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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

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The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part.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 and Reviews.

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

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

1.3 Prior Publication

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

1.4 Patents and Intellectual Property

Authors need to resolve all patent and intellectual property issues. Acceptance

and publication will not be delayed for pending or unresolved issues of this type. Note that Accepted manuscripts and online manuscripts are considered as published documents.

1.5 Professional Ethics

Editors, reviewers, and authors are expected to adhere to internationally accepted criteria's for scientific publishing.

1.5.1 Author Consent. Submitting authors are reminded that consent of all coauthors must be obtained prior to submission of manuscripts. If an author is removed after submission, the submitting author must have the removed author consent to the change by e-mail or faxed letter to the assigned Editor.

1.5.2. Plagiarism. Manuscripts must be original with respect to concept, content, and writing. It is not appropriate for an author to reuse wording from other publications, including one›s own previous publications, whether or not that publication is cited. Suspected plagiarism should be reported immediately to the editorial office. Report should specifically indicate the plagiarized material within the manuscripts. Acta Pharmaceutica Sciencia uses iThenticate or Turnitin software to screen submitted manuscripts for similarity to published material. Note that your manuscript may be screened during the submission process.

1.5.3. Use of Human or Animal Subjects. For research involving biological samples obtained from animals or human subjects, editors reserve the right to request additional information from authors. Studies submitted for publication approval must present evidence that the described experimental activities have undergone local institutional review assessing safety and humane usage of study subject animals. In the case of human subjects authors must also provide a statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the studies' Material and Methods.

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.

The responsibility for all aspects of manuscript preparation rests with the authors. Extensive changes or rewriting of the manuscript will not be undertaken by the Editors. A standard list of Abbreviations, Acronyms and Symbols is in section 5.

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

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

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

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

Authors may find the following sources useful for recommended nomenclature:

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

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

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

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

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

· Once in the abstract.

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

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

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

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

2.2 Manuscript Organization

2.2.1 Title Page. Title: The title of the manuscript should reflect the purposes and findings of the work in order to provide maximum information in a

computerized title search. Minimal use of nonfunctional words is encouraged. Only commonly employed abbreviations (e.g., DNA, RNA, ATP) are acceptable. Code numbers for compounds may be used in a manuscript title when placed in parentheses AFTER the chemical or descriptive name.

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

2.2.2 Abstract and keywords. Articles of all types must have an abstract following the title page. The maximum length of the Abstract should be 150 words, organized in a findings-oriented format in which the most important results and conclusions are summarized. Code numbers may be used once in the abstract.

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

2.2.3 Introduction. The rationale and objectives of the research should be discussed in this section. The background material should be brief and relevant to the research described.

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. Number literature references and notes in one consecutive series by order of mention in the text. Numbers in the text are nonparenthesized superscripts. The accuracy of the references is the responsibility of the author. List all authors; do not use et al. Provide inclusive page numbers. Titles may have capitalization of first word only (excluding, for example, acronyms and trade names) or standard capitalization as shown below. The chosen style should be used consistently throughout the references. Double-space the references using the following format.

· For journals: Rich, D. H.; Green, J.; Toth, M. V.; Marshall, G. R.; Kent, S. B. H. Hydroxyethylamine Analogues of the p17/p24 Substrate Cleavage Site Are Tight Binding Inhibitors of HIV Protease. *J. Med. Chem.* **1990**, *33*, 1285-1288.

· For online early access: Rubner, G.; Bensdorf, K.; Wellner, A.; Kircher, B.; Bergemann, S.; Ott, I.; Gust, R. Synthesis and Biological Activities of Transition Metal Complexes Based on Acetylsalicylic Acid as Neo-Anticancer Agents. *J. Med. Chem.* [Online early access]. DOI: 10.1021/jm101019j. Published Online: September 21, 2010.

· For periodicals published in electronic format only: Author 1; Author 2; Author 3; etc. Title of Article. *Journal Abbreviation* [Online] **Year**, *Volume*, Article Number or other identifying information.

· For monographs: Casy, A. F.; Parfitt, R. T. *Opioid Analgesics*; Plenum: New York, 1986.

· For edited books: Rall, T. W.; Schleifer, L. S. Drugs Effective in the Therapy of the Epilepsies. In *The Pharmacological Basis of Therapeutics*, 7th ed.; Gilman, A. G., Goodman, L. S., Rall, T. W., Murad, F., Eds.; Macmillan: New York, 1985; pp 446-472

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.

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 dropdown 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 re-

quired. 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 150. 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 infor-

mation and bridges related to the sent articles, revision required articles and the articles that are not completed to be sent.

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

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

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

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

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

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

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.

ORIGINAL ARTICLES

Acta Pharm. Sci. Vol 58 No: 2. 2020
DOI: 10.23893/1307-2080.APS.05808

Antipsychotic effects of *Philenoptera cyanescens* (Schum. & Thonn.) Roberty (Leguminosae) Leaf Extract and Fractions against Ketamine-induced Psychosis in Mice

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2 NeuroPharmacology Unit, Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan

ABSTRACT

This study investigated the antipsychotic potentials of *Philenoptera cyanescens* (PC) methanol extract and fractions against ketamine-induced psychosis in Balb/C mice.

The methods used were the hyper-locomotion, stereotype behaviour, Y-maze and Forced Swim Test models. The extrapyramidal effect of the active fractions was tested using the catalepsy model.

The crude extract and all fractions at varying doses significantly reduced ketamine-induced hyperactivity and stereotype behaviour with dichloromethane fraction being the most active compared with the standards. Dichloromethane and ethyl acetate fractions (250 mg/kg) reversed the cognitive impairments enhanced by ketamine at the 1st, 5th, and 10th day of the experiments and also decreased the immobility time in the FST model at 11th and 15th days post treatment. The two fractions did not induce catalepsy and also inhibited the cataleptic effect induced by haloperidol.

The results established that *Philenoptera cyanescens* possesses antipsychotic effect against positive, negative and cognitive symptoms of psychosis.

Keywords: *Philenoptera cyanescens,* Antipsychotic activity, Hyper-locomotion, Stereotypy, Forced swim test

INTRODUCTION

Psychosis is a mental health problem involving loss of contact with reality. It affects as many as 450 million people worldwide, about a million people en-

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danger their lives by committing suicide every year and one out of four families has one member with mental disorder[1]. Its symptoms include schizophrenia, hallucination, social withdrawal, incoherent behaviour/speech, difficulty in concentration, anxiety, depressed mood, and suicidal thoughts. Despite the burden of this illness to the society, the treatment is still poor among patients, hence, patients especially from less developed countries like Nigeria prefer to seek treatment from traditional healers. They believe traditional medicine is safer, less expensive and easily assessable than orthodox antipsychotic drugs such as haloperidol, chlopromazine (typical antipsychotics), risperidone and clozapine (atypical antipsychotics). The long-term use of these drugs results into extra pyramidal effects, high oxidative damage[2], and this could result in agranulocytosis, cardiovascular disorders, diabetes and some other severe conditions[3-5].

The models (Hyper-locomotion, stereotypy, Y-maze test, Forced Swim test and catalepsy) used in this study have been reported to mimic psychosis in human. Locomotion is mostly increased by central nervous system (CNS) stimulants and reduced by CNS depressants[6]. Enhanced locomotory activity has been linked to the dopaminergic hyper activation in striatal areas of mouse brain[7]. Stereotypy mostly seen in people with mental disorder is usually in the form of repetitive performance of a set of strange behaviours such as making the same kind of comments or asking the same questions[8, 9]. In experimental animals, it is usually observed as persistent sniffing, abnormal sideways movements of the head, intense licking, and chewing behaviours[10]. Cognitive dysfunction such as attention deficits, short- and long-term working memory are symptoms seen in psychotic patients[11]. The Y-maze test has previously been used to investigate the effectiveness of antipsychotic drugs on memory and learning function in rodents[12]. It involves measuring the spatial working memory, which is based on rodent's willingness to explore new environments. Rodents prefer to explore new arm of the maze, rather than return to a previously visited arm. In addition, chronic administration of ketamine has been reported to enhance immobility in forced swim test and used as a model for negative symptoms of psychosis[13] such as avolition (severe decrease in motivation to initiate and perform self-directed purposeful tasks). Catalepsy has been used to predict safe tranquillizing activity and also to access motor effects of drugs, especially effects related to extrapyramidal symptoms (EPS) [14]. It is one of the major side effects related to the use of orthodox antipsychotic drugs[15]. The EPS has been linked to the decrease of striatal dopamine D1 and D2 receptors[16, 17]. The great burden of the various side effects associated with the use of antipsychotics has therefore led to a continuous search for novel and well tolerated molecules

from natural resources to control severe mental disorders[18-22].

High chemical diversity, biochemical specificity, and several other medicinal properties such as anti-inflammatory, anti-psychotic, antioxidant properties among others are characteristics of natural products, which enable them to possess favourable lead structures for the treatment of various disorders, including psychosis. Some plants like *Hypericum perforatum* L. (Saint John's Wort) [23-27], *Psidium guajava* L.[28], *Newbouldia laevis* Seem. [29], *Bacopa monniera*[30-32], *Synedrella nodiflora*[33] and many others have been screened for their neuroleptic activities. In line with this, *Lonchocarpus cyanescens* (Schumach and Thonn.) Benth. now called *Philenoptera cyanescens* (PC) (Schum. & Thonn.) Roberty, is a medicinal plant used traditionally in south western Nigeria to treat mental disordered patients[42], and we have reported the antipsychotic properties of this plant in amphetamine and apomorphine-induced psychotic models[34, 35]. Both amphetamine and apomorphine used in the study to induce psychosis work by blocking the D_2 receptor (positive symptoms only). In this study, the antipsychotic potential of the crude extract and fractions of *P. cyanescens* was investigated on ketamine-induced psychosis in mice. Ketamine is known to affect positive, negative and cognitive functions of the brain[36] alongside the extrapyramidal side effects usually found in orthodox antipsychotics. Since PC possesses antipsychotic properties against apomorphine and amphetamine induced psychosis, our hypothesis is that it may be effective against ketamine models of psychosis. Thus, the present study investigates the effect of the extract and fractions of *P. cyanescens* against positive, negative and cognitive symptoms in ketamine-induced experimental psychosis in mice.

METHODOLOGY

Preparation of crude extract and fractions

Philenoptera cyanescens leaves were collected at Kajola, Ibadan, Oyo State, Nigeria and identified at the Forest Herbarium Ibadan (FHI) by Dr. O. A. Osiyemi of the Forest Research Institute of Nigeria where specimen was deposited with voucher number FHI 109689. Shade-dried whole plant (3 kg) was pulverized and macerated using methanol for 72 h. The methanol extract was filtered and concentrated *in vacuo* at 40°C. The residue was re-extracted twice for optimum yield of the extract. The crude extract was dissolved in methanol: water (1:1) and partitioned into HEX, DCM, EtOAc, BuOH and aqueous (Aq) fractions. The crude extract and fractions of PC were stored in the refrigerator until ready for use.

Experimental Animals

Balb C albino mice of both sex between 7 – 9 weeks old (30-35 g) used for the study were purchased from National Institute of Health (NIH), Islamabad, Pakistan. They were housed ten to twelve per cage (L X B X H: 16 cm x 10 cm x 6 cm) at room temperature, and 12 h to 12 h light-dark cycle (8:00 a.m-8:00 p.m.). The cages were lined with soft wood shavings, used as beddings to absorb waste products from the animals, and changed after 3 days. They were allowed to acclimatize for minimum of one week before the start of experimental procedures. The animals were also acclimatized to each of the experimental procedures after which they were randomly distributed into different treatment groups (n = 6 per group, in ratio 1:1 of both male and female animal) of both sex. There were eighteen experimental groups and three control groups for the acute study, as shown in Table 1. They had free access to constant food pellets and water *ad libitum*. The experiments were performed after approval of the protocol by the Ethics Committee of the COMSATS Institution of Information Technology, Abbottabad, Pakistan, according to the National Institutes of Health Guide for care and use of laboratory animals.

Drugs and Treatments

Ketamine was purchased from Indus Pharma, Karachi, Pakistan, haloperidol (injection) from The Searle Company Limited, Karachi, Pakistan and risperidone (risperidal tablet) from West-Coast Pharmaceutical, India. Haloperidol and risperidone (0.2 mg/kg) were used so as to compare the effect of the plant extract and fractions with typical and atypical antipsychotics, respectively. All drugs (plant extracts) were administered orally at a dose of 125, 250 and 500 mg/kg; these doses were used based on the results obtained from preliminary studies. Experiments were carried out in the laboratory, between the hours of 8 a.m. – 4 p.m., using different observation chambers as required by each model.

Table 1. Animal Grouping

Group	Treatment
1	Ketamine (30 mg/kg) + Distilled water (10 mL/kg)
2	Ketamine (30 mg/kg) + Haloperidol (0.2 mg/kg)
3	Ketamine (30 mg/kg) + Risperidone (0.2 mg/kg)
4,5,6	Ketamine (30 mg/kg) + PC crude extract (125, 250, 500 mg/kg)
7,8,9	Ketamine (30 mg/kg) + PC Hexane (125, 250, 500 mg/kg)
10,11,12	Ketamine (30 mg/kg) + PC Dichloromethane (125, 250, 500 mg/kg)
13,14,15	Ketamine (30 mg/kg) + PC Ethyl acetate (125, 250, 500 mg/kg)
16,17,18	Ketamine (30 mg/kg) + PC Butanol (125, 250, 500 mg/kg)
19,20,21	Ketamine (30 mg/kg) + PC Aqueous (125, 250, 500 mg/kg)

Behavioural Assays

Hyper-locomotion and stereotypy were performed based on previously described methods[35, 36], Forced Swim test and Y-maze test were performed according to described methods[36, 14].

Hyper-locomotion in mice

The open field test model was employed to observe hyper-locomotion in animals. The total number of animals used (126) was obtained by the formula; $Ta = Na \times Ng$,

Where *Ta* is the total number of animals, *Na* is the number of animal per group and *Ng* is the number of groups in the study.

The chamber was made of wood with dimensions of 35cm x 30cm x 23cm, Here, animals (n = 6 per group) were administered with crude extract, HEX, DCM, EtOAc, BuOH or Aq fractions (125 - 500 mg/kg) of PC orally 1 h before the injection of ketamine (30 mg/kg, i.p), thereafter; they were placed at the centre of an open field chamber (35cm x 30cm x 23cm), immediately after ketamine injection. The number of lines crossed and time at which it did not move (time of ambulation) were recorded for 5 min using a mobile camera.

Stereotype behaviour in mice

The antagonistic effect of the crude extract and fractions of PC were tested on ketamine-induced stereotype behaviour using the same mice that were used for hyper-locomotion study. Animals were placed in transparent observation chamber (L X B X H: 16 cm x 10 cm x 6 cm) 5 min after ketamine injection

immediately after the hyper-locomotion experiment. Thereafter, stereotype parameters were observed and recorded for a period of 2 min at 5, 10, 15, 20, 30, 45, and 60 min, respectively. Stereotypy parameters were scored as 0 = absence of stereotypy parameters, 1 = presence of stereotyped movements of the head, 2 = intermittent sniffing, 3 = chewing, and 4 = intense licking.

Forced Swim Test (FST)

The forced swim test was carried out on the bioactive fractions, PC DCM and PC EtOAc. It is a measure of behavioural despair and was carried out according to[36] with little modification. In this test, mice were treated with distilled water, haloperidol 1 mg/kg, risperidone 0.2 mg/kg, p.o., PC DCM (125, 250 and 500 mg/kg, p.o.), and PC EtOAc (125, 250 and 500 mg/kg, p.o.), 60 min before administration of ketamine 30 mg/kg, i.p. for 10 days. FST was then performed 24 h after the last treatment day. Effect of chronic treatment of the fractions and standards were also tested at day 15 after withdrawal. Briefly, mice were placed individually in the center of a transparent glass cylinder (height – 23 cm, diameter – 40 cm) which contained water at room temperature to a depth of 16 cm and acclimatized for 2 min (trial phase), 24 h after the trial phase, mice were again placed in the cylinder, and swimming activity was recorded for 5 min after 1 min of acclimatization. Duration of immobility was observed and recorded; mice were considered immobile when they were floating motionless in water.

Y-maze Test

The effect of the bioactive fractions of PC (DCM and EtOAc) on cognitive dysfunction by measuring the correct alternation were tested in the Y-maze paradigm in mice, according to[14, 19], with little modification. Sixty mice were divided into (and treated with) control group (distilled water 10 mL/kg, p.o.), negative control group (ketamine 30 mg/kg, i.p.), positive control groups (haloperidol 1mg/kg and risperidone 0.2 mg/kg, p.o.), PC DCM (125, 250 and 500 mg/kg, p.o.), and PC EtOAc (125, 250 and 500 mg/kg, p.o.) groups, containing six mice per group, with an hour interval between the oral and intraperitoneal administration. Treatments were given for 10 days and behavioural experiment was carried out at day 1, day 5 and day 10. Three wooden arms (Length 30 cm x Breadth 5 cm x Height 10 cm), symmetrically separated at 120° each was used for the study. On day 1, animals were placed at the closed end of arm A, 1 hour after treatment, and allowed to explore all the three labelled arms (A, B, C) freely for 5 min, whereas on day 5 and day 10, experiments were carried out after 24 h of treatment. Parameters recorded were sequence of alternation and number of arm entry. The Y-maze apparatus was cleaned with 70% ethanol to remove residual odour after each mouse session.

Percentage correct alternation was calculated using the formula:

Sequence of alternation / number of arm entry – 2 multiplied by 100.

Catalepsy

The cataleptic side effects of antipsychotic drugs have been routinely measured using the standard bar test[37]. Haloperidol (1.0 mg/kg. i.p) was used to induce catalepsy and the time (sec) the mouse maintain an imposed position with both forepaws resting on a 4 cm high glass horizontal bar (1.0 cm diameter) was recorded. Time recording was terminated when both front paws were removed from the bar or if the mouse moves its head in an exploratory way, and a cut-off time of 5 min was applied. Observations were taken at 0, 30, 60, 90, and 120 min after administration of drug[38, 39]. Group 1 received distilled water (10 mL/kg, positive control), group 2 received haloperidol (1 mg/kg; i.p, negative control) only, groups 3 and 4 received either DCM or EtOAc fractions of PC (125 mg/kg and 500 mg/kg; p.o), groups 5 and 6 received either haloperidol (1 mg/kg; i.p) with DCM fraction or haloperidol (1 mg/kg) with EtOAc fraction at lower (125 mg/kg) and higher doses (500 mg/kg), respectively. In all, 60 mice were used for the study, derived from the formula above.

Statistical analysis

One and two – way analysis of variance (ANOVA) was employed to compare all groups against the negative control group after which Dunnett's and Bonferroni post hoc test was used. Values were presented as mean ± standard error of mean, and a p-value of 0.05, 0.01 or 0.001 was considered statistically significant.

RESULTS AND DISCUSSION

Inhibition of ketamine induced hyper-locomotion by crude extract of PC

The group of mice that received crude extract of PC showed reduction in the locomotion activity in ketamine-induced psychotic mice at 125 mg/kg *b.w*. when compared with the mice induced with ketamine only (negative control group) (Figure 1). The antagonism of hyper-locomotion at a lower dose of 125 mg/kg encouraged us to go further by fractionating the crude extract and tested the different fractions on locomotion and other models of psychosis.

