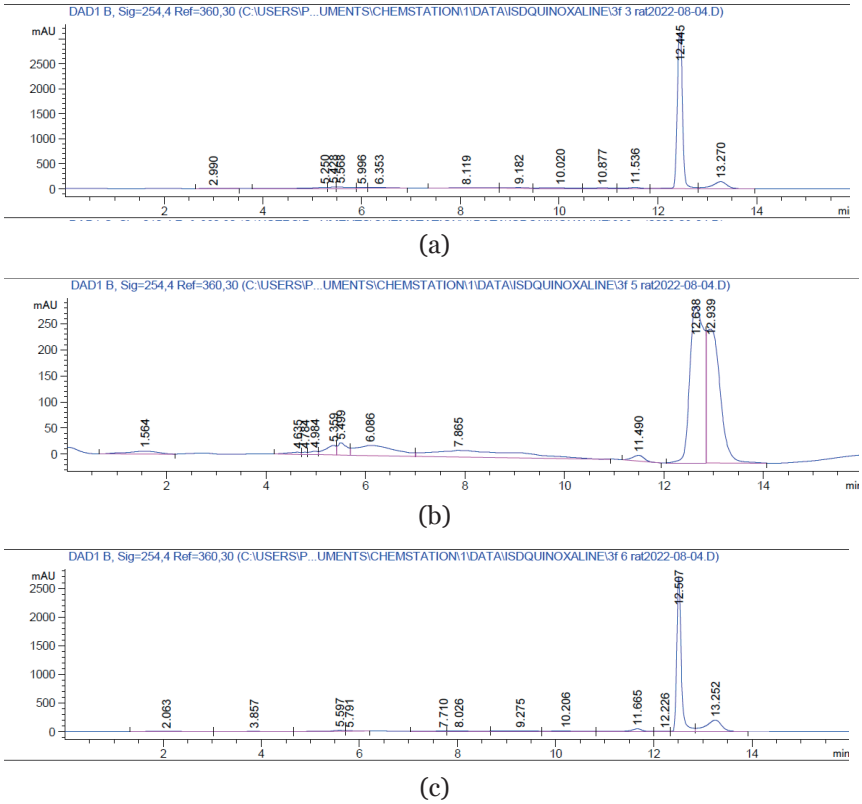
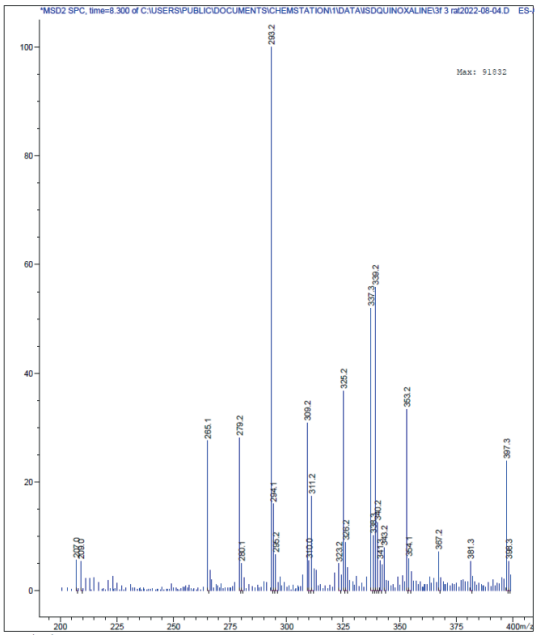


Figure 3. HPLC chromatogram obtained from (a) metabolism of substrate (in the presence of microsomes and co-factors) (Rt (min) metabolically formed N-oxide: 8.11, substrate:12.44 and all other peaks resulted from microsomal environment); (b) control with boiled (denatured) microsomes (Rt (min) substrate: 12.63-12.93 and all other peaks resulted from microsomal environment) and (c) control with no co-factor (Rt (min) substrate: 12.50 and all other peaks resulted from microsomal environment) with rat microsomal preparations

The MS spectrum for all samples revealed the formation of *N*-oxide metabolite. The m/z peaks in the spectrum were detected in negative ions in 8.3 min. The same spectrum was not detected in all the control experiments. The results revealed that the *N*-oxide formation can only be observed with both microsomes and co-factors in biological conditions (Figure 4-5).