All the doses of the hexane and DCM fractions of PC (125 mg/kg, 250 mg/kg, and 500 mg/kg) significantly reduced the hyper-locomotion (PC Hex $F_{3,20} = 12.57$, *** $P < 0.001$, $R^2 = 65\%$, PC DCM $F_{3,20} = 13.99$, ***$P < 0.001$, $R^2 = 68\%$) induced by ketamine (30 mg/kg) with a comparable effect to the standards: haloperidol and risperidone (0.2 mg/kg) [$F_{2,15} = 28.22$, ***$P < 0.001$, $R^2 = 79\%$]. The EtOAc

fraction of PC also showed significant reduction at 250 mg/kg (*P < 0.05), while BuOH fraction was significant at 250 mg/kg and 500 mg/kg (***P < 0.001 and *P < 0.05, respectively). When compared with the negative control group, the aqueous fraction of PC did not show any significant reduction in locomotion.

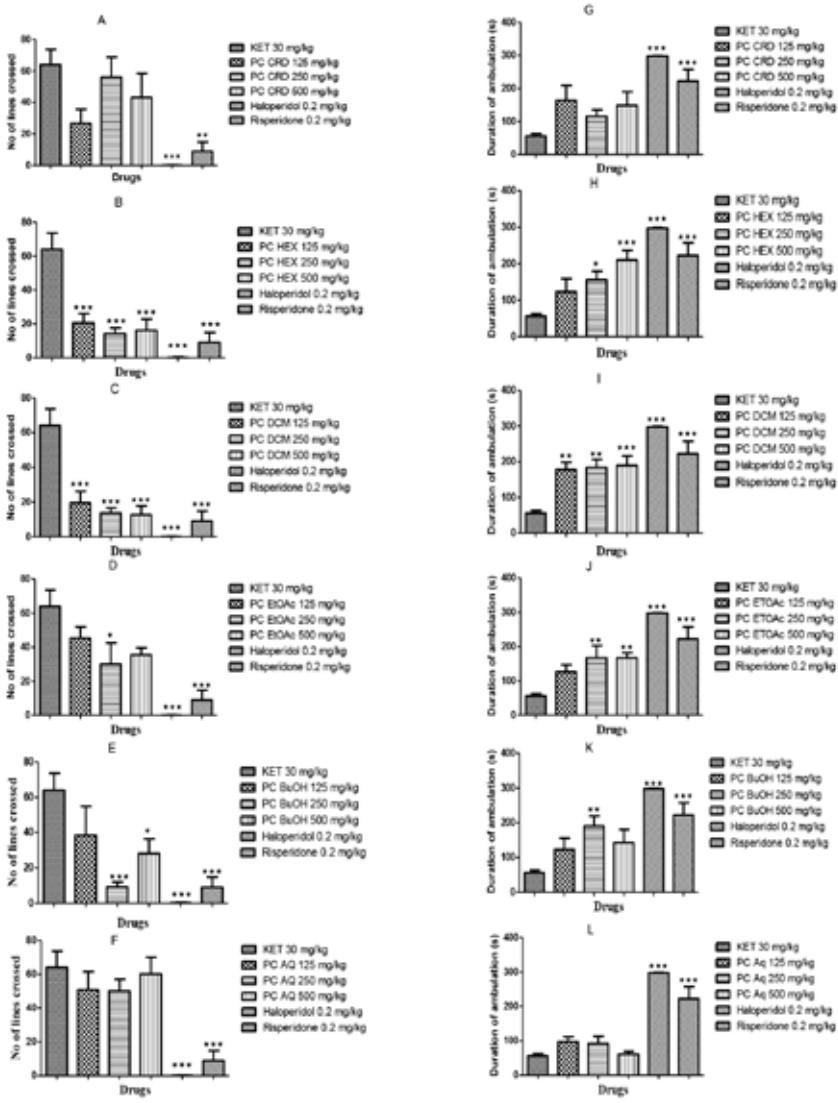

Figure 1. Number of lines crossed (A-F) and duration of ambulation (G-L) in mice administered with PC crude extract and fractions (HEX, DCM, EtOAc, BuOH and Aq, respectively; 125-500 mg/kg; orally) 1 h before ketamine (30 mg/kg; i.p.) administration. Data's were mean ± SEM (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 compared with negative control group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

Antagonism of stereotype behaviour by PC crude extract and fractions in ketamine-induced Stereotypy

Treatment with ketamine in mice is known to cause stereotypy. Ketamine-induced stereotype behaviour is characterized by intermittent sniffing, head movement and chewing. The group of animals treated with PC crude extract significantly reduced the stereotypy behaviour induced by ketamine at 500 mg/kg ($F_{3, 24}$ = 5.54, **P < 0.01), likewise haloperidol and risperidone (0.2 mg/kg) the standard typical and atypical antipsychotics, respectively (Figure 2a). The fractions of PC were also observed for their effect in antagonizing ketamine-induced stereotype behaviour, one way ANOVA results showed that Hex fraction was significant (*P < 0.05, $F_{3,24}$ = 3.31) although not significant at Dunnett's multiple comparison test when the doses were compared each with the negative control (Figure 2b). The DCM fraction significantly (***P < 0.001) antagonized stereotype behaviour in the mice and when compared with Dunnett**'s** post hoc test, it was also significant at 250 mg/kg (**P < 0.01) as shown in Figure 2c. At 500 mg/kg, the EtOAc fraction significantly (*P < 0.05) reduced stereotypy behaviour (Figure 2d). However, the BuOH fraction did not reduce stereotype behaviour significantly in the mice (Figure 2e), while Aq fraction at 125 mg/kg significantly (**P < 0.01) reduced stereotype behaviour (Figure 2f). All animals used in the study were used for analysing the result.

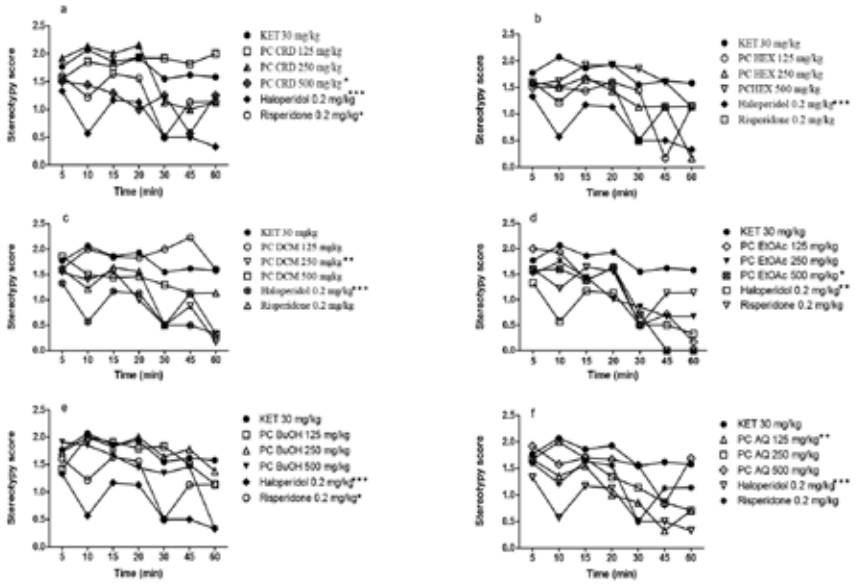

Figure 2. Effect of Philenoptera cyanescens crude extract-a, and fractions (b-Hex, c-DCM, d-EtOAc, e-BuOH and f-Aq; 125-500 mg/kg; orally), Haloperidol, Risperidone and negative control on stereotype behaviour, 5 min after ketamine (30 mg/kg, i.p) treatment in a transparent

observation chamber. Data were mean ± SEM (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001, compared with negative control group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc

Chronic pre-treatment effects of PC DCM and EtOAc on ketamine enhanced immobility time in FST

The protective chronic effects of the DCM and EtOAc fractions of PC against ketamine-induced negative symptoms of psychosis were studied, using the FST model. We observed that repeated ketamine treatment (30 mg/kg/day, i.p. for 10 days) increased the immobility time, and significantly (**P < 0.01) persisted for 5 days after ketamine withdrawal. Pre-treatment with the DCM fraction of PC (500, 250, 125 mg/kg, p.o. for 10 days) revealed significant protection against ketamine-induced immobility (***P < 0.001) on day 11, which persisted up to the 15th day (***P < 0.001) of the experiment. The EtOAc fraction also gave a dose-dependent significant [500 mg/kg, p.o. (*P < 0.05); 250 & 125 mg/kg, p.o. (***P < 0.001)] decrease in all the three doses and persisted up to the 15th day (***P < 0.001) of the experiment. Furthermore, risperidone and haloperidol treatment for 10 days significantly (***P < 0.001) reduced the duration of immobility on days 11 and 15, respectively, as against ketamine treated groups (Figure 3 a & b).

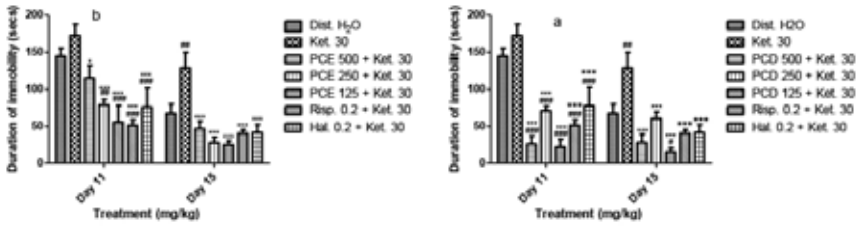

Figure 3. Effect of pre-treatment with DCM and EtOAc fractions of PC on ketamine-enhanced immobility in FST. Bar chart indicates day–dependent effect of the fractions, risperidone, and haloperidol against ketamine – enhanced immobility in mice. Data were mean ± SEM (n = 6). *P < 0.05, ***P < 0.001, compared with negative control group and #P<0.05, ##P < 0.01, ###P < 0.001, compared with distilled water (two-way ANOVA showed significant difference between interaction, treatment groups and duration of immobility among the experimental days, followed by a Bonferroni multiple comparison post hoc test)

Effect of DCM and EtOAc fractions of PC on ketamine induced cognitive dysfunction in Y-maze Test

Pre-treatment effect of PC fractions (DCM and EtOAc) on reversal of ketamine-induced cognitive dysfunction was evaluated by the number of arm entries and sequence of arm entry using Y-maze apparatus. Chronic treatment with ketamine (30 mg/kg, i.p.) induced memory impairment with a decrease in percentage correct alternation on days 1, 5 and 10, respectively as compared to the group that received distilled water only (10 mL/kg). Pre-treatment with the DCM (day 5) and EtOAc fractions of PC (day 10) at 250 mg/kg p.o. 1 h before administration of ketamine, significantly (*P < 0.05) reversed the ketamine effect by increasing the percentage correct alternation on experimental days. In addition, risperidone and haloperidol showed increase in percentage correct alternation as compared to ketamine treated groups (Figure 4a and 4b).

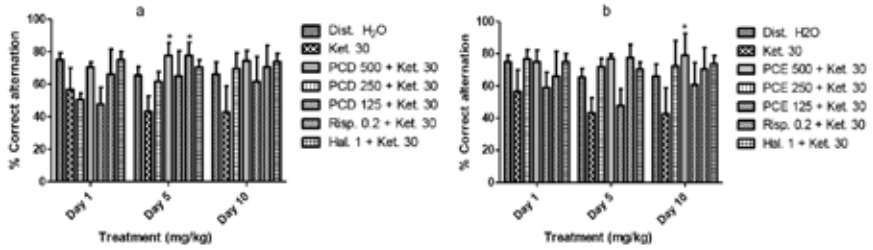

Figure 4. Effect of pre-treatment with dichloromethane and ethyl acetate fractions of PC on ketamine-induced cognitive dysfunction. Bar chart indicates day – dependent effect of the fractions, risperidone, and haloperidol against ketamine- enhanced immobility in mice. Data were mean ± SEM (n = 6). *P < 0.05, compared with negative control group (two-way ANOVA revealed significance difference between all treatment groups, followed by a Bonferroni multiple comparison post hoc test)

Catalepsy

The active fractions of PC (DCM and EtOAc) were tested for cataleptic effect at higher and lower doses of 500 mg/kg and 125 mg/kg, respectively, singly and combined with haloperidol. It was observed that the group of animals treated with the DCM fraction at 500 mg/kg was not showing catalepsy up till 60 min after administration as compared to mice treated with 125 mg/kg. Also, when combined with haloperidol, the DCM fraction (500 mg/kg) reduced the cataleptic effect induced by haloperidol (Table 2). Furthermore, the administration of the EtOAc fraction only, did not show catalepsy at both 500 and 125 (mg/kg) up till 120 min of administration, it also reduced the catalepsy caused by haloperidol. However, the 500 mg/kg dose was more effective (Table 3). The

two active fractions of PC (DCM and EtOAc) did not induce catalepsy when administered singly and also reduced the cataleptic effect induced by haloperidol when it was coadministered with haloperidol. This implies that the DCM and EtOAc fractions of PC are good candidates of antipsychotics with little or no side effect.

Philenoptera cyanescens has been reported for various pharmacological properties[40, 41]. It has been traditionally used in Nigerian ethnomedicine for the treatment of mentally derailed people[42]. We previously reported the effect of the crude extract and fractions of this plant on amphetamine and apomorphine induced behavioural paradigms[34, 35], but in this study, the effect of the crude and fractions of PC against ketamine-induced psychosis was investigated. This is important because ketamine is known to affect dopaminergic, serotonergic and cholinergic receptors[43], while apomorphine and amphetamine are stimulants of dopaminergic receptors[44, 45]. Hence, the major goal of this study was to investigate the antipsychotic potential of *Philenoptera cyanescens* (PC) crude extract and fractions against ketamine-induced experimental psychosis models in mice.

Table 2. Effect of Haloperidol and DCM fraction of *Philenoptera cyanescens* on catalepsy

Group	Treatment	Time (min)/Mean Catalepsy Score ± SEM, n=6				
		0 min	30 min	60 min	90 min	120 min
1	Distilled water 10 mL/kg	3.50 ± 0.85	5.50 ± 1.06	10.0 ± 4.14	46.83 ± 18.79	58.83 ± 19.28
2	Haloperidol 1 mg/kg	137.30 ± 28.86	224.00 ±30.19	209.70 ± 31.67	249.50 ± 18.99	209.70 ± 21.74
3	PC DCM 500 mg/kg	8.00 ± 2.77	3.33 ± 0.62	8.00 ± 3.36	28.83 ± 20.16	37.67 ± 21.01
4	PC DCM 125 mg/kg	6.67 ± 2.23	22.33 ± 12.09	80.83 ± 35.93	49.17 ± 23.33	61.83 ± 20.01
5	Hal.+PC DCM 500 mg/kg	32.50 ± 26.52	65.00 ± 29.17	105.70 ± 39.92	115.50 ± 38.84	97.33 ± 39.40
6	Hal.+PC DCM 125 mg/kg	7.67 ± 2.80	109.3 ± 39.03	134.80 ± 27.70	156.30 ± 29.27	158.50 ± 24.32

Table 3. Effect of Haloperidol and EtOAc fraction of *Philenoptera cyanescens* on catalepsy

Group	Treatment	Time (min)/Mean Catalepsy Score ± SEM, n=6				
		0 min	30 min	60 min	90 min	120 min
1	Distilled water 10 mL/kg	3.50 ± 0.85	5.50 ± 1.06	10.0 ± 4.14	46.83 ± 18.79	58.83 ± 19.28
2	Haloperidol 1 mg/kg	137.30 ± 28.86	224.00 ±30.19	209.70 ± 31.67	249.50 ± 18.99	209.70 ± 21.74
3	PC EtOAc 500 mg/kg	1.00 ± 0.00	1.17 ± 0.17	1.50 ± 0.34	4.83 ± 2.69	2.33 ± 0.62
4	PC EtOAc 125 mg/kg	1.33 ± 0.21	3.50 ± 1.34	3.83 ± 1.20	5.83 ± 1.96	6.17 ± 2.02
5	Hal.+ PC EtOAc 500 mg/kg	3.00 ± 1.18	23.67 ± 7.99	82.83 ± 23.58	148.80 ± 35.06	127.00 ± 25.00
6	Hal. + PC EtOAc 125 mg/kg	2.33 ± 0.80	83.33 ± 34.26	155.50 ± 34.07	198.00 ± 27.92	218.20 ± 21.91

Reports from literature revealed that ketamine, an antagonist of N-methyl-D aspartate (NMDA) receptor induces psychotic-like symptoms in healthy human beings[45-47] and exacerbates psychosis in patients with schizophrenia[48-51]. Furthermore, animal and human cognitive functions are disrupted[13], thereby producing schizophrenia like deficits[52]. Chatterjee et al. (2011a) tried to characterize a ketamine-induced model in mice, with certain symptoms observed in schizophrenia using selected behavioural phenotypes, especially stereotypy behaviour and locomotor activity, and they reported increase in the 3, 4-Dihydroxyphenylacetic acid/Dopamine (DOPAC/DA) ratio in the striatal areas of the brain both after short and long term use of ketamine administration. They also demonstrated that ketamine induced anxiety of various neurotransmitters level such as glutamate, glycine, noradrenalin and related receptor systems (dopaminergic, serotoninergic, and cholinergic systems), which are implicated in the pathophysiology of schizophrenia[43].

In experimental animals, locomotion activity is mostly increased by CNS stimulants and reduced by CNS depressants[7]. Enhanced locomotor activity has been linked to the dopaminergic hyper activation in striatal areas of mouse brain[8]. This inhibitory effect has been shown to increase activity of the neurons and cause excess release of dopamine in the limbic striatal regions. Acute ketamine treatment has been reported to cause 60% increase in dopamine level in the cortex of animal, and 130% increase in the striatal region of the brain[43]. *Bacopa monniera* was shown to regulate the dopamine pathway in the striatum of mice, suggesting its protective action against positive symptoms of psychosis[36]. In this study the locomotion deficits induced by ketamine were significantly reduced after being exposed to the crude extract and fractions of PC. The Hex (***P < 0.001), DCM (***P < 0.001), EtOAc (***P < 0.001) and BuOH (***P < 0.001, *P < 0.05) fractions significantly reduced the hyper-locomotion induced by ketamine. An average dose of 250 mg/kg was more effective and was found comparable to the standard drug, risperidone. This may be due to partial restoration of striatal dopamine levels[43], thus, suggesting it may mediate its protective effect against the positive symptoms through regulation of the dopamine pathway in the striatum.

Ambulation in the animals supports the result of hyper-locomotion. When there was an increase in hyper-locomotion, the duration of ambulation was reduced. A significant difference was not observed between the negative control group and PC crude extract-treated mice at various doses (125, 250, and 500 mg/kg), in the time animals spent in ambulation (walking around). The hexane (*P < 0.05, ***P < 0.001), DCM (**P < 0.01, ***P < 0.001) fractions at all

doses (125, 250, and 500 mg/kg), were significantly different from the negative control group compared to the standard drugs, haloperidol (***P < 0.001) and risperidone (***P < 0.001), indicating that there was a reduction in ambulation. Also, at 250 mg/kg and 500 mg/kg, the EtOAc fraction (**P < 0.01) was significantly different from the control group. Likewise, BuOH (**P < 0.01) at 250 mg/kg differed significantly from control group, but the aqueous fraction showed no significant difference at all doses.

Stereotypy, one of the most prominent symptoms seen in people with mental disorder, is usually seen in the form of repetitive performance of a set of strange behaviours such as making the same kind of comments or asking the same questions[9, 10]. In experimental animals, stereotypy is usually observed as persistent sniffing, abnormal sideways movements of the head, intense licking, and chewing behaviours[10]. The acute administration of the crude extract and fractions of PC significantly suppressed the stereotypy behaviour induced by ketamine indicating its neuroleptic properties[53]. This investigation is in line with earlier research on the inhibition of ketamine-induced stereotypy by ethanol extract of *Terminalia ivorensis*[14]. Also, Amoateng et al. (2017) reported the antagonism of apomorphine-induced stereotypy by *Synedrella nodiflora* whole plant.

We minimized the use of animals in the behavioural studies by using the same animals for both hyper-locomotion and stereotype behaviour paradigm. The behavioural results showed that the PC fractions inhibited hyper-locomotion and stereotypy induced by ketamine (compared to risperidone) and might be due to its atypical mechanistic effects through the NMDA receptor[54], which resulted into the modulation of dopamine, hence, its efficiency against positive symptoms of psychosis.

Chronic administration of ketamine has been reported to enhance immobility in forced swim test, and used as a model for negative symptoms of psychosis[13], such as avolition (severe decrease in motivation to initiate and perform self-directed purposeful tasks). We observed that chronic administration of DCM and EtOAc fractions of PC gave a significant decrease in the duration of immobility. Twenty-four hours after treatment, all the three doses of DCM fraction significantly reduced the immobility time (*P < 0.05, ***P < 0.001), with 125 mg/kg being more active than the standard drugs (haloperidol and risperidone). This activity was also consistent at day 15 post treatment. The EtOAc fraction gave a dose-dependent decrease in the duration of immobility in the FST model at day 11, with 125 mg/kg being the most active and consistent on day 15 of the study**.** The efficiency of antipsychotics such as clozapine, risperidone in negative symptoms of psychosis has been credited to its serotonin (5HT-2) receptor

blockage, Chatterjee *et al.* (2012) have reported that chronic administration of ketamine induces an increase in the serotonin content in brain cortex, and that pre-treatment of animals with *Bacopa monniera* normalised the serotonergic receptor, thereby protecting the animals against negative symptoms of psychosis[36]. This is in support of our study whereby the DCM and EtOAc fractions of PC gave protective effect against ketamine-enhanced immobility in negative symptoms of psychosis.

Cognitive dysfunction such as attention deficits, short- and long-term working memory are symptoms seen in psychotic patients[11]. Learning and memory are mostly severe in patients[55] and are known to be a major factor in determining the level of disabled patients with psychotic experience in personal living, social and occupational functions[56].

The Y-maze test has previously been used to investigate the effectiveness of antipsychotic drugs on memory and learning function in rodents[12]. It involves measuring the spatial working memory, which is based on rodent's willingness to explore new environments. Rodents prefer to explore new arm of the maze, rather than return to a previously visited arm. It is mostly used to measure the cognitive deficiencies in transgenic mice and investigate effects of novel principles on cognition. In this study, we also observe the memory dysfunction properties of ketamine, which was restored by the DCM and EtOAc fractions of PC. The DCM fraction at 250 mg/kg consistently gave high percentage correct alternation of 70.5%, 77.5% and 74.3% on days 1, 5 and 10, respectively. Also, the EtOAc (250 mg/kg) gave 75.2%, 77.0%, and 79.2% percentage correct alternation on days 1, 5 and 10, respectively. These results are comparable to the standards, risperidone (day 1 – 66%, day 5 – 77.7%, day 10 – 70.5%) and haloperidol (day 1 – 75.0%, day 5 – 70.3%, day 10 – 73.8%). Chatterjee *et al.,* (2012), had reported that ketamine-disrupted memory has been linked to the abnormal functioning of the acetylcholine and glutamate neurotransmitters. Acetylcholine plays an important synaptic role in the initial stages of memory formation[57], while glutamate mediates long-term potentiation and memory formation through the NMDA receptors. These two neurotransmitters were inhibited due to the administration of ketamine by enhancing the cholinesterase levels which is an enzyme that metabolizes acetylcholine, thus reducing its synaptic level. Our observation showed that PC DCM and EtOAc may mediate their protective effect against ketamine by antagonizing the cholinesterase activity and restoring glutamate levels in the brain.

Catalepsy has been used to predict safe tranquillizing activity and also to access motor effects of drugs, especially effects related to extrapyramidal symptoms

(EPS) [14]. It is one of the major side effects related to the use of orthodox antipsychotic drugs[15]. The EPS has been linked to the decrease of striatal dopamine D1 and D2 receptors[16, 17]. Also, some other neurotransmitters such as serotonin, acetylcholine, opiods or adenosine have been implicated[58]. Therefore, testing for cataleptic behaviour in experimental animals forms an integral part of the discovery and development of antipsychotic drugs. The active fractions from the hyper-locomotion and stereotypy studies (DCM and EtOAc) were tested for catalepsy. Apparently, the absence of cataleptic effect in the DCM fractions of PC at 500 mg/kg (up till 120 min) and the ability to reduce the catalepsy induced by haloperidol suggest that the extract at higher doses may not produce any significant motor side-effects. Also, the EtOAc fraction exhibited no cataleptic effect at both 500 and 125 mg/kg suggesting that this fraction does not possess any significant motor side effects at both lower and higher doses. These doses were also able to reduce haloperidol-induced catalepsy. This is in support of a previously reported study in which black tea extract effectively reduced haloperidol-induced catalepsy[58]. The appearance of cataleptic event in naïve mice at 60 min post-DCM fraction (125 mg/kg) administration and its significant increase in haloperidol-induced catalepsy also at the 30th min may suggest that motor side effects are likely to develop with lower doses of the extract[38]. The DCM and EtOAc fractions having shown a protective effect against ketamine-induced positive symptoms, without any extrapyramidal side effects, could possess enormous antipsychotic potential. The DCM fraction of *Philenoptera cyanescens* was not reported earlier, it shows good activity alongside the ethyl acetate fraction, which also potentiated antipsychotic activities when compared to reference antipsychotic drugs; haloperidol and risperidone, suggesting that it may contain typical and/atypical compound(s) responsible for inhibiting psychosis.

These findings provide supportive evidence demonstrating that methanol crude extract and fractions of *Philenoptera cyanescens* leaves possess safe antipsychotic properties with no extrapyramidal effects when tested in ketamine-induced positive, negative symptoms as well as cognitive impairments, and catalepsy models of psychosis. This efficacy may be due to its dopaminergic, serotonergic and cholinergic neurotransmission normalisation effects. The DCM fraction has not been reported earlier. The isolation, purification and identification of the bioactive constituents of *Philenoptera cyanescens* is on-going, as it may lead to the discovery of new molecular entities for future development of antipsychotic drugs, which could serve as an alternative safe therapy for treating psychosis in humans.

AUTHORS' CONTRIBUTION

Author MAS took part in research concept and design, supervision, analysis, interpretation of the result findings, writing of manuscript, ITA carried out the laboratory work, analysed, interpreted the findings from the results and wrote the draft manuscript, KR was the host supervisor in whose lab the research was carried out and also supervised the study. All authors have read and approved the manuscript.

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Dose-dependent Efficacy of the N-acetylglucosamine and Quercetin Combination in Rats with Renal Failure

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ABSTRACT

The study was devoted to the evaluation of dose-dependent efficacy of N-acetylglucosamine and quercetin combination in the treatment of renal failure. The combination was studied in injectable dosage form at the doses of 10, 20, 40 and 60 mg/kg in rats with chromium-induced nephropathy. The efficacy of combination was assessed by animal survival, renal excretory function, nitrogen metabolism and nephroprotective activity. Also, the ED_{50} index was calculated by Probit Analysis method. The efficacy of combination at doses of 40 and 60 mg/kg was most expressed with insignificant differences ($p>0.05$) between them. There was a significance increase ($p<0.05$) in animal survival, renal excretory function and normalization of nitrogen metabolism. This led to the nephroprotective activity of 67.8 and 70.4%, respectively. The ED_{50} index of combination was 30.2 ± 6.3 mg/kg. Thus, this test combination at a dose of 30.2 mg/kg is the promising drug for experimental treatment of kidney diseases.

Keywords: N-acetylglucosamine, Quercetin, Nephroprotective effect, Median effective dose, Renal failure

INTRODUCTION

Improving the efficacy of the renal diseases treatment is an important problem in the medical and pharmaceutical practice. The first place in this group of diseases occupied by chronic kidney disease (CKD), since it is not only the most common pathology of the urinary system, but also has a great medical

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and social significance[1]. Prevalence of CKD is 8-16% of the total population and reaches 47% among people over 70 years old[2-3]. At the same time, this pathology affects more than 500 million adults in the whole world[3].

The course of CKD leads to severe complications such as renal failure (RF), which is accompanied by a decrease of renal excretory function, development of azotemia, oxidative stress, electrolyte imbalance, and other manifestations[4]. Patients with CKD are quickly disablement and lose their social activity[1-3]. Therefore, there is an ever-increasing number of patients requiring renal replacement therapy, and this population increases by about 7% each year[4], and it is over 2.5 million people in the world[1]. In this regard, the search for drugs to improve the efficacy of CKD treatment and reduce the rate of its progression, as well as to expand the list of effective nephroprotective agents is an important task of the pharmaceutical science.

A promising approach in the solution of this problem may be the implementation of combined drugs based on membrane protects and antioxidants of natural origin, among the properties of which nephroprotective effects on different mechanisms of action are present. Based on this, the research of pharmaceutical combination on the basis of quercetin and glucosamine derivative – N-acetylglucosamine (NAG) in injectable dosage form is a great scientific interest.

Quercetin is a well-known flavonoid of plant origin with a wide range of pharmacological effects. The most significant among them are antioxidant, antihypoxic, membrane stabilizing and anti-inflammatory[5-6]. As a result, quercetin has angioprotective properties and reduces the permeability of glomerular capillaries. This complex of effects is useful in the treatment of renal diseases.

Glucosamine is a natural human metabolite almost safe for the body[7-8]. It is a part of glycosaminoglycans and glycoproteins of the biological membranes, including the glomerular basement membrane[9], which causes its nephroprotective properties. Glucosamine realizes its physiological effects through the biologically active form – NAG and it is added to the damaged membranes in this form[10]. Therefore, potentially NAG has a more expressed nephroprotective effect due to direct mechanism of action.

Based on the peculiarities of the pharmacological properties of quercetin and NAG, the combined drug on their basis is promising for the renal diseases treatment, since both components mutually complement each other's pharmacodynamics with the effects necessary for the therapy of kidney diseases. In previous experimental studies, we have proved the high efficacy of oral combinations of quercetin with some glucosamine derivatives on different models of kidney injury in rats[11].

In this regard, the scientific interest was to study the combined injection drug, which may be more effective not only in the latent course of kidney diseases, but also in acute injuries and exacerbations of chronic nephropathies. The implementation of this combination requires the investigation of dose-dependent efficacy in RF, in order to determine the optimal dose for further in-depth preclinical and clinical studies. The aim of this study was to research the efficacy of the injection combination of NAG and quercetin in different doses in rats with RF.

METHODOLOGY

Animals

Experimental study was performed using 58 randomly selected male albino rats weighing 170-190 g, which were obtained from the *vivarium* of the Central Research Laboratory of the National University of Pharmacy (Kharkiv, Ukraine). The animals received standard rat diet and water *ad libitum*. The rats were housed under standard laboratory conditions in a well-ventilated room at 25 ± 1°C and with a relative humidity of 55 ± 5% with a regular 12 h light / 12 h dark cycle[12-13]. All studies were conducted in accordance with the EU Council Directive 2010/63/EU dated 22 September 2010 on the protection of animals used for scientific purposes[14]. The experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy.

Test object and its preparation

Research object was the combination of NAG and quercetin in the injectable dosage form in a ratio of 1:1. NAG was used as a 6% solution for injections, which was developed and manufactured as a pilot series by PJSC SIC "Borschahivskiy CPP" (Ukraine). Quercetin was used as the Corvitin® (COR) medication (PJSC SIC "Borschahivskiy CPP, Ukraine), which is a freeze-dried powder for injections. COR was diluted with a solution of NAG to achieve a 1:1 ratio and added 0.9% sodium chloride solution for injections to a concentration of 20 mg/mL (for the sum of active substances) to prepare the combination immediately before use.

Experimental design

All animals were randomly divided into 6 experimental groups as follows.

Group 1 – intact control (healthy rats receiving vehicle, n = 8).

Group 2 – control pathology (untreated rats receiving vehicle, n = 10).

Group 3 – rats with RF treated with NAG/COR at 10 mg/kg (n = 10).

Group 4 – rats with RF treated with NAG/COR at 20 mg/kg (n = 10).

Group 5 – rats with RF treated with NAG/COR at 40 mg/kg (n = 10).

Group 6 – rats with RF treated with NAG/COR at 60 mg/kg (n = 10).

Chromium-induced nephropathy was used as RF model[15]. It was induced by subcutaneous injection of 2.5% potassium chromate solution (Sigma-Aldrich, USA) in an original modification at a dose of 0.7 mL/kg on the first day of experiment[16]. After this, animals received the test NAG/COR combination at doses of 10, 20, 40 and 60 mg/kg (for the sum of active substances). All test samples were injected intramuscularly daily for 10 days. Animals of control groups were received simultaneously intramuscular injections of equivalent dose of 0.9% sodium chloride solution. The functional state of the kidneys was evaluated 10 days after the pathology induction.

Biological samples preparation and storage

The animals were sacrificed under anesthesia with ketamine/xylazine (75/10 mg/kg, i.p.) at the end of experiment[17]. Blood samples were collected from the inferior vein cava and centrifuged at 1500 g at +4°C for 10 minutes using refrigerated centrifuge Eppendorf 5702R (Eppendorf, Germany). Urine samples were collected using individual metabolic cages and centrifuged at 500 g for 10 min. The supernatants were separated and used for biochemical assays. All biological samples were frozen and stored at -80 °C.

Evaluation of the functional state of kidneys

Spontaneous daily diuresis was determined with individual metabolic cages at the end of experiment in all animals. The protein content and its daily excretion were determined in the collected urine[18]. Glomerular filtration rate (GFR) was evaluated as endogenous creatinine clearance, tubular reabsorption (TR) and urea clearance (UC) were also calculated, using the standard equations[2-3,18]:

$$GFR = U_{cr} \times V / P_{cr}$$
(eq 1)

$$TR = (1 - P_{cr} / U_{cr}) \times 100\%$$
(eq 2)

$$UC = U_{ur} \times V / P_{ur}$$
(eq 3)

Where U_{cr} is the urine creatinine concentration, V is the daily diuresis, P_{cr} is the plasma creatinine concentration, U_{ur} is the urine urea concentration and P_{ur} is the plasma urea concentration.

Biochemical assays

Biochemical studies were performed using commercial kits "Creatinine FS" (cat. 117119910021), "Urea FS" (cat. 131019910021) and "Total protein UC FS" (cat No 102109910021) manufactured by DiaSys Diagnostic Systems GmbH (Germany) using the automatic biochemical analyzer Express Plus (Bayer Diagnostics, Germany) to evaluate the parameters of excretory renal function and nitrogen metabolism. The creatinine and urea blood and urine levels were determined using a kinetic test without deproteinization according to Jaffe method and urease glutamate dehydrogenase enzymatic UV test respectively[18]. Urinary excretion of creatinine and urea was also calculated. The protein urine concentration was determined by photometric reaction with pyrogallol red[18].

Calculation of nephroprotective activity

The nephroprotective activity (NA) of the test combination was evaluated for its ability to reduce glomerular filtration dysfunction. The NA index was assessed by the degree of GFR amplification in comparison with untreated animals and calculated by the formula:

$$NA = (GFRt - GFRc) / (GFRi - GFRc) \times 100\% \qquad \text{(eq 4)}$$

Where GFRt is the value of GFR under the influence of the test sample, GFRc is the control pathology group GFR value and GFRi is the intact control group GFR value.

Calculation of the median effective dose (ED_{50})

Indicator ED_{50} of the test combination was calculated based on the dose-dependent NA with Probit Analysis method[19]. To do this, we used Probit Analysis according to the Bliss-Finney method in the modification of Prozorovskii[20] and MS Excel 2016 software (Microsoft Corp., USA).

The calculations were carried out in the next way. The percentages of activity in each group were converted to probits (y) and their weighing factors (B) according to special tables. The dose points (x) and intermediate variables were determined with necessary calculations. The relationship between dose points (x) and probits (y) was reflected by the equation:

$y=A_0 + A_1x$ (eq 5)

Coefficients A_0 and A_1 were calculated by following equations:

$A_0 = [(\Sigma B) - (\Sigma xB)A_1] / \Sigma B$ (eq 6)

$\Sigma xyB = \Sigma xB / \Sigma B \times [\Sigma yB - (\Sigma xB)A_1] + (\Sigma x^2B)A_1$ (eq 7)

The solution of these equations allowed constructing a Probit Analysis chart of the "activity-dose" dependence. Then the dose points and dose variables were found by equation 5 for ED_{16}, ED_{50} and ED_{84}, taking into account values of probits, which are 4 for ED_{16}, 5 – ED_{50} and 6 – ED_{84}, respectively.

The standard error (SE) of ED_{50} was determined by the equation:

$SE = (ED_{84} - ED_{16}) / 2\sqrt{n}$ (eq 8)

Where ED_{84} is the dose corresponding to the drug activity of 84%, ED_{16} is the dose corresponding to the drug activity of 16% and n is the number of observations.

Statistical analysis

All the results were processed by descriptive statistics and presented as the mean ± standard error of the mean (SEM) excluding the survival rate. Statistical differences between groups were analyzed using one-way ANOVA followed by Dunnett`s post-hoc test and using Fisher's exact test for survival analysis[21-22]. Utilized computer software included IBM SPSS Statistics v. 22 (IBM **Corp**., USA) and MS Excel 2016 (Microsoft Corp., USA). The level of statistical significance was considered as $p<0.05$.

RESULTS AND DISCUSSION

A high mortality was observed in the control pathology group 10 days after chromium-induced nephropathy with animal survival rates of only 50% (Figure 1). Rats were in poor physical condition, with reduced motor activity, edema and ascites.

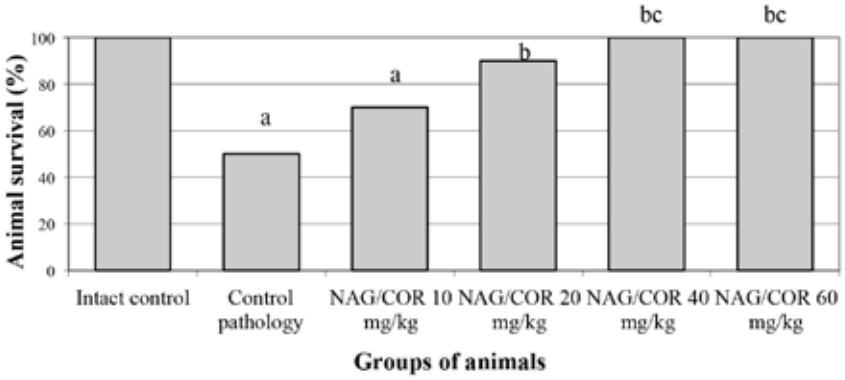

Figure 1. Influence of NAG/COR combination at different doses on the survival of rats with RF.