*MSD2 SPC, time=8.300 of C:\USERS\PUBLIC\DOCUMENTS\CHEMSTATION\1\DATA\ISDQUINOXALINE\3f 3 rat2022-08-04.D ES-
Max: 91832
293.2
265.1
279.2
309.2
325.2
337.3
339.2
353.2
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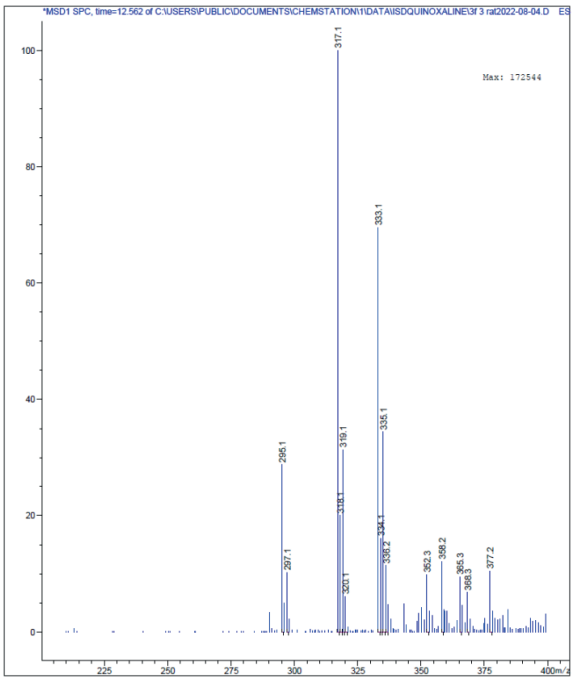
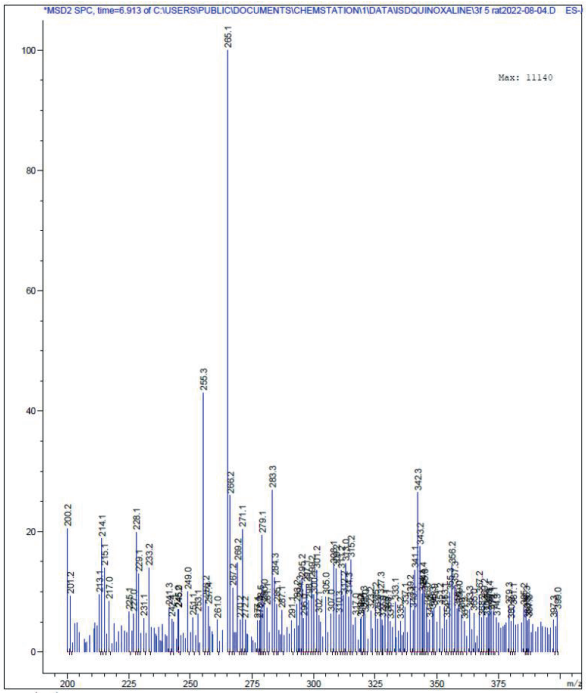
294.1
311.2
280.1
323.2
364.1
367.2
381.3
386.3
343.2

(a)


*MSD1 SPC, time=12.562 of C:\USERS\PUBLIC\DOCUMENTS\CHEMSTATION\1\DATA\ISDQUINOXALINE\3f 3 rat2022-08-04.D ES
Max: 172544
317.1
333.1
295.1
319.1
336.1
318.1
334.1
297.1
338.2
352.3
356.2
365.3
377.2
321.1
368.3