Data are presented as percentage of animals survived in each group. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg (Fisher's exact test).

The renal excretory function was expressly deteriorated. Daily diuresis was 1.6 times and GFR – 2.7 times lower (p<0.05) compared to intact animals and TR was increased by 1.1% (Table 1). In addition, proteinuria was observed, which reached 41.3 mg/day (Figure 2).

Table 1. Effect of NAG/COR combination at different doses on the renal excretory function in rats with RF.

Groups of animals	Daily diuresis (mL/day)	GFR (mL/day)	TR (%)
Intact control	6.7 ± 0.2	402.0 ± 16.8	98.32 ± 0.06
Control pathology	4.1 ± 0.2 [a]	150.5 ± 7.9 [a]	97.24 ± 0.16 [a]
NAG/COR 10 mg/kg	4.9 ± 0.2 [abde]	219.3 ± 5.6 [abde]	97.76 ± 0.09 [abde]
NAG/COR 20 mg/kg	5.5 ± 0.1 [abcde]	255.3 ± 9.0 [abcde]	97.80 ± 0.12 [ab]
NAG/COR 40 mg/kg	6.2 ± 0.2 [ac]	320.9 ± 11.8 [abc]	98.06 ± 0.04 [abc]
NAG/COR 60 mg/kg	6.4 ± 0.3 [ac]	327.5 ± 14.5 [abc]	98.03 ± 0.04 [abc]

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

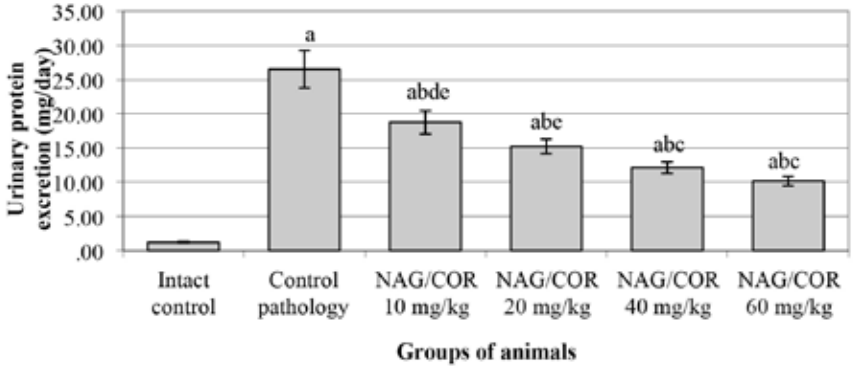

Figure 2. Influence of NAG/COR combination at different doses on the urinary protein excretion in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The kidney dysfunction led to a decrease in the excretion of nitrogenous compounds and an increase in the blood residual nitrogen level. The blood creatinine and urea were 3.1 and 3.2 times higher (p<0.05) than in intact rats, respectively (Table 2). Their urinary excretion was increased, which can be regarded as an organism compensatory reaction to the nitrogen compounds retention and autointoxication (Table 2). But this was not enough to rebalance nitrogen metabolism.

Table 2. Influence of NAG/COR combination at different doses on the nitrogen metabolism in rats with RF.

Groups of animals	Blood level		Urine excretion	
	Creatinine (μmol/L)	Urea (mmol/L)	Creatinine (μmol/day)	Urea (mmol/day)
Intact control	60.6 ± 2.7	4.7 ± 0.3	24.1 ± 0.5	0.78 ± 0.05
Control pathology	188.8 ± 8.8 [a]	15.1 ± 0.8 [a]	28.2 ± 0.7 [a]	0.83 ± 0.06
NAG/COR 10 mg/kg	148.3 ± 7.6 [abde]	12.3 ± 0.4 [abde]	32.4 ± 1.3 [ab]	0.93 ± 0.06 [de]
NAG/COR 20 mg/kg	132.1 ± 6.1 [abde]	10.6 ± 0.5 [abcde]	33.6 ± 1.7 [ab]	1.07 ± 0.05 [ab]
NAG/COR 40 mg/kg	107.1 ± 5.5 [abc]	8.5 ± 0.4 [abc]	34.0 ± 1.3 [ab]	1.12 ± 0.05 [abc]
NAG/COR 60 mg/kg	99.6 ± 5.5 [abc]	8.2 ± 0.4 [abc]	32.1 ± 1.2 [ab]	1.10 ± 0.04 [abc]

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

Corresponding changes were also observed in UC, which reflects the rate of blood purification from urea. In untreated rats, this index dropped to 54.9 mL/day, which was 3.0 times lower (p<0.05) than in intact rats (Figure 3).

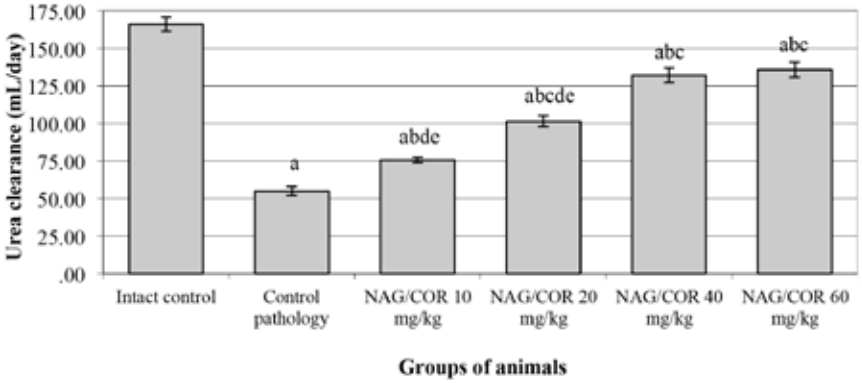

Figure 3. Effect of NAG/COR combination at different doses on the urea clearance in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The described pattern is typical for chromium-induced nephropathy, which develops as a result of toxic effect of chromium compounds on the proximal nephron tubule, induces tubular necrosis and subsequent RF[15].

Test NAG/COR combination showed a positive dose-dependent effect on the course of RF. Under its influence at a dose of 10 mg/kg, the functional state of rats was improved, and survival increased to 70%, which, however, was unreliably (Figure 1). There was a significant increase (p<0.05) in the renal excretory function compared to untreated animals: diuresis was increased by 19.5% and GFR – by 45.7% (Table 1). Also, there was a significant decrease (p<0.05) in urinary protein excretion by 29.2% (Figure 2). Additionally, creatinine and urea blood levels were significantly decreased (p<0.05) by 21.5% and 18.5%, respectively (Table 2). The UC index was significantly increased (p<0.05) by 37.7% (Figure 3). As a result, the NA index was 27.3% (Figure 4).

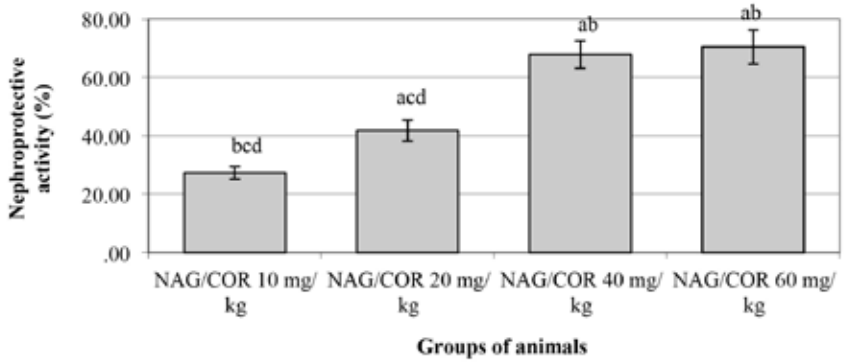

Figure 4. Nephroprotective activity of NAG/COR combination at different doses in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [b]p<0.05 compared to group treated with NAG/COR at 20 mg/kg, [c]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The use of test combination at a higher dose level of 20 mg/kg led to increased efficacy, which was reliably in the most cases. The animal survival rate was 90% unlike the previous group (Figure 1). Daily diuresis and GFR were significantly increased ($p<0.05$) by 34.1% and 69.6% compared to untreated animals, respectively (Table 1). The proteinuria level was 1.7 times lower (Figure 2). This test sample significantly reduced ($p<0.05$) the creatinine and urea blood levels by 30.0%, and also increased their excretion by 19.1% and 28.9%, respectively (Table 3). In addition, UC was 1.8 times higher ($p<0.05$) than in untreated animals (Figure 3). The NA index reached to 41.7%, which was credibly higher ($p<0.05$) than at the dose of 10 mg/kg (Figure 4).

An increase in the NAG/COR dose level up to 40 mg/kg resulted in a significant enhancing of efficacy, which was reliable ($p<0.05$) compared to dose level of 20 mg/kg in the most of assessment parameters. Under the influence of NAG/COR at 40 mg/kg, the functional state of rats normalized, and mortality disappeared. The survival rate was at the level of intact group – 100% (Figure 1). Renal excretory function was significantly increased ($p<0.05$) compared to the control pathology group: diuresis was 1.5 times higher, GFR was 2.1 times higher and TR was increased by 0.84% (Table 1). Also, the level of proteinuria was 2.2 times lower (Figure 2). This dose reliably increased ($p<0.05$) urinary creatinine and urea excretion. As a result, the blood creatinine and urea were 1.8 times lower ($p<0.05$) than in untreated animals (Table 2), and there was a

significant 2.4-fold increase in UC (Figure 3). The obtained results allowed to calculate the NA index, which was 67.8% (Figure 4).

A similar level of influence on RF was observed with the test combination at a dose of 60 mg/kg. At the same time, differences in all indicators of efficacy compared to the dose of 40 mg/kg were insignificant, despite a 1.5-fold higher dose. The NA index in this case was 70.4%, which was significantly higher ($p<0.05$) than in doses of 10 and 20 mg/kg and did not differ ($p>0.05$) from the dose of 40 mg/kg (Figure 4).

These results are expected and correspond to scientific data. Both components of the combination have a positive effect on the course of experimental renal pathology. Thus, the efficacy of quercetin has been confirmed in some studies on various models of kidney injury in different dosage forms, including injections[11,23-25]. The glucosamine derivatives also showed the expressed nephroprotective effect in the experiment. It was proved that glucosamine is embedded to the damaged renal tissue and increases the content of endogenous hexosamines[26]. These results correlate with another studies, which showed the efficacy of glucosamine derivatives in the treatment of kidney fibrosis in mice[27], contrast-induced acute kidney injury[28] and renal ischemia/reperfusion injury in rats[29].

The high efficacy of the combination is due to the fact that quercetin and NAG have nephroprotective effect with different mechanisms of action. The injection route of administration brings advantages for both components, since it allows to avoid the first pass metabolism and to ensure that the total dose of active substances reached the blood circulation and renal tissue in non-metabolized form.

At the next stage of the study, we calculated the ED_{50} index for NAG/COR combination under condition of RF based on the dependence of test drug activity on the administered dose by Probit Analysis.

Using the special table data, the percentages of activity were converted to probits (y) and their weighing coefficients (B). The dose points (x), intermediate variables were determined with necessary calculations. All the results obtained are presented in Table 3.

Table 3. Doses, activity levels and intermediate variables for calculating ED_{50} of NAG/COR combination using Probit Analysis.

Dose (mg/kg)	NA (%)	Dose point (x)	Probit (y)	Weighing factor (B)	xB	x^2B	yB	xyB
10	27.3	1	4.39	4.3	4.3	4.3	18.88	18.88
20	41.7	2	4.82	4.8	9.6	19.2	23.14	46.27
40	67.8	4	5.47	4.5	18.0	72.0	24.62	98.46
60	70.4	6	5.52	4.5	27.0	162.0	24.84	149.04
Sum				18.1	58.9	257.5	91.47	312.65

The results of calculations allowed to construct a chart of Probit Analysis of the "activity-dose" dependence (Figure 5). Final results of calculations are presented in Table 4.

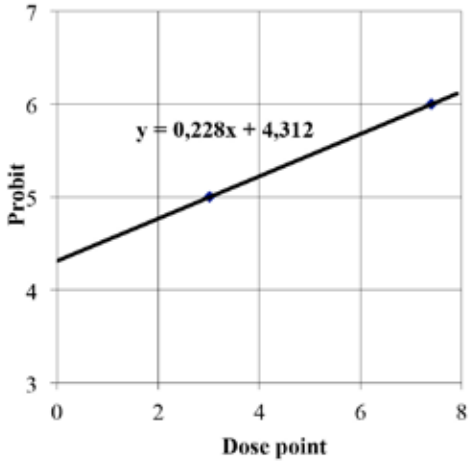

Figure 5. Chart of Probit Analysis of "activity-dose" dependence of NAG/COR combination.

Table 4. Results of calculations for determining ED50 of NAG/COR combination using Probit Analysis.

A_1	A_0	Equation of „probit-dose" dependence	Dose point ED_{50}	Dose point ED_{16}	Dose point ED_{84}	ED_{50} (mg/kg)	SE (mg/kg)
0.228	4.312	y = 0.228x + 4.312	3.02	-1.37	7.41	30.2	6.3

As a result of calculations, the ED_{50} index of test combination was 30.2 ± 6.3 mg/kg (Table 4).

The daily dose for clinical application of the combination was determined based on observed ED_{50} with conversion to humans following the FDA recommendations[30]:

Daily Dose = (30.2 mg/kg / 6.2) x 60 kg = 292.3 mg

Thus, the recommended daily dose of NAG/COR combination is 292.3 mg (for a median patient body weight of 60 kg) or 4.9 mg/kg.

ED_{50} index of the combination of quercetin and some glucosamine derivatives in oral dosage form is 80 mg/kg, determined in a previous experimental study on chromium-induced nephropathy in rats[11]. The result obtained in this study was 2.7 times lower, so the injection solution of NAG/COR combination is much more effective. These data have a great value for clinical nephrology, since they discover wide perspectives for the use of a new nephroprotective drug for CKD treatment – the NAG/COR combination in injectable dosage form.

Combination of NAG and quercetin in the injectable dosage form in a ratio of 1:1 leads to a significant increase in efficacy under conditions of RF in rats, which causes the expressed positive effect on the course of nephropathy. The NA of this combination has an expressed dose-dependence in the dose range of 10-40 mg/kg. The ED_{50} of the test combination is 30.2 ± 6.3 mg/kg, and it is the most optimal dose for further preclinical in-depth studies in order to justify its use in CKD therapy. The starting dose of the combination for clinical application extrapolated from the experimental data is 292.3 mg/day or 4.9 mg/kg/day, and it is the most appropriate for clinical trials as a kidney diseases treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Quercetin Enhances Human Sperm Motility in a Dose and Time Dependent Manner

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ABSTRACT

The aim of this study was to investigate the effect of quercetin on the motility of ejaculated human spermatozoa in asthenozoospermic cases by using different doses and exposure times. Semen samples of 94 men were incubated with quercetin at different doses and durations. Sperm motility was analysed in each group, and the results were compared. Compared to control, Quercetin improved sperm motility in each molarity and each interval except 1M. Statistically significant increase was assessed at 0.05 M after 1 hours of incubation, and 0.1 M after two hours of incubation ($p<0.05$). According to our results, it can be suggested that quercetin has a positive effect on sperm motility on a dose and time dependent manner. This study provides evidence for the potential use of quercetin for sperm preparation to be used in assisted reproduction techniques especially in cases of asthenozoospermia.

Keywords: Sperm, motility, quercetin, asthenozoospermia

INTRODUCTION

Infertility is defined as inability to conceive after one years of unprotected course. Male factor infertility occupies 40% of the infertility causes and semen quality occupies 70% of total male infertility reasons. Decreased values of sperm concentration, motility and morphology defined by WHO is known to impair sperm function thus effects fertility.[1] There is no prov-

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en treatment strategies to overcome the sperm problems. Therefore the couples facing the situation is advised to undergo an assisted reproduction technique including intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI). Motility comprises an important problem in both of the techniques especially in IUI cycles where the sperm fertilizes the oocyte spontaneously. There have been numerous studies in the literature regarding sperm motility enhancement strategies whose results are controversial.[2] Pentoxifyllin is a synthetic dimethylxanthine derivative which is one of the most widely used agent to improve sperm motility[3] but it is reported to be toxic in longer exposure times.[4]

Flavonoids are polyphenolic compounds that are found in many plant-based foods, including fruits, vegetables, and tea which have been reported to prevent from a wide variety of diseases such as cancer and cardiovascular diseases by acting as an antioxidant.[5] Amongst the flavonoids, Quercetin is one of the most studied one because of its free radical scavenging and metal chelating abilities.[6] Quercetin acts as an antioxidant by scavenging ROS and thus suggested to have anticarcinogenic, antiinflammatory and antiviral roles.[7] Moreover, Quercetin is suggested to act by protecting against DNA damage[8] and may be considered as an effective motility enhancement factor for treating male factor infertility.

The effect of quercetin on the sperm cells of several animal species show conflicting results. It was shown that quercetin inhibited rat sperm motility, but a contrary result was obtained with bovine spermatozoa. The aim of this study was to investigate the effect of quercetin on the motility of ejaculated human spermatozoa. We analyzed for the first time the effect of quercetin on sperm motility by using different doses and different exposure times.

METHODOLOGY

Semen samples of 94 men were obtained from the IVF Center of Medistate Hospital, that applied the clinic because of infertility from August 2018 to January 2019. Patients's semen analysis were performed according to the World Health Organization (WHO) semen analysis guideline. The exclusion criteria were as follows: presence of azoospermia/cryptozoospermia, presence of any kind of chromosomal abnormalities and/or point mutations including AZFy deletions, varicocele, patients with smoking history or alcohol consumption and evidence of infection suggested by the presence of leukocytes on semen analysis.

Semen samples were analyzed according to WHO criteria.[9] Sperm samples were collected after 3–7 days of sexual abstinence by masturbation and semen analysis was performed as previously reported.[10] Shortly, after determining liquefaction time, volume, appearance, Ph and viscosity of semen samples, sperm concentration (mil/mL), forwardly progressive sperm motility (A motility) and total motility rates were assessed. At least 100 spermatozoa were scored for motility assessment and motility patterns were classified into four grades as follows: A motility for forward progressive; B motility as, slow non-progressive; C motility as, sluggish and D motility as non-motile motility. Total motility rate was calculated as the sum of A, B and C motility rates.

Sperm samples were divided into 6 aliquots and incubated with different quercetin concentrations of 0,05 - 0,1 - 0,2 - 0,5 – and 1M with a final mixture of 1:1 (semen + quercetin) respectively. No quercetin was added to one of the semen aliquots which is classified as the control group. Motility rates of each group were assessed in the first, second and third hours and were compared for each other.

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS, Version 21 for Windows; SPSS, Inc., Chicago, IL, USA). Mann–Whitney-U test were conducted to compare the quantitative variables. The data were expressed as mean percentage. All tests were conducted using a p-value ≤ 0.05 defining statistical significance.

RESULTS AND DISCUSSION

We observed a time and dose dependent change in motility patterns of each group (Figure 1). Quercetin improved sperm motility in each of the groups and each interval except 1M when compared with the control but the statistically significant increase was assessed at 0.05 M after 1 hours of incubation and 0.1 M after two hours of incubation ($p<0.05$). 1M quercetin showed a toxic effect as assessed by a significant decrease in motility patterns (Figure 1).

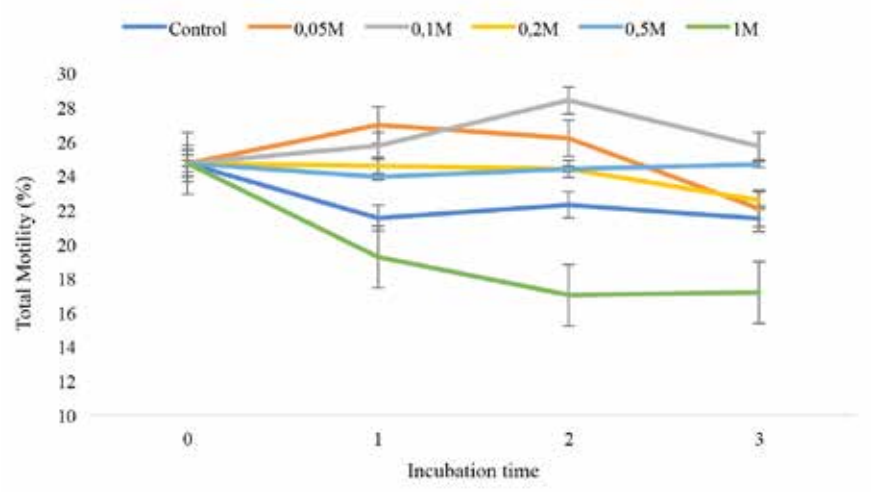

Figure 1. Sperm total motility values after different doses and periods of quercetin exposure

The other semen parameters including sperm concentration, normal morphology and acrosomal index were not changed after the addition of quercetin (Table 1).

Table 1. Semen parameters of groups (Control and quercetin supplemented 0,05 - 0,1 - 0,2 - 0,5 – and 1M) after different time intervals (0, 1, 2, 3 hours). Values are presented as mean percentage.

Variable	Control				0,05 M Q				0,1 M Q				0,2 M Q				0,5 M Q				1M Q			
	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h
Concentration ($x10^6$ sperm/mL)	26				25				26,1				25				23				24,4			
Total motility (%)	24,71	21,52	22,28	21,47	24,71	26,94	26,18	22,07	24,71	25,73	28,39	25,71	24,71	24,55	24,39	22,55	24,71	23,92	24,39	24,65	24,71	19,23	17	17,15
Normal morphology (%)	4,2				4				4,2				4,2				4				4,1			
Normal acrosomal index (%)	64,2				64,2				64,2				64,2				64,2				64,2			

The use of flavonoids for human health as a preventive and/or therapeutic have attracted increasing attention nowadays. Quercetin which is a flavonol-type flavonoid is one of those whose biological effects seem to be associated with its antioxidant role with a wide variety of biological activities, including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-inflammatory, anti-hypertensive, cardio-, neuro-, gastro- hepato-protective and anti-carcinogenic effects[11] although its cytotoxic effects including apoptosis induction, cell cycle arrest and anti-proliferative effects. Some studies show that quercetin acts as pro-oxidant or antioxidant depending on its concentration. The protective effects of quercetin is suggested to be correlated with the inhibition of lipid peroxidation which is assessed by malondialdehyde (MDA) level measurement.[12] However, harmful effects of quercetin were also shown which is suggested to be caused by mutagenic and DNA-damaging activities.[13] Male infertility is responsible for 40% of all infertility cases in which abnormalities in semen parameters comprise 60% of them. Motility is one of the most important semen parameters which is accepted to be classified as abnormal below 50% according to WHO criteria. The studies focusing on sperm motility enhancement mostly focus on antioxidants which have proven to be beneficial in treating several aspects of male infertility.[14]

The effects of quercetin on sperm viability and motility were studied in several animal and human studies with controversial results. Some of these studies concluded that quercetin has a protective or beneficial effect on sperm functions and fertility preservation in several species including mouse[15], human[16], buffalo[17], rooster[18], rats[19], rabbit[20], bull[21], goat[22], rabbit[23], while the others have shown no effect in equine[24] or negative effect in humans.[8] Most of these studies have focused on the effect of quercetin as a cryoprotectant during freezing or cold storage. There are only 3 studies that included fresh samples in humans which suggested positive effect in one[25] and negative effect in two[8] in which the study of Khanduja et al was the only one that observed motility rates in sperm cells[26] while the other two analyzed oxidative stress and lipid peroxidation.[25] This is the second study in the literature that mainly focus on the effect of quercetin on sperm motility rates.

We found a dose and time dependent positive effect of quercetin on sperm motility. Quercetin improved sperm motility in final concentrations of 0,05 - 0,1 - 0,2 - 0,5 M, and up to three hours of incubation. Higher concentrations (1M) were found to have toxic effect and inhibited motility.

Statistically significant increase was assessed at 0.05 M after 1 hours of incubation and 0.1 M after two hours of incubation. The result obtained in the recent study is not in accordance with the only study of Khanduja et al who reported a dose-dependent fall in sperm motility. The difference obtained may be because of the different concentrations used in this study which is lower than the concentrations used in our study 5-200 μM. Moretti et al. (2012) reported that quercetin is effective at low concentrations which has a limited effect on sperm motility and viability but showed to decrease lipid peroxidation[25] which may partly confirm the findings of our study.

Other studies observing the effect of quercetin on cryopreservation or cold-storage reported its protective and positive effect on post thaw semen parameters including motility, viability, ROS concentration and DNA integrity[27] which support our results by providing data of frozen sperm samples.

Animal studies including a wide variety of species including buffalo, rooster, rat, rabbit, bull and goat also found a positive effect of quercetin on sperm cells[16,18,19,20,21,22,23,24] except 2 studies including equine in which they observed no effect [24] and mice in which it is suggested to induce sperm abnormalities.[28] Abdallah et al. reported that quercetin may prevent the adverse effects of oxygen radicals, improve the functional parameters of spermatozoa, reduce the levels of lipid peroxidation and increase antioxidant levels in rats.[29] Quercetin produced a limited positive effect on sperm parameters, but it produced a protective effect by decreasing DNA breaks in sperm cells. However some studies revealed different findings including Jamalan et al.'s study who reported that quercetin do not have a protective effect[30] against lipid peroxidation induced by metal toxicants; rather, it had inhibitory effects on sperm motility.

According to our results, it can be suggested that quercetin has a positive effect on sperm motility on a dose and time dependent manner. This study provides evidence for the potential use of this flavonoid for sperm preparation to be used in assisted reproduction techniques including Intrauterine insemination (IUI) and Intracytoplasmic Sperm Injection (ICSI) cycles especially in cases of asthenozoospermia.

The findings should be verified by further studies with larger study populations. The molecular mechanisms causing these results and the toxicity assays should be performed before clinic use.

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Microbiological and Histological Characteristics of Interactions Between Carvacrol and Fluconazole in a Systemic Candidiasis Animal Model

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ABSTRACT

The objectives of the study were to explore the effect of carvacrol on *Candida tropicalis in vitro* and in animal model, alone and in combination with fluconazole. The activity of carvacrol alone and in combination with fluconazole were tested on *C. tropicalis* using the CLSI reference method and in murine models. Carvacrol in combination with fluconazole showed satisfactory activity, with FICI mainly in the range of 0.375-1.00. Microbiologically, treatment with carvacrol alone in combination with fluconazole decreased the fungal load of kidneys of infected animal at negligible level of the number of colony counts of *C. tropicalis*. Histologically, in treated animals, no *Candida* organisms were found in the kidney tissues; this was in contrast to control groups in which many yeasts mixed with hyphae were observed. Carvacrol alone in combination with fluconazole as an alternative approach could be considered as the extensive opportunities for the treatment and prevention of systemic candidiasis.

Keywords: Animal models; *Candida tropicalis*; Candidiasis; *in vitro*

INTRODUCTION

The rapid emergence of non-*albicans Candida* species are major public health importance, notably *Candida tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata*. *Candida* species account for about 80% of fatal systemic infection,

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where the most frequent cause of fungal infections in immunocompromised persons. The distribution of *Candida* species influenced by geographical location and healthcare factors[1-6]. In this context, *C. tropicalis* emerged as the most common non-*albicans Candida* species[7]. The reasons for dominance of *C. tropicalis* and its resistance to fluconazole have been difficult to clear[8]. Evidence suggests that marked shift in the epidemiological profile of *Candida* species are associated with increasing use of antifungal drugs[1,9].

The currently available antifungal drugs which are used for fungal infections, have the limitation of drugs resistance and high toxicity of the compounds[10]. Novel therapeutic strategies are needed to counter non-*albicans Candida* species infections. Natural products are the most important source for promising antimicrobials, the majority of which are surprisingly found in plants[11,12]. To combat the continual emergence of non-*albicans Candida* species, there is a critical need to develop new strategies. Combination therapies are the language of antimicrobial interactions, evolved to mediate non-*albicans Candida* species[3,13,14]. Within natural products, carvacrol (2-methyl-5-[1-methylethyl] phenol), a phenolic monoterpenoid with its pharmacological ability including antimicrobial, antioxidant, anti-inflammatory, antitumor activities, antispasmodic and antiangiogenic, have become promising therapeutic potential[11,12,15,16].

Antifungal activity of carvacrol against *C. tropicalis* has also been reported[17-20]. However, the activity of carvacrol against *C. tropicalis* in animal model is not known. Recent reports showed that combinatorial therapy of antifungal drugs and carvacrol is very effective to eradicate *Candida* infections[21,22]. In fact, this armamentarium of therapeutic drugs provides substantial benefits in terms of rapid effect of the antifungal therapy, wide drug spectrum and potency of drug activity, synergy, lowered toxicity and reduced risk of antifungal resistance[10,23].

We hypothesized that natural products in combination with antifungal drugs inhibit yeast cells growth. In this study, we explored the effect of carvacrol on C. tropicalis in vitro and in animal model, alone and in combination with fluconazole.

METHODOLOGY

Candida tropicalis

Two clinical isolates of *C. tropicalis* SN1 and SN2 and *C. tropicalis* ATCC 750 were used in this study. Clinical isolates from the vagina of patients with recurrent vulvovaginal candidiasis in Yasooj, were kindly provided by Microbiology Laboratory, Cellular and Molecular Research Center, Yasuj University of Medical Sciences (Iran). *C. tropicalis* cells were subcultured in Sabouraud

Dextrose Agar (SDA; Difco Laboratories, USA) and chromogenic culture media (CHROMagar Company, France). All isolates were stored at –80 °C in Sabouraud Dextrose Broth (SDB; Difco Laboratories, US) and supplemented with 300 μg/ml of chloramphenicol and isolates sterile maintained in 20% (v/v) glycerol.

In vitro Studies

Suspensions of *C. tropicalis* ($5 \times 10^2 - 2.5 \times 10^3$ cells/ml) were incubated in the U-bottomed 96-well polystyrene microtiter plate at 35 °C for 24 to 72 h with RPMI 1640 medium without sodium bicarbonate and with L-glutamine (Sigma-Aldrich Chemicals Co. St. Louis, MO, USA) [buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich)] and added a range of carvacrol (Sigma-Aldrich) concentrations between 0.049 and 100 μg/ml and fluconazole (Merck, Darmstadt, Germany) concentrations between 0.03125 and 64 μg/ml in Dimethyl sulfoxide (DMSO, Sigma-Aldrich) alone or in combination (1 : 1 ratio). Yeast cell- and drug-free controls were used as sterile and growth controls, respectively. The minimum inhibitory concentrations (MICs) were determined for each isolate in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M27-A3). Referring to the MICs of carvacrol and fluconazole alone and in combination, fractional inhibitory concentration index (FICI) was calculated for clinical isolates of *C. tropicalis* as described earlier[24]. The combining effect of carvacrol with fluconazole against clinical isolates of *C. tropicalis* interpreted as follows: Synergy, FICI≤0.5, partial synergy FICI> 0.5 but < 1.0, additive FICI= 1.0, Indifference FICI> 1.0 but < 4.0, and antagonism FICI≥ 4.0.

In vivo Murine Studies

Female BALB/c mice (Animal Breeding Stock Facility of Pasteur Institute of Iran, Karaj, Iran), weighing 25 g to 30 g, were used according to experimental protocol approved by the Islamic Azad University Animal Ethics Committee (IR.IAU.YASOOJ.REC.1396.12), which adheres to international procedures for animal care.

The mice (n = 10 per group) were infected with 5×10^6 yeast cells/ml suspensions of *C. tropicalis* ATCC 750 diluted to 200 μl with sterile PBS by intravenous (i.v.) injection. At 1 h following the infection, mice were treated with 5 mg/kg/day of i.v. injections of carvacrol and intraperitoneal (i.p.) injections of fluconazole alone or combination in a final volume of 200 μl/mouse for 5 days. The untreated control animals received 200 μl/mouse of sterile PBS.

For the systemic candidiasis study, at 2, 4, 7, 14 and 30 days after infection,

mice were sacrificed and kidneys was collected for evaluation of microbiological characteristics following the methods described by Khodavandi et al[25]. Briefly, kidney tissues were homogenized in 1 ml of sterile normal saline and serial dilutions plated on SDA to obtain the numbers of viable *C. tropicalis* ATCC 750 per gram of tissue and identified the fungal loads.

At day 14 after infection, mice were sacrificed and their kidneys were harvested and formalin fixed, dehydrated and embedded in paraffin wax using a Shandon Automated Tissue Processor (ThermoShandon, PA, USA). Sectioning of tissue samples was done at 4-5 μm and samples were stained with haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS). Final slides were then mounted and viewed under Leica microscope (DMRA II, Germany). Images were captured at 400× and 1000× magnification to visualize fungi and the intensity of the inflammatory response.

Ethical Approval

All procedures performed in studies involving human participants, which are obtained from Microbiology Laboratory, Cellular and Molecular Research Center, Yasuj University of Medical Sciences, were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration. The animal studies were approved by Islamic Azad University Animal Ethics Committee, which adheres to international procedures for animal care.

Statistical Analysis

Data were normalized with Kolmogorov–Smirnov test. Differences between means of values were compared for significance with one way ANOVA and Tukey's post-hoc test. ANOVA with repeated measure and nonparametric tests including Kruskal-Wallis test followed by Bonferroni's post hoc test were used when appropriate. Data shown reflect means ± standard division of the means. Significant findings are denoted as: $*p < 0.01$; and $**p < 0.001$ in each figure. SPSS Statistics (SPSS Inc., Chicago, IL, USA) v. 24 software was used for statistical analysis.

RESULTS AND DISCUSSION

In order to investigate the antifungal activity of carvacrol or fluconazole alone and in combination against clinical isolates of *C. tropicalis*, FICI of a combination of carvacrol and fluconazole was determined based on the MICs of carvacrol and fluconazole alone and in combination. Through antifungal activity assay, result show that carvacrol in combination with fluconazole exhibited significantly greater inhibitory activity towards clinical isolates of *C. tropicalis*

(Table 1). Specifically, the MIC range for carvacrol alone and in combination with fluconazole which exhibited antifungal inhibition, were 4.00±0.00 μg/ml to 10.00±0.00 μg/ml and 1.00±0.00 μg/ml to 2.50±0.00 μg/ml, respectively for C. tropicalis. Calculation of the FICI produced values of 0.375-1.00, indicating significant synergism, partial synergism and indifferent. The MIC with synergistic and partial synergistic effects of carvacrol and fluconazole were markedly decreased by 4- and 8-fold in *C. tropicalis* ATCC 750 and *C. tropicalis* SN2, respectively compared to the MICs of carvacrol and the fluconazole alone.

Table 1. The combined antifungal effects of carvacrol with fluconazole against *Candida tropicalis* evaluated by FICI.

Antifungal agents	Isolates	MICs (μg/ml)	FICI	Interpretation
Carvacrol	*C. tropicalis* ATCC 750	10.00±0.00		
	C. tropicalis SN1	4.00±0.00		
	C. tropicalis SN2	8.00±0.00		
Fluconazole	*C. tropicalis* ATCC 750	0.50±0.00		
	C. tropicalis SN1	0.25±0.00		
	C. tropicalis SN2	0.125±0.00		
Carvacrol/ Fluconazole	*C. tropicalis* ATCC 750	2.5±0.00/0.0625±0.00	0.375	Synergy
	C. tropicalis SN1	2.00±0.00/0.125±0.00	1.00	Indifference
	C. tropicalis SN2	1.00±0.00/0.0625±0.00	0.75	Partial synergy

Data are means ± standard deviation of three independent experiments.

To determine whether these effects operated in an *in vivo* setting, we conducted the impact of a carvacrol or fluconazole alone and in combination in systemic candidiasis animal model. The efficacy of combination therapy was evaluated based on tissue fungal burden of kidneys and tissue histopathology. We found that mice exposure to carvacrol or fluconazole alone and in combination caused a decrease in the fungal load of kidneys (p <0.01 and p <0.0001) (Figure 1).

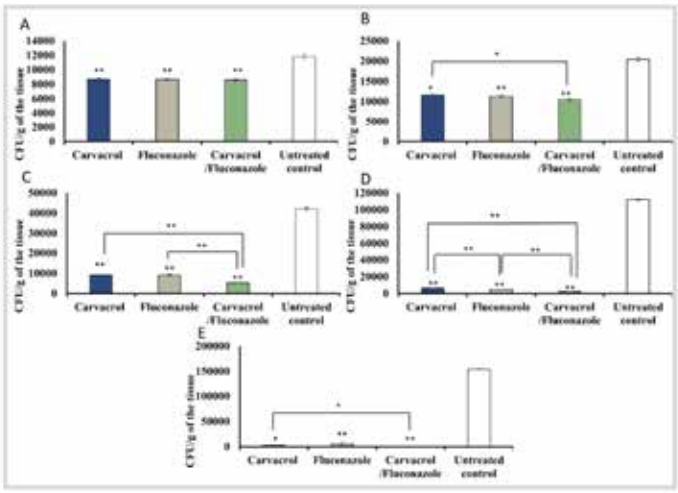

Figure 1. Fungal load of kidneys in systemically infected BALB/c mice treated or not with carvacrol or fluconazole alone and in combination, at days 2 (A), 4 (B), 7 (C), 14 (D) and 30 (E) after infection with *C. tropicalis* ATCC 750. Data are means ± SD of three independent ($*p$ <0.01 and $**p$ <0.0001).

Carvacrol or fluconazole alone and in combination inhibited systemic candidiasis during the first 2 days of infection comparing to their control groups (p <0.0001). There was no statistically significant difference between the two treated groups with carvacrol or fluconazole alone and in combination in the fungal load of kidneys (Tukey post hoc, $p > 0.05$). We also noted a significant difference in Kruskal-Wallis test on the fungal load of kidneys; as expected, carvacrol or fluconazole alone and in combination exposure elicited a decrease in number of the fungal load of kidneys after 4 days of infection (p <0.0001). Post hoc Bonferroni's multiple comparison test indicated a significant reduction of the fungal load of kidneys in carvacrol and combination of carvacrol and fluconazole treated groups ($p < 0.01$). While carvacrol or fluconazole alone and in combination reduced the fungal load of kidneys between treated groups after 7 days of infection (p <0.0001), there was no statistically significant difference in the fungal load of kidneys between the carvacrol and fluconazole treated groups (Tukey post hoc, $p > 0.05$). At 14 days after infection, one-way ANOVA indicated a statistically significant difference between groups (p <0.0001). Interestingly, significant difference between the two treated groups were observed in fungal load of kidneys (Tukey post hoc, p <0.0001). In the kidneys at 30 days after infection indicated a significant reduction of fungal load in treated groups comparing to their control groups. Repeated measures ANOVA indicated considerable difference between two groups of carvacrol alone and

in combination with fluconazole at different time points (p <0.0001). No significant differences were found between two groups after treatment with fluconazole alone (p = 0.058).