(b)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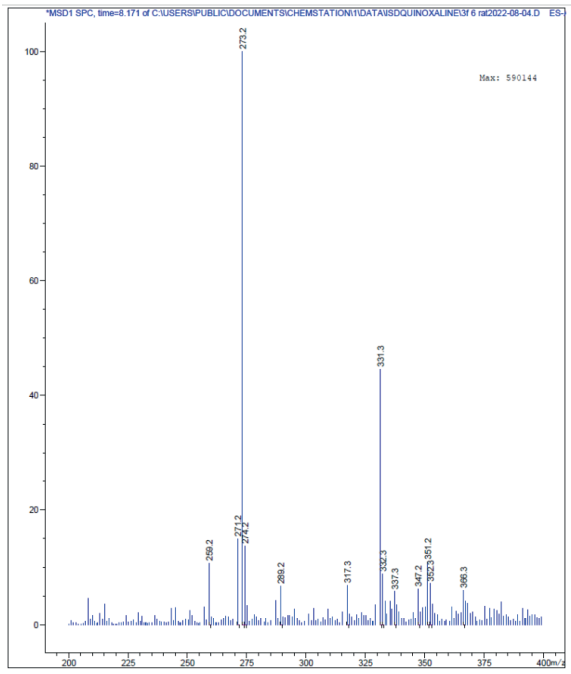
Max: 11140
265.1
255.3
342.3
283.3
200.2
214.1
228.1
271.1
279.1
233.2
m/z

(c)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Max: 590144
273.2
331.3
271.2
274.2
289.2
317.3
332.3
337.3
347.2
351.2
366.3
400m/z

(d)

Figure 4. Mass Spectrum results; (a) Mass Spectrum of substrate test (min 8.3, negative ion 309 was observed for *N*-oxide metabolite); (b) Mass spectrum of substrate test (min 12.56, positive ion 295 was observed for substrate); (c) Mass spectrum of substrate control with denaturized microsomes (no *N*-oxide ion peak was observed); (d) Mass spectrum of substrate control with no co-factor (no *N*-oxide ion peak was observed)

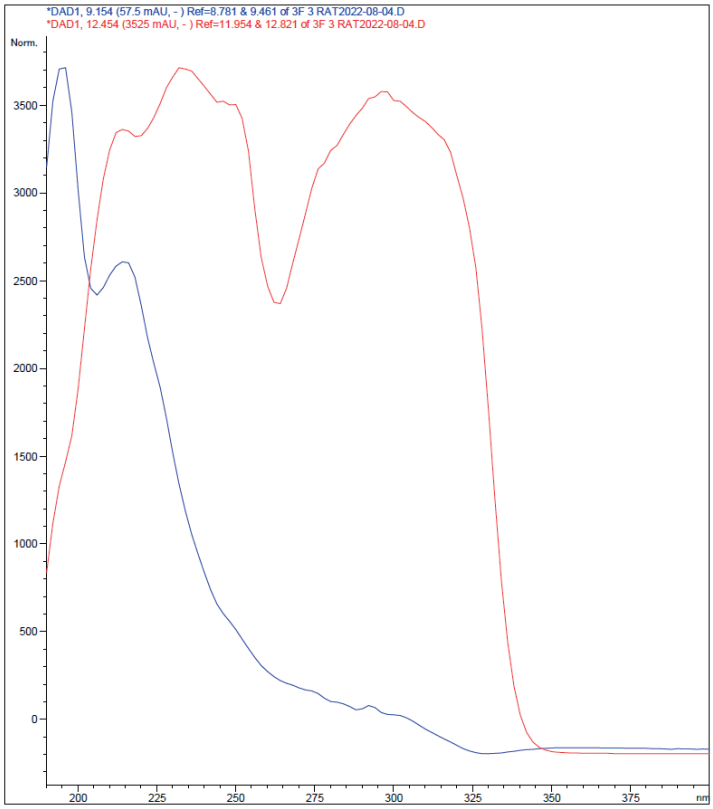
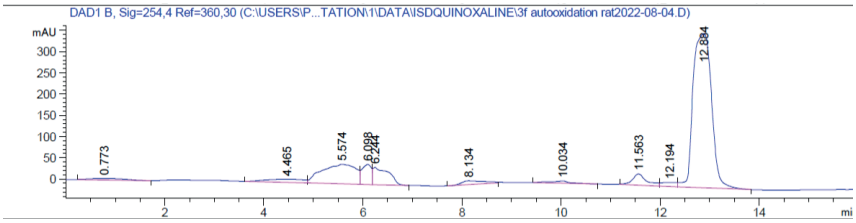
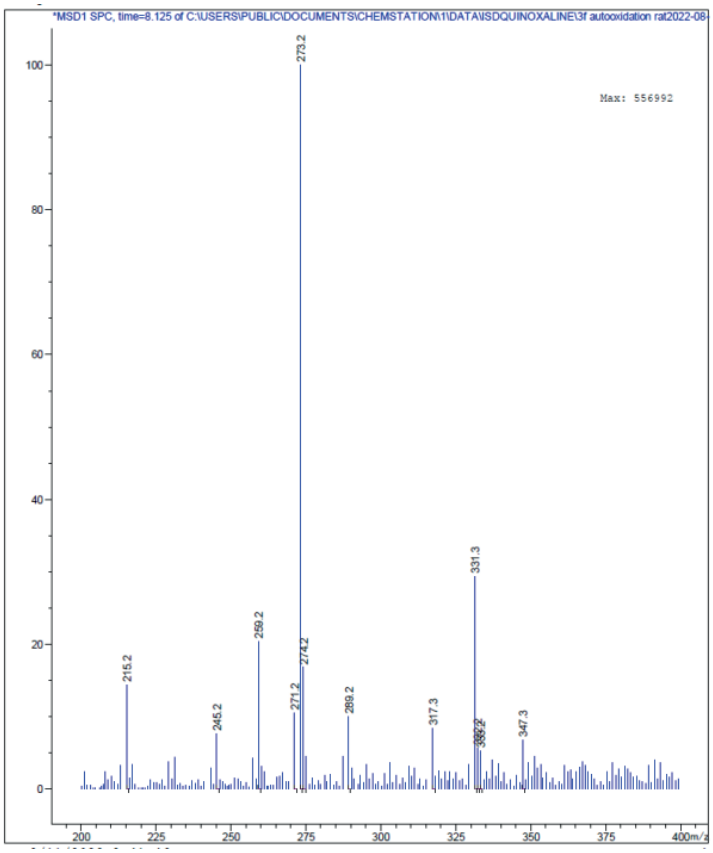