The presence of microabscesses with fungal elements was confirmed in kidney tissue in the untreated group. The most predominant forms of *C. tropicalis* in the kidney tissue were yeast mixed with hyphae (Figure 2).

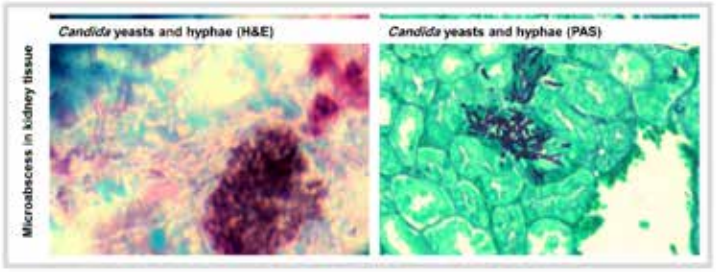

Figure 2. The presence of microabscesses with yeast mixed with hyphae of C. tropicalis in kidney tissue from BALB/c (H&E and PAS ×400).

The kidneys showed moderate congestion and cellular density in the untreated group. Treatments of systemically infected BALB/c mice with carvacrol and fluconazole alone and in combination presented a normal appearance of kidney tissue (Figure 3).

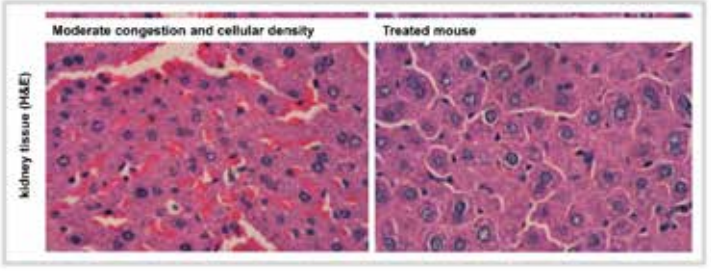

Figure 3. Moderate congestion and cellular density in systemically infected BALB/c mice and treated mouse with carvacrol and fluconazole alone and in combination (H&E ×400).

Through our *in vitro* and *in vivo* assessment, we show carvacrol in combination with fluconazole present a promising effect in the antifungal armamentarium. Our application of carvacrol in combination with fluconazole displayed an increase of their antifungal activity against *C. tropicalis*. The unique chemical defenses of plant phenolic compounds, such as carvacrol, have been a fruitful discovery resource in the past few years[11,26,27]. Inhibition of *C. tropicalis* high-

lights the value of carvacrol as a source of bioactive molecules[17,20,28]. Importantly, carvacrol alone and in combination with fluconazole retain high efficacy in mouse models of systemic *C. tropicalis* infection.

Several research studies have shown that the plant phenolic compounds triggered attachment of the cell surface and penetrate the phospholipid bilayer of the cell membrane afterwards. The accumulation of plant phenolic compounds disturbed structural integrity of cell membrane, and detrimentally affect the overall cell metabolism, eventually leading to cell death. *p*-Cymene is an aromatic monoterpene consists of a benzene ring without any functional groups on its side chains which is the precursor of carvacrol. *p*-Cymene is not an effective antimicrobial when used alone, but it potentiate the activity of compounds like carvacrol[11,27,29]. This effect has been ascribed to the accumulation of carvacrol and *p* □ cymene in the lipid phase of the membrane and caused an expansion of the phospholipids bilayer increasing spaces through which ion leakage might occur[30,31]. In *Escherichia coli,* carvacrol and *p*-cymene impact protein synthesis and cell motility[30].

Carvacrol alone and in combination with fluconazole appear a particularly valuable source of antimicrobials *in vitro* and in systemic candidiasis mouse model. High overall mortality and high rate of antifungal resistance to many known antifungals have made *Candida* infections a global health burden. Combinatorial antifungal therapy has emerged as a promising alternative approach in the treatment of *Candida* infections[32]. Fluconazole block ergosterol biosynthesis by inhibiting fungal cytochrome P_{450}-dependent enzyme lanosterol 14-α-demethylase[33,34]. Many mechanisms involved in the synergistic activity of antifungal agents. The inhibition of different stages in the fungal intracellular pathways that are essential for cell survival, increase uptake of an antifungal agent resulting from the action of another antifungal agent on the fungal cell wall or cell membrane and reach their target fungal DNA, the inhibition of carrier proteins and the simultaneous inhibition of different cell targets[14,35]. Studies demonstrated that carvacrol combined with fluconazole suggested as an approach to achieve synergy and expand fluconazole spectrum[21,22]. The synergistic combination of carvacrol with fluconazole might be explained by the carvacrol promoting the effects of fluconazole, mainly on the cell wall, cell membrane and other membrane structures of yeast. Additionally, carvacrol damage the fungal cell membrane may facilitate the entrance of fluconazole to the cell, thereby leading to a higher effect on inhibition of ergosterol biosynthesis. Moreover, this issue might induce apoptosis in yeast cell by reactive oxygen species accumulation[33;36,37]. This raises the possibility that carvacrol may also turn the fungistatic action of fluconazole into a fungicidal action[33;36].

Many murine models of systemic candidiasis have been reported[25,38,39]. Carvacrol alone and in combination with fluconazole inhibited *C. tropicalis* growth in a murine systemic candidiasis model. Microbiological and histopathological changes suggest that carvacrol alone and in combination with fluconazole can prevent candidiasis by inhibiting growth and morphogenesis of *C. tropicalis*. Carvacrol was previously reported to inhibit hyphal growth[40-43]. The results of our present study were in agreement with the previous report that carvacrol significantly reduced the number of CFU sampled from the oral and vaginal candidiasis in immunosuppressed rats. In addition, no hyphal colonization of the epithelium and lumina of the vagina were seen in animals treated with carvacrol[40,44,45]. Similar results were obtained from Manohar et al[42] experiments.

Antifungals developed from combinatorial therapy are the foundation of modern medicine and have saved millions of lives. The promise of carvacrol in combination with fluconazole as a source of antifungals has the potential to reinvigorate the stagnated antifungal discovery pipelines. Our validation of carvacrol in combination with fluconazole as an alternative approach demonstrates the extensive opportunities for the treatment and prevention of systemic candidiasis after further investigations and sufficient clinical trials.

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Chemical Composition and Anti-scabies Activity of Essential Oil of *Elettaria Cardamomum* Maton. Leaves

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ABSTRACT

The objective of this study was to investigate the chemical constituents of essential oil of *Elettaria cardamomum* leaves and assess its anti-scabies potential.

Essential oil obtained by hydrodistillation of fresh leaves of *E. cardamomum*, subjected to gas chromatography, gas chromatography-mass spectrometry for identification and quantification of components. Anti-scabies potential of essential oil of *E. cardamomum* leaves against *S. scabiei* was investigated by contact bioassay method.

GC and GC-MS analysis results revealed the presence of 44 compounds, representing 96.42% of the oil. The volatile components in leaves of *E. cardamomum* were made up of largely of oxygenated monoterpenes including terpinen-4-ol, eucalyptol, p-cymene, trans-phytol and cis-sabinene. The anti-scabies study revealed that 10% *E. cardamomum* oil showed 100% mortality within 60 min.

This study demonstrated the potential of *E. cardamomum* leaves essential oil as a scabicidal agent, therefore can be used as an alternative for the cure and effective control of *S. scabiei*.

Keywords: *Elettaria cardamomum*, Essential oil, GC, GC-MS, Anti-scabies

INTRODUCTION

Sarcoptes scabiei var hominis "itch mite", family Sarcoptidae, causes a contagious pruritic skin infestation in animals and humans (Scabies). According to WHO, it affects more than 130 million people ubiquitously at any time and prevalence varies from 0.35 to 46% [1]. In tropical regions, it is epidemiologi-

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cally evidenced that, scabies generally cause pyoderma and eventually serious illness due to invasion by opportunistic bacteria. Such infections can lead to cellulitis, bacteraemia and sepsis, kidney and heart disease that will increase health burden in resource-poor communities [2]. Due to suboptimal efficacy of few available therapies, the treatment of *Sarcoptes scabiei* infection is getting hindered in human being [1].

Elettaria cardamomum Maton., cardamom, universally known as "queen of spices", is dried fruit of a herbaceous perennial shrub belonging to the family Zingiberaceae. Cardamom is endemic to South Asia but is mercantily cultivated in Southern India on the shady slopes of Ghats (mostly in Tamilnadu, Kerala and Karnataka), Nepal, Sri Lanka, Guatemala, Thailand, Mexico, Tanzania and Central America [3]. In Indian Ayurvedic system of medicine, it is used for alleviating skin and urinary problems [4]. Cardamom is commonly used as antiseptic, carminative, expectorant, diuretic, breath freshener, desiccant, stomachic, anti-emetic and as an aphrodisiac [5-7]. *E. cardamomum* have been reported to have various biological potential such as antimicrobial [8-9], anti-inflammatory [10], bronchodilator [11], blood pressure lowering [12], gastroprotective[13-17], sedative and anticonvulsant [18], anticancer, antihypertensive, antioxidant and anti platelet aggregation [19].

Due to biological and medicinal importance, the present study is carried out to analyse and characterise the bioactive constituents present in essential oil of leaves of *E. cardamomum* by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) technique and further to assess the *in vitro* anti-scabies potential of essential oil against *Sarcoptes scabiei.*

METHODOLOGY

Plant material

The leaves of *E. cardamomum* were collected from Botanical Garden, Mallah, Morni Hills, India. The leaf samples were identified and authenticated by Dr. Satish Kumar, Taxonomist at Department of Botany, Government College of Girls, Bhodia Khera, Fatehabad, Haryana. A voucher specimen (GJUP-COG160015 I) was preserved in the Herbarium of Department of Pharmaceutical sciences, Guru Jambheshwar University of Science and Technology, Hisar, Haryana, India. The fresh leaves (100g) of were distilled for 6 h to obtain oil by hydro-distillation method using Clevenger's apparatus. The oil was collected over anhydrous sodium sulphate in a glass vial to obtain pure oil, without any traces of moisture and stored at 40 ^{o}C until used [20].

Analysis of the essential oil

The gas chromatographic (GC) analysis of essential oil was carried out using a Shimadzu GC-2010 Gas chromatography equipped with flame ionization detector using Rtx 5 MS capillary column (RESTEK Company: crossbond 5% diphenyl/ 95% dimethyl polysiloxane) having dimensions 30m (Length) x 0.25mm (diameter) x 0.25 μm df (film thickness). The sample (0.2 μL) was injected into the column with a split ratio of 1:100. The analytical conditions were: carrier gas (N 1.21 mL/min, 69.0 kPa), injector temperature 260 ºC, detector (FID) temperature 280 ºC, oven temperature 50 ºC (2 min hold) to 280 ºC (9 min hold) at 3 ºC/min. The retention indices (RIs) were in relation to homologous series of n-alkane (C_9 to C_{33}) on the Rtx 5 MS capillary column under the same chromatographic condition.

GC-MS analysis was performed using GCMS-QP2010 Plus, Shimadzu, system equipped with mass selective detector, having ion source temperature 230 ºC, Interface Temp. – 270 ºC, Solvent Cut Time – 2.50 min threshold of 1000ev and mass range was 40-650 m/z, Rtx 5 MS capillary column and aforementioned chromatographic conditions, with He used as a carrier gas.

Compounds were identified using two methods, one of the methods was based on comparison of mass spectra with the data in NIST or Wiley library. The other one was by comparison of their retention indices (RIs) with those which reported in literature for Rtx 5 MS capillary column.

In vitro anti-scabies activity

Collection of mites

The *Sarcoptes scabiei* mites were isolated from scabes and ear cerumen of infested legs and ears of rabbits under clinical examination by Dr. Snahil Gupta, Assistant Professor, Department of Veterinarian Parasitology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. The morphologically characterized mites were placed in petri dishes and motile adult mites were collected for testing.

Contact bioassay

The essential oil was diluted with paraffin oil to get concentrations of 1%, 5% and 10%. Ten mites were placed in each petri dish and then in petri dishes 1 mL of diluted solution was added in direct contact with adult mites. Three replicates were performed for each concentration of oil. Permethrin 5% was used as a positive control and liquid paraffin was used as a negative control. The mites were inspected under stereomicroscope (Olympus) 20, 40, 60, 80 min after

inoculation. Mites were considered dead when no movement was seen even after touching it with needle and no gut movement was observed over 2 min [1].

Statistical analysis

% Mortality was calculated and expressed as mean±SEM and significance of difference was determined by two-way ANOVA test using Graph Pad Prism.

RESULTS AND DISCUSSION

Essential oil analysis

Hydrodistillation of leaves of *E. cardamomum* generated yellowish green liquid with a yield of 2%. The GC and GC-MS analysis of essential oil of leaves of *E. cardamomum* allowed the identification and quantification of 44 components which accounts for 96.42% of the total oil, as presented in Figure 1. The identified components of the essential oil of *E. cardamomum* as well as their percentage area and retention indices are reported in Table 1. The oxygenated monoterpenes (65.62%) dominated in essential oil, with terpinen-4-ol (32.99%), eucalyptol (19.82%), p-cymene (10.17%), trans-phytol (5.26%), cis-sabinene (2.53%), linalool (2.41%), as the most abundant constituents, followed by oxygenated sesquiterpenes (9.45%) with caryophyllene oxide (6.59%), β-eudesmol (0.99%), trans-nerodilol (0.59%). Sesquiterpenes hydrocarbons (6.04%) slightly prevailed over monoterpenes hydrocarbons (4.30%). The monoterpenes hydrocarbons were mainly represented by β-pinene (1.54%), β-ocimene (0.85%), α-thujene (0.75%), α-pinene (0.53%). β-Farnesene (3.45%) was the major constituent among sesquiterpene hydrocarbons in essential oil of *E. cardamomum* leaves. The findings obtained were compared with those reported earlier on *E. cardamomum* seeds, fruits or seed coat essential oil analysed by GC-MS.

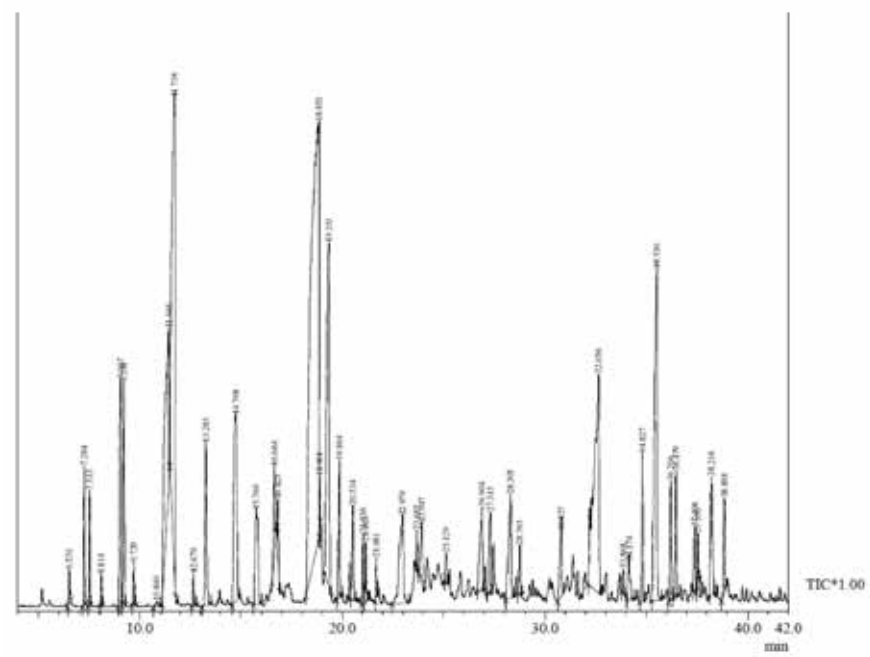

Figure 1. GC-MS chromatogram for essential oil of *Elettaria cardamomum* leaves

Table 1. Volatile components in essential oil of leaves of *Elettaria cardamomum*

Peak No.	Name	Area%	RI[a] (Lit.)	RI[b] (Exp.)	R. Time[c]
1	Heptan-2-ol	0.21	896	903	6.530
2	α-Thujene	0.76	928	928	7.284
3	α-Pinene	0.53	941	930	7.533
4	Camphene	0.12	946	945	8.114
5	cis-Sabinene	2.53	972	970	9.067
6	β-Pinene	1.54	976	975	9.234
7	Myrcene	0.22	993	988	9.729
8	δ-3-Carene	0.11	1011	1015	10.840
9	p-Cymene	10.17	1025	1029	11.446
10	Eucalyptol	19.83	1031	1035	11.738
11	γ-Terpinene	0.19	1063	1057	12.679
12	β-Ocimene	0.85	1050	1070	13.285
13	Linalool	2.41	1102	1104	14.768
14	cis-Menth-2-en-1-ol	0.91	1122	1126	15.769
15	Camphor	0.34	1143	1145	16.664
16	Pinocarvone	1.43	1146	1148	16.825
17	Borneol	1.56	1165	1170	17.703
18	Terpinen-4-ol	32.99	1177	1174	18.851
19	Cryptone	0.76	1192	1189	18.901
20	α-Terpineol	2.37	1193	1203	19.353
21	trans-Piperitol	0.61	1208	1214	19.864
22	Nerol	0.55	1226	1229	20.534
23	Ascaridole	0.22	1237	1240	21.036
24	4-Phenyl-2-butanone	0.21	1251	1243	21.163
25	trans-2-Decenal	0.20	1265	1254	21.681
26	Bornyl acetate	0.59	1286	1283	22.979
27	Carvenone	0.29	1252	1298	23.685
28	Isoledene	0.38	1377	1304	23.947
29	cis-Methyl-cinnamate	0.78	1380	1372	26.904
30	Methyl cinnamylate	0.37	1394	1381	27.315
31	Caryophyllene	0.86	1417	1415	28.765
32	α-Bergamotene	0.43	1433	1465	30.827
33	β-Farnesene	3.45	1455	1510	32.656
34	β-Bisabolene	0.16	1506	1540	33.868

35	γ-Cadinene	0.34	1513	1543	33.927
36	trans-Nerodilol	0.59	1569	1548	34.174
37	Caryophyllene epoxide	0.20	1580	1565	34.827
38	Caryophyllene oxide	6.06	1582	1583	35.530
39	Carotol	0.45	1587	1600	36.206
40	Humulene epoxide	0.51	1594	1607	36.479
41	Caryophylladienol II	0.27	1631	1632	37.404
42	β-Eudesmol	0.99	1641	1636	37.550
43	Guaiyl acetate	0.36	1712	1654	38.216
44	trans-Phytol	5.26	2099	1988	38.858

[a]RI, programmed temperature retention index as determined on Rtx 5 MS capillary column using a homologous series of n-alkanes (C_9 to C_{33});
[b]RI, Identification was based on the compound of retention indices with those of published data (NIST);
[c]Retention Time.

Ashokkumar et al. [21]characterised essential oil content of four varieties of *E. cardamomum* capsules, of which 1,8-cineole (28.94%–34.91%), sabinene (11.17%–13.50%), α-terpineol (12.47%–14.89%) and α-terpinyl acetate (26.68%–29.60%) constituents were detected as major constituents and also reported their use in aroma, food, pharmaceutical and cosmetic domains. Han and Parker [22] showed the presence of α-terpinyl acetate (38.00%), linalyl acetate, 1,8-cineole/eucalyptol (36.00%) in *E. cardamomum* essential oil and demonstrated anti-inflammatory and immune modulatory potential due to presence of eucalyptol. Iranian *E. cardamomum* essential oil showed the presence of α-terpineol acetate (11.78%), nerolidol (8.82%), linalool (10.15%), α-pinene (8.11%), 1,8-cineole (4.25%), geranyl acetate (3.47%), γ-terpinene (3.88%), according to Asadollahi-Baboli and Mani-Varnosfaderani[23]. Kaskoos et al.[24] characterised essential oil of *E. cardamomum* fruits and recorded monoterpenes (87.60%) of total volatiles such as 1,8-cineole (35.60%), α-terpineol (4.90%), α-terpinylacetate (27.10%), thujyl alcohol, linalool (4.10%) and sesquiterpenes as valencene (1.00%), t-caryophyllene (0.80%).

Gradinaru et al. [25] analysed *E. cardamomum* fruit essential oil and α-terpinyl acetate (39.59%), 1,8-cineol (31.27%) were found as major constituents. Study also revealed that oxygenated monoterpenes (84.54%) were dominant over monoterpenes (8.27%) and also investigated combination effect of *E. cardamomum* fruit essential oil with amoxicillin or ciprofloxacin against methicllin-resistant clinical isolates. The volatile oil of seed of *E. cardamomum* was reported to have pinene (2.80%), sabinene (1.60%), myrcene (36.00%), 1,8-cineole, graniol and terpinyl acetate as the major constituents [26-27]. These

differences in chemical composition could be due to different distillation technique used, climate or growing conditions of plant.

In vitro anti-scabies activity

The *E. cardamomum* essential oil was evaluated for their *in vitro* anti-scabies potential against *S. scabiei* mites. % Mean mortality for the mites treated with three concentrations of oil is presented in Table 2. *E. cardamomum* essential oil demonstrated scabicidal potential as its 10% concentration caused 100% mortality within 60 min whereas 5% diluted solution took 80 min to kill all the mites. Based on % mean mortalities study, it was found that Permethrin (reference) killed all the mites within 60 min but in negative control group, mortality was only 1.58% and most of mites remained alive after 80 min of treatment. The scabicidal effects produced by *E. cardamomum* essential oil were significant ($p < 0.0001$) as compared with the respective control groups as demonstrated in Figure 2.

Table 2. *In vitro* anti-scabies activity of *E. cardamomum* essential oil against S. *scabiei*

Test agent	Conc.	% Mortality (mean± SEM)			
		20 min	40 min	60 min	80 min
E. cardamomum essential oil	1%	30.00± 0.57***	42.66± 0.56***	57.33± 1.00***	80.34± 0.59***
	5%	39.44± 1.53***	48.14± 1.15***	86.14± 0.57***	100.00± 0.00***
	10%	47.81± 1.15***	85.33± 0.99***	100.00± 0.00***	100.00± 0.00***
Positive control		68.00±0.20	89.00± 0.40	100.00± 0.00	100.00± 0.00
Negative control		00.00 ± 0.00	00.00 ± 0.00	01.58±0.08	01.58± 0.08

Data are expressed as mean±SEM; n=3
***, $p < 0.0001$ indicates highly significant results

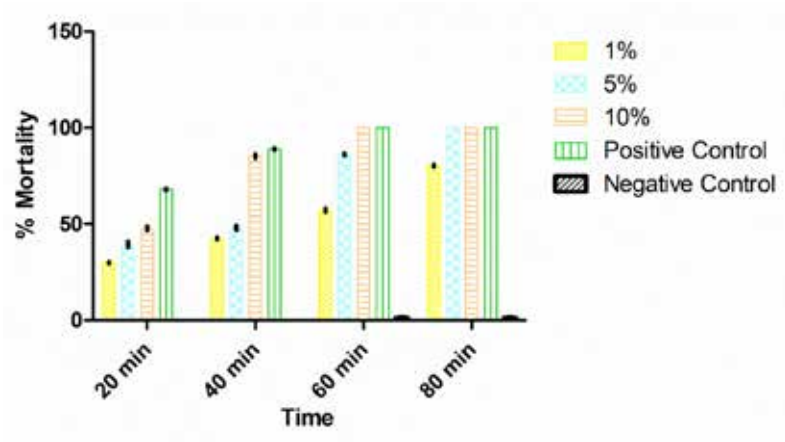

Figure 2. Anti-scabies activity of *E. cardamomum* leaves essential oil against adult *S. scabiei.* Data are analysed by two-way ANOVA and expressed as mean±SEM, n=3.

Adupa et al. [28] reported the toxicant, fumigant and repellent potential of eucalyptol against the maize weevils. Abbassy et al. [29] study inferred the pronounced insecticidal activity of terpinen-4-ol and γ-terpinene against tested insects *Spodoptera littoralis* and *Aphis fabae* L.

It was also found that eucalyptol enhances the superoxide dismutase and glutathione-s-transferase enzymatic activity, which play a role in protection mechanism of *S. scabiei* mites [30]. The natural components i.e. terpinen-4-ol, γ-terpinene and eucalyptol have been reported to exhibit insecticidal activity may be responsible for anti-scabies potential of *E. cardamomum* oil. Fang et al. [1] studied ten essential oils and reported that 1% clove and palmarosa oil killed all the motile mites within 20 and 50 min, respectively. Using contact bioassay, clove oil (1.56%) killed all the mites after exposure of 15 min while nutmeg oil showed moderate toxicity against scabies mites [31]. Aboelhadid et al. [32] stated that 20% lemon oil caused 100% mortality of mites after 24 h and also investigated the elevation in hydrogen peroxide level that leads to considerable cellular damage. *Elsholtzia densa* also found to possess acaricidal potential against *S. scabiei* and at 16 mg/mL concentration killed all the mites within 16 h period [33].

A number of previous studies have been performed on fruits and seed of *E. cardamomum*. The present study is an attempt made to identify the constituents of volatile oil of *E. cardamomum* leaves and find its utilization for anti-scabies activity. The chemical composition of the leaves is more or less similar to fruits and seeds though the concentrations of the constituents vary. Furthermore, in

view of its action against *S. scabiei* mites, it may prove to be beneficial to the patients with scabies disease and could be explore as alternative to current medicines.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Intralesional Microemulsions for Effective Dermal Delivery of Chondroitinase: Formulation, Characterisation and Evaluation

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ABSTRACT

The rationale behind the microemulsion (ME) formulation was to achieve the efficacy of intralesional delivery and permeability of chondroitinase (CHASE) along with enhanced stability over conventional delivery mode and thus patient compliance. The formulation proceeded with the pseudo-ternary phase diagrams construction to identify microemulsions regions and to establish unique ratio of oil surfactants and aqueous phase. The components used for microemulsion formulation were oleic acid as oil phase, Tween 80 and propylene glycol as surfactant-cosurfactant mixture and phosphate buffer pH 7.4 as aqueous phase. The concentration of CHASE in microemulsions was 2.96 U/mL which was stabilised with 1M trehalose solution (w/w). The physicochemical properties of microemulsion were determined. The localization of CHASE within the dermal membrane was determined by in vitro permeability study using excised porcine ear skin. The permeation and the penetration properties of CHASE loaded microemulsion were compared with stabilised aqueous solution of CHASE for varied duration.

Keywords: Chondroitinase, intralesional, dermal delivery, *in-vitro* permeability.

INTRODUCTION

Chondroitinase ABC (*chondroitinase, cABC, ChABC or CHASE*) is an enzyme with systematic name *chondroitin ABC lyase* that specifically acts as chondroitin depolymerizer and also effective on other proteoglycans *viz.* dermatan sulfate, keratan sulfate and hyaluronate in lowering their levels in irregular extracellular matrix (ECM) formation during cartilage repair process in burns and accident, without disturbing other ECM components. Proteoglycans are the molecules with strong negative charge that attract positive ions

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and drag water ions into ECM during cartilage repair process. However, over-production of proteoglycans inhibits the production of collagen II. The high levels of proteoglycans and low collagen II levels results in cartilage formation with poor biochemical and mechanical strength compared to native cartilage. The treatment of cartilage constructs with CHASE results in more collagen II production, thus the mechanical properties of the repaired cartilage would be closer to that of native articular cartilage. This concept is also applicable for efficient ECM mass formation with the optimum level of proteoglycans and collagen in case of skin recovery after burns and accident.

Intralesional drug delivery is an approach in which medications are injected percutaneously into skin lesion more specifically, at epidermal and dermal membrane with minimal systemic effects and for faster therapeutic effect. The sub-epidermal depot establishment to bypasses the superficial barrier zone is the rationale of this therapy. Here skin enable to deposit medication in the dermal layer as a reservoir and medicaments would be delivered over duration of time, thus achieving prolonged therapy with the avoidance of adverse effects of systemic therapy.[1]

Microemulsion (ME) is drug delivery systems with several advantages such as high stability, enhanced drug solubility, manufacturing ease including enhanced percutaneous penetration of drugs.[2] These microemulsions loaded with specific drugs if given intralesionally, could exert the potential enhancing effect than the conventional therapy to treat lesions like keloids and hypertropic scars (HSc) till deeper part of skin via percutaneous route.[3,4] The interaction of oil with the lipids in the *stratum corneum* results in enhanced fluidity thus increased drug mobility and penetration of surfactants through the skin enhances cutaneous delivery of drug by increasing the partition coefficient of the drug between skin and formulation medium so that drug can be incorporate efficiently at target site[5]. Based on these facts work was aimed to formulate an intralesional microemulsion system for percutaneous delivery of CHASE enzyme. Thus, based on our earlier studies, a stable colloidal system was composed of oleic acid as oil phase, Tween 80 and propylene glycol as surfactant-cosurfactant mixture and phosphate buffer (pH 7.4) as aqueous phase along with stabilised CHASE enzyme as drug entity[6]. The prepared formulation were evaluated for its physicochemical properties including *in vitro* release and *ex vivo* skin retention studies, physical and microbiological stability were evaluated and compared with CHASE solution in phosphate buffer pH 7.4.

METHODOLOGY

Materials

The enzyme chondroitinase ABC (CHASE), reported here, was isolated from *Proteus penneri* SN5, a novel and non-virulent strain. It was further purified, lyophilised and characterised as initial study performed previously.[7] Sodium phosphate buffer solution pH 7.4 (PBS) was procured from Fresenius Kabi, Pune (India). All substances, chemicals and solvents used during study were analytical grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai (India).

Development of Microemulsion Formulations

Selection of oil, surfactant, and cosurfactant

Based on the preformulation and solubility studies performed previously.[6] Oleic acid, Tween 80 and propylene glycol (PG) and phosphate buffer solution (PBS) pH 7.4 was used as components in microemulsion formulation. These excipients are listed under GRAS category (generally regarded as safe) for injectable formulations by US FDA.

Construction of pseudo-ternary phase diagrams

Pseudo-ternary phase diagrams were constructed using CHEMIX school 7.0 software. The surfactant and cosurfactant used were Tween 80 and PG, having nonionic and hydrophilic nature. The pseudo-ternary phase diagrams of oil, surfactant, cosurfactant, and PBS pH 7.5 were constructed using phase titration method to obtain the components and their concentration ranges resulted as microemulsion region with large existence.[8] The blend of surfactant with cosurfactant was prepared in specific weight ratios (1:1 and 2:1). The blend of surfactant and cosurfactant (S_{mix}) was then mixed with oil at ambient temperature (25°C). The ratios of oil to Smix were varied as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (w/w) for each phase diagram. By using magnetic stirrer (Remi Equipments Pvt. Ltd. Mumbai, India) vigorous stirring of each mixture was performed with the drop wise addition of PBS pH 7.4 at same time at 25°C. The heating was avoided during the preparation and it was followed with visual observation of each mixture for transparency. These samples were remarked in the phase diagram as points. The region occupied by these points was named as the microemulsion region. All trials were performed in triplicates.

Process optimisation of o/w type microemulsion

The microemulsion formulations were selected at desired component ratios in view of oil in water type microemulsion formulation with the identification of microemulsion region as per ternary phase diagram. The specific concentration range implying o/w type microemulsion was found to be 1:5 for oil and S_{mix} as per preliminary trial batches. Certain formulation within this ratio with Oil–S_{mix}–PBS pH 7.4 was prepared and the process for the preparation of microemulsion was optimized by central composite design method. The impact of variable factors such as stirring speed (200 to 800 rpm) and time (5 to 20 min for each stirring speed), over the globule size was considered as response[9].

Central Composite Design as Experimental Design in process optimisation of microemulsion

Response surface methodology (RSM) based central composite design **(**CCD) is a combined technique of mathematics and statistics in designing the experiments and to investigate the factors of process including their interaction between and among variables to find optimum suitable condition to design the model.[10] A factorial CCD for different factors along with their replicates at the centre point was used for optimisation studies. The variables used were stirring speed (200 to 800 rpm) and time (5 to 20 min for each stirring speed) at five coded levels ($-\alpha$, –1, 0, +1, $+\alpha$) as shown in Table 1.

Table 1. Real and coded variables used for process optimisation in central composite design (CCD).

Name of independent variables	Levels				
	Axial ($-\alpha$)	Lower (-1)	Central	Higher (+1)	Axial ($+\alpha$)
Stirring speed (rpm)	75.74	200	500	800	924.26
Time (min)	1.89	5	12.5	20	23.11

Preparation of microemulsions

To prepare the drug loaded microemulsions required quantity of *CHASE* (370.38 U/mg activity) at the concentration of 2.96 U/mL [CS degrading unit, CSU] in presence of 1M trehalose was dissolved in the mixture of Tween 80 and propylene glycol (2:1). It was followed with slow addition of oleic acid to form a transparent blend. Then the PBS pH 7.4 was added to the clear oil-surfactant solution drop by drop. The o/w microemulsions containing *CHASE* were obtained under stirring the mixture using magnetic stirrer (Remi Equip-

ments Pvt. Ltd. Mumbai, India) at 25°C with optimized stirring speed and duration.[11] The evaluation of developed microemulsion was performed further on the basis of zeta potential, globule size, % permeability and % drug release.

In order to obtain an aqueous solution *CHASE* (370.38 U/mg activity) at the concentration of 2.96 U/mL [CS degrading unit, CSU] with 1M trehalose was dissolved in PBS pH 7.4.

Characterization of Microemulsion Formulations

Surface Morphology by TEM

Surface morphology of globules was observed by means of transmission electron microscopy (TEM) (LVEM5, Delong Instruments, Canada) at 100 kV. The optimized microemulsion was diluted 10 times with distilled water. A drop of diluted sample was placed on a 200-mesh film grid to dry at room temperature. The sample was further stained with 2% phosphotungstic acid solution. Further, it was allowed to dry for 5 min before observation under the electron microscope.[12]

Globule Size, zeta potential and Polydispersity Index (PDI)

The average globule size and PDI of microemulsions were determined by a laser-scattering method (Nano ZS® 90, Malvern). To avoid multi-scattering phenomena, the samples were diluted suitably with distilled water. The droplet size of the diluted microemulsions was not significantly changed. These investigations were performed as per manufacturer's specifications.

Viscosity, pH and Refractive Index (RI)

The viscosity of the formulation was measured by using Oswald-type viscometer (Techniko 841, D) while the pH of microemulsions was determined using a pH meter (Systronics, MK VI). The procedure was repeated three times and the average was taken for calculation. The refractive indices were determined to evaluate the isotropic nature of microemulsions by means of Digital Abbe refractometer (Rudolph Research J257, USA). The refractive index was measured under by placing a drop of microemulsion on the lens of refractometer and observation was done. All measurements were performed at 25±0.5 °C.

Dye solubility test and dilutability

This test specifically performed to determine the type of microemulsion. About 4 to 5 drops of methylene blue (water-soluble dye) was added to the test tube containing micro emulsion formulation and visual observation was done after 5 min.[13] The microemulsion were further diluted in 1:10 and 1:100 ratios with double distilled water to check if the system shows any signs of separation.[14]

Syringeability and injectability

The evaluation of syringeability and injectability was performed on qualitative basis by using insulin syringe. The ease with which the formulation pass through the needle was considered as the basis to qualify syringeability while injectability was evaluated on the basis of the ease with which the formulation was injected into excised porcine ear skin tissue weighing about 2.0 g which was earlier processed under in vitro permeation.[15-16] About 1 mL of formulation was drawn into a insulin syringe with a 30 gauge needle (AccuShot, SPM Medicare) and injected into the muscle. The assessment of syringeability and injectability was performed based on the following scores:

- +++Easily passed/injected
- ++Moderate
- +Difficult

Sterilization and Control of the Sterility of the Microemulsions

Blank and CHASE loaded microemulsions were sterilized by aseptic filtration method through 0.22 μm pore-size disposable syringe filter (Minisart, Sartorius) under Class II Laminar airflow Cabinet (Cleanair, Chennai).[17] The sterilized formulations were kept in sterile vials until completion of all experiments. After sterilization process and at the end of the 12 months the sterility testing of the microemulsions was checked to determine the presence of viable forms of microorganisms. To carry out sterility test, 100 μl of formulation was inoculated on Blood and Eosin Methylene Blue agar medium (Himedia) by using Drigalski's loop. *Escherichia coli* ATCC 25922 strains was used to control the agar mediums for positive control. The plates were incubated in the incubator (ILECO, Chennai) for 48 h at 37°C and bacterial colony counting techniques were used to evaluate sterility. All the experiments were carried out in triplicates.[18]

Thermodynamic Stability

Centrifugation

The microemulsion formulations were centrifuged at 3500 rpm using centrifuge (REMI, Mumbai, India) for 30 min and the phase separation is detected if any.[19]

Freeze - thaw Cycle

These microemulsions were further subjected for six alternate heating and cooling cycles between 25°C and –4°C with storage at each temperature for not less than 24 h and assessed for their physical instability like phase separation, precipitation or any colour change.[19]

Drug content estimation

The microemulsions were diluted with PBS to obtain required drug concentration; the absorbance was determined using UV spectrophotometer (Systronics 2202) at 232 nm. Against placebo formulation treated in the similar manner as blank.[6]

Concentration of sample = (Absorbance of sample * Concentration of standard) / Absorbance of standard.

Skin penetration and percutaneous delivery

Collection and processing of skin tissue

The skin penetration and percutaneous delivery of CHASE microemulsions were assessed in an in vitro model of porcine ear skin, as per earlier performed studies.[20] Briefly, the porcine ear skin was collected anonymously from local abattoir. For anonymously collected pig skin samples from slaughter house, approval of institutional Review Board is not required. The tissue was transported to the laboratory in transporting fluid Dulbecco's Modified Eagle Medium (DMEM). It was further thoroughly washed with water and wrapped in cover of aluminium foil and stored in deep freezer. The skin was processed within 24 h. The skin tissue was washed with water and further it was mounted on a Franz diffusion cell (diffusion area of 1.44 cm^2), with the *stratum corneum* facing donor compartment and the dermis facing the receptor compartment.[21] The latter compartment was filled with saline solution (0.9% NaCl) with a water jacket 32 ± 1°C.