Figure 5. Comparison of UV spectra of metabolically formed *N*-oxide and substrate

In order to understand the metabolic profile of the substrate, an autoxidation experiment was also performed. The results indicated that the substrate was stable in buffer. No oxidation or any other metabolite was detected in LC-MS analysis.



(a)



(b)

Figure 6. Autoxidation of substrate with buffer (a) HPLC chromatogram of autoxidation of substrate with buffer; (b) Mass spectrum of autoxidation of substrate with buffer in min 8 (no *N*-oxide was observed)

The current preliminary study indicated that the drug candidate 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline (substrate) was converted into the corresponding *N*-oxide by rat liver microsomes *in vitro*. The LC-MS results indicated that the compound most probably undergoes metabolic *N*-oxidation rather than hydroxylation (Figure 3-4). The amount of the metabolically formed *N*-oxide was relatively low, therefore the *N*-oxide formation was more favorable than aromatic hydroxylation. As a result, we assumed that *N*-oxidative product was formed in the presence of NADPH. The UV spectrum of this *N*-oxide was also different from that of the substrate (Figure 5). The autoxidation experiment showed that the substrate was stable in buffer (Figure 6). No further metabolite was detected in LC-MS analysis. Experiments are under way to synthesize the corresponding *N*-oxide and further confirm its metabolic formation by comparison.

Ethics Approval and Consent to Participate

The rat livers were donated by Acibadem University, Animal Laboratory Centre from the Project by Dr. Mehmet Emin Aksoy; laparoscopic and robotic surgery, with the 2021-01 ethical approval number. The liver tissue was obtained from the euthanized rats at the end of course.

Human and Animal Rights

No humans were used in this study. All animal research procedures were followed in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) and/or the declaration of Helsinki promulgated in 1964 as amended in 1996.

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