In vitro skin permeation (percutaneous) study

The 10 µl of the CHASE microemulsion formulations was injected just beneath of *stratum corneum* using an insulin syringe with needle of 30 gauges. At 6 and 12 h post-injection of microemulsion, removal of excess formulation on skin surfaces was washed thoroughly with distilled water and wiped by means of cotton swab. The *stratum corneum* (SC) was separated from the remaining epidermis (E), dermis (D) and skin sections by performing tape stripping technique. The skin was stripped with 10-12 pieces of adhesive tape, the initial one was discarded, and the next one containing the SC were immersed in Eppendorf containing 4 mL PBS pH 7.4, vortexed and bath sonicated for 15 min and this solution was filtered using a 0.22µm membrane. Further, the remained [E + D] membrane were cut in small pieces, vortexed and sonicated for 30 min. The resulting mixture was then filtered using a 0.22µm membrane, and these both filtrate (SC) and [E + D] was further assayed to determine CHASE content at 232 nm by UV spectrophotometer. Aqueous solution of CHASE (0.3% w/w) stabilised with 1M trehalose in PBS pH 7.4 was used as control formulation.[21]

Stability Studies

The formulation under investigation includes the enzyme as biological entity. Hence CHASE microemulsions prepared in the present study were refrigerated for storage. Stability on storage under the following conditions was assessed according to ICH guidelines for stability studies on biopharmaceuticals.[6] The optimised formulation was packaged in glass vials and stored at 5±1°C in the refrigerator and 25±2°C at room temperature for 2 months. Samples were withdrawn at the end of 1st and 2nd month from both storage conditions. They were investigated for changes in clarity, precipitation, phase separation, viscosity, pH, globule size, zeta potential, and drug content.[22] The experiments were repeated in triplicates.

RESULTS AND DISCUSSION

Selection of oil, surfactant, and cosurfactant

The HLB value of Tween 80 is 15 and for propylene glycol it is almost not applicable. As per concern o/w microemulsion, usually high HLB surfactant should match with low HLB co-surfactant.[23] In this study, oleic acid used as oil phase, tween 80 as surfactant and propylene glycol as cosurfactant and PBS pH 7.4 solutions as water phase in o/w type microemulsions formulation. Oleic acid has been approved for clinical use in parenteral based formulations for a long time.[24-25] due to its tissue compatibility and high skin permeability. Tween 80 and propylene glycol were selected as an ideal surfactant since non ionic surfactants and cosurfactants do not undergo ionisation at any extent in solution. They are also approved for their use in parenteral formulations by US FDA. The presence of cosurfactants also contributes its role in maintaining the efficient flexibility of the interfacial film.[26]

Construction of pseudoternary phase diagrams

In the present study, phase diagrams were constructed to select the concentration range of components for the microemulsions, and the marked areas indicate the clear and viscous microemulsion region (Fig. 1). The major driving force for the release and the penetration of the drug into skin was appreciated with lower surfactant mixture content and this was demonstrated by the thermodynamic activity of drugs in microemulsion.[27-28] The lower concentration of surfactant in dispersed systems essentially enhances drug release rate and skin permeability.[29] The individual studies also reported that the formulation containing maximum amount of water with optimum surfactant mixture ratio contributes in highest skin flux and permeability coefficient. Thus, the water content ranged between 60-80% and 10-30% of S_{mix} probably would be the

efficient ratio to obtain cutaneous delivery of microemulsion. The domain of microemulsion was investigated by visual inspection in terms of clarity and fluidity. It is well known fact that by increasing concentration of Tween 80 incorporation of water can be increased but drug solubility decreases while by increasing concentration of PG drug solubility increases but incorporation of water decreases. Hence the highest microemulsion area was obtained with ratio of 2:1 and thus selected for further studies. The phase diagrams of different microemulsions are shown in Figure 1.

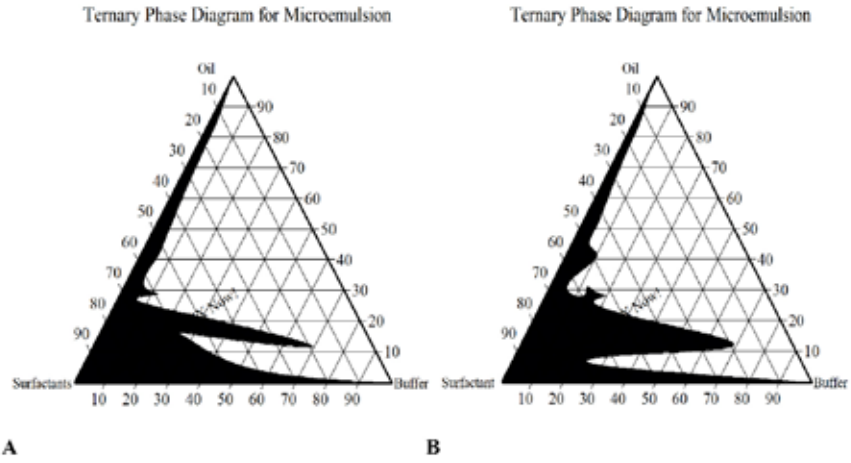

Figure 1. Pseudo-ternary phase diagrams of the oil-surfactant-water system 1:1 (A) and 2:1 (B) weight ratios of Tween 80 to propylene glycol at 25ºC. The dark area represents microemulsion regions.

Process optimisation of o/w type microemulsion by central composite design (CCD)

After applying the CCD design for the process optimization of microemulsion, the results indicated that the magnetic stirring speed and time is having profound effect on the globule size reduction in microemulsion. The magnetic stirring speed and duration were found the crucial parameters in effective globule size for o/w type microemulsion with considered ratio. The results of this process are summarised as below in Table 2.

Table 2. The experiments design trial used for process optimisation for effective globule size

No.	Stirring speed (rpm)	Time (min)	Globule size (nm)
1	500	12.5	126.54
2	500	23.11	123.86
3	500	12.5	126.12
4	800	20	118.36
5	500	10.8	124.28
6	500	12.5	125.94
7	924	12.5	117.88
8	500	12.5	126.74
9	76	12.5	138.44
10	200	20	133.16
11	200	5	130.25
12	800	5	123.88
13	500	12.5	126.27

Analysis of variance and fitting of data to the model

In order to clarify the impact of the independent variables according to the results, response surface and 3D plots of the quadratic polynomial model were created by varying two of independent variables (within the experimental range) for studying the combination effect on response. It showed the statistical significance of adding new terms step by step in increasing order. It provided accounts of variation and associated P-values (Prob > F). The model was selected based on the highest order that was significant (P-value small) and not aliased, on lack of fit (P-value > 0.10) and the Pred-R^2 value of 0.9289 was in rational agreement with the Adj. R^2 of 0.9815. The difference was less than 0.2. Value of lack of fit was 0.159 > 0.05 (not significant) showing that the hypothesis of significance of fitting was rejected for RSM method. ANOVA results are illustrated in Table 3.

Table 3. Analysis of variance of the regression coefficients of the quadratic equation for globule size.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Model significancy
Model	403.027	5	80.61	128.38	< 0.0001	significant
A-Stirring speed	381.518	1	381.52	607.65	< 0.0001	
B-Time	0.403	1	0.40	0.64	0.4493	
AB	2.941	1	2.94	4.68	0.0672	
A^2	3.225	1	3.22	5.14	0.0578	
B^2	12.945	1	12.94	20.62	0.0027	
Residual	4.395	7	0.63			
Lack of Fit	3.983	3	1.33	12.90	0.159	not significant
Pure Error	0.412	4	0.10			
Corrected Total	407.422	12				

The analysis result by ANOVA was with 95% confidence displaying F value model was 128.38 and if the value of P lower than 0.05, that means the predicted model was substantial. Moreover, the lowest Press value 28.97 of this model in comparison with others ensures fitting of this model. All the responses observed for nine runs were fitted to various models using response surface methodology (Design Expert software). It was observed that the best fitted model was polynomial quadratic. In primary observation, the 3D surface plot behaviour gives information that the globule size could be minimised by incresing stirring rate for longer time (Fig. 2).

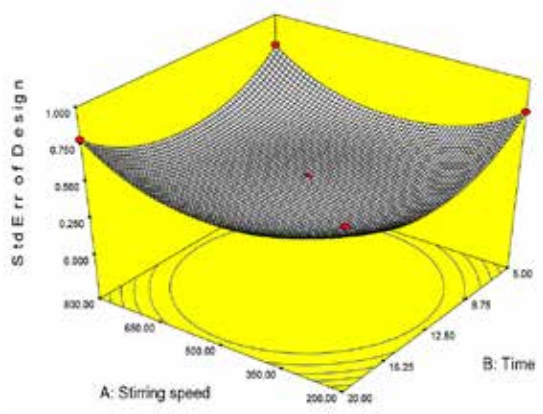

Figure 2. The 3D surface plot behaviour indicating interaction between stirring rate and time.

Optimised Condition Specifications

Obtaining the best conditions for the o/w microemulsion formulation with effective globule size was obtained by setting each variable in range via numerical optimization. Accordingly, the first most solution was selected as per higher desirability resulted with minimal globule size. Thus, the experimental value of optimised stirring speed of 800 rpm with 20 min duration was 118.78±0.27 nm (predicted value: 118.10 nm) as given in Table 4.

Table 4. Validation of most desirable solution obtained while optimization.

No.	Stirring speed (rpm)	Time (min)	Globule size (nm)		Desirability	Remark
			Predicted value	Experimental value		
1	800	20.0	118.10	118.78±0.27	0.9893	Selected
2	800	18.5	118.72		0.9593	
3	800	5.0	119.37		0.9277	
4	800	6.3	119.69		0.9119	
5	800	11.4	120.16		0.8890	

Development of CHASE microemulsion formulations

Parenteral microemulsions may be utilized for the drug targeting and to decrease the toxic effects to tissues.[30] The potential of parenteral microemulsions has been explored so far primarily to prolong the mean residence time of drugs administered intralesionally [24]. The administration of oil-in-water (o/w) formulations could be beneficial in the parenteral drug delivery, since the attendance of surfactant and co-surfactant raises permeability, thus improving drug uptake. From pseudo ternary study, surfactant and co-surfactant having 2:1 ratio selected for micro emulsion batches formulation. CHASE which acts as core enzyme drug (activity: 370.38 U/mg) was freshly thawed from -20° C by putting it on ice and about 5 mg was immediately dissolved in 5 mL of 1M trehalose solution. From this stock solution enzyme drug equivalent to 2.96 units was utilised in microemulsion formulation. According to constant oil:S_{mix} ratio (1:5) the batches were developed and evaluated for various tests. The clear microemulsion was obtained without phase segregation after addition of drug and additive. The developed microemulsions were filtered through 0.2 mm sterile syringe filters under sterile conditions. The sterile microemulsions were kept in sealed sterile vials until the experiment (Fig. 3). The CHASE microemulsions with different composition phases along with their % weight is summarised (table 5) with pictorial view.

Table 5. The percentage weight and batch composition of each microemulsion formulation.

Formulation code	Oleic acid (%)	Tween 80: Propylene glycol (2:1) (%)	Phosphate Buffer Saline (PBS) pH 7.4 (%)
CHASE ME1	3.0	15	82
CHASE ME2	3.5	17.5	79
CHASE ME3	4.0	20	76
CHASE ME4	4.5	22.5	73
CHASE ME5	5.0	25	70

**The final amount of CHASE in each microemulsion formulation was 2.96 U/mL [CS degrading unit, CSU].*

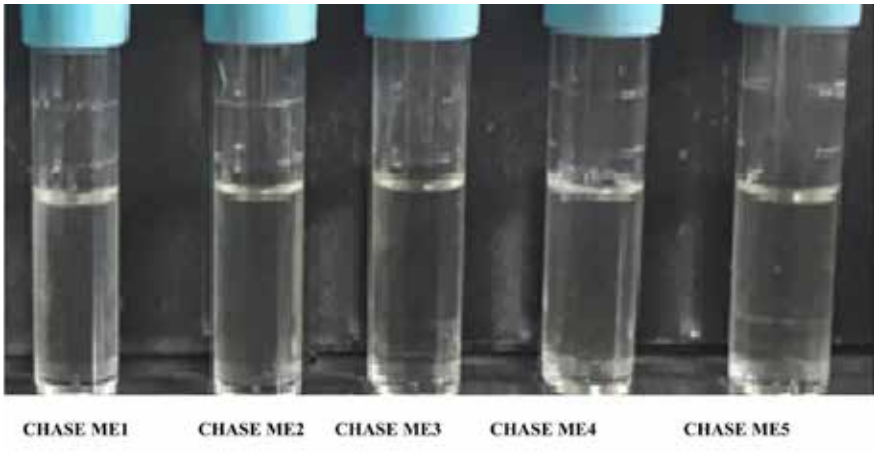

Figure 3. Image of CHASE ME formulations with different phase and % weight ratio

Characterization of Microemulsion Formulations

Surface Morphology by TEM

In principle, when a drop of microemulsion is place on a TEM copper grid and allowed to dry, rearrangement of particles may takes place during drying process. TEM analysis revealed that globules of formulation were almost homogenous and spherical shaped. The globules in the CHASE microemulsion were appeared to be dark spheres with bright background (Fig. 4). These results indicates that a large number of globules were in range of nano size (less than 200 nm). It was further concluded that these oil globules were well dispersed in continuous phase without any aggregation. However; the globule size distribution of microemulsion was not uniform.

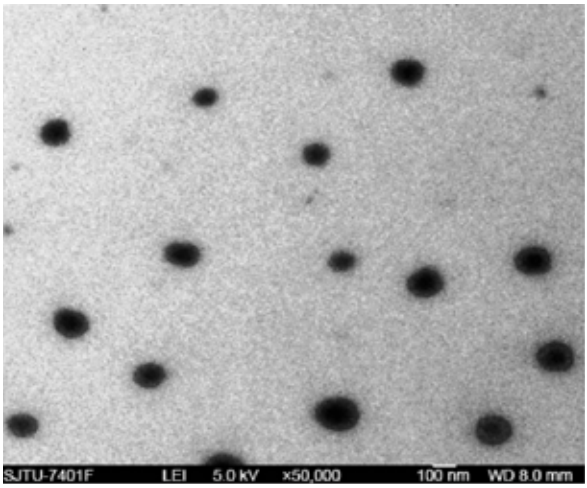

Figure 4. TEM photograph of CHASE ME formulation.

Globule Size, zeta potential and Polydispersity Index (PDI)

Globule size of microemulsions was found in the range of 108-111 nm (Table 6). This indicated a uniform microemulsion with a narrow size distribution. In addition, droplet size increased in proportion with the oil content while surfactant mixture lowers its content. This phenomenon may be attributed to the expansion of the microemusion oil core, and the high surfactant concentration forcefully reducing the oilwater interfacial tension to reduce droplet size. It also can be seen the range of ζ-potential of -0.117 to -0.123. This negative charge value of ζ-potential was resulted probably due to carboxyl groups of oleic acid, also due to several functional groups with high electronegativity such as hydroxyl, amide and carboxyl groups of CHASE enzyme. The negativity of ζ-potential is a sign of longer stability of microemulsion due to electrostatic repulsion of individual particles.[31]

The polydispersity index (PDI) of the microemulsions was observed in the range of 0.186-0.198 that indicates narrow size distribution of water globules In general, PDI range from 0.01- 0.5 is considered as the narrow size distribution of globules and with PDI > 0.7 indicates very broad size distribution.[33]

Viscosity, pH and Refractive Index (RI

The stability of the microemulsion is often governed by its viscosity, i.e., is an expression of the resistance to flow. The viscosity refers the tendency of microemulsion system to form agglomeration. It is fundamental fact that the viscosity of parenteral formulation may also affect the syringeability particularly in case of intralesional route.[15] Using the equation (1), the viscosity of the

developed formulated was calculated to be 8.76 to 10.79 cP. The viscosity was calculated from the equation:

$$\frac{\eta 1}{\eta 2} = \frac{\rho 1 t 1}{\rho 2 t 2} \quad \text{---------- equation (I)}$$

where η_1 and η_2 are the viscosities of the test and the standard liquids, ρ_1 and ρ_2 are the densities of the liquids, and t_1 and t_2 are the respective flow times in seconds.

The pH values of microemulsions were varied from the range 5.99 to 6.40 (Table 6). For any therapeutic delivery, such pH values could be considered in the tolerable/acceptable range. The pH of the formulation was in an acceptable range for intralesional administration.[34] The RI of microemulsions was determined which were found in range of 1.3126 and 1.3602 (almost nearer to the RI of the oils used) which demonstrates the isotropy (homogeneity) of the formulation.[32] These characterisation parameters are detailed in Table 6.

Table 6. Characterisation studies of different CHASE microemulsions (mean ± SD, n=3).

Formulation code	Globule size (nm)	Zeta potential (mV)	Polydispersity index	pH	Viscosity (cP)	Refractive index
ME1	108.86±1.23	-0.117±0.005	0.186±0.006	6.40±0.01	8.7611±0.023	1.3126±0.0001
ME2	109.18±1.59	-0.120±0.003	0.194±0.009	6.34±0.01	9.2343±0.014	1.3202±0.0001
ME3	110.17±0.21	-0.118±0.002	0.195±0.004	6.24±0.01	9.7888±0.011	1.3416±0.0002
ME4	110.79±0.75	-0.123±0.006	0.196±0.004	6.05±0.05	10.2665±0.006	1.3584±0.0002
ME5	109.83±0.75	-0.118±0.005	0.198±0.006	5.99±0.02	10.7936±0.012	1.3602±0.0002

Dye solubility test and dilutability

It is also called as the stain test in which specific dye is sprinkled on the surface of the emulsion to confirm the nature of continuous phase. In case of an o/w emulsion there is rapid incorporation of a water-soluble dye into the system whereas w/o emulsion generates microscopically visible clumps. The reverse happens on addition of an oil soluble dye.[13-14]

The microemulsion under examination was transparent and looked blue and almost the same colour as water dyed with methylene blue. This is an indication of uniform and fine dispersion of oil globules in water; to form o/w type microemulsion. There were no signs of phase separation upon dilution of microemulsion formulations in 1:10 and 1:100 ratios with double distilled water.

Syringeability and injectability

Syringeability is the ability or an ease withdrawal of formulation from vial before dispensing and injectability refers to the solution performance during injection. The factors such as force or pressure required for injection is also considered. Both these parameters are influenced by the viscosity of the formulations. Viscosity contributes significant challenges in injectability since high viscosity requires high injection force that ultimately leads to cause inevitable pain. High viscous products sometime cause deterrent in completeness of the injection and thus percentage of dose delivered.[16] No formulation from F1 to F5 showed resistance to syringeability and injectability, moreover these formulations were able to withdraw easily into syringe and injected into the tissue, thereby reducing the potential to create problems during withdrawal of doses or produce pain on injection. The results of syringeability and injectability of all microemulsions are given in Table 7.

Table 7. Data for syringeability and injectability of formulations

Formulation code	Syringeability	Injectability
CHASE ME1	+++	+++
CHASE ME2	+++	+++
CHASE ME3	+++	+++
CHASE ME4	+++	+++
CHASE ME5	+++	+++

Sterilization and Control of the Sterility of the Microemulsions

Sterilization of the microemulsion formulation for intralesional administration is an important parameter and it can be universally applied to microemulsion formulations with aseptic filtration methods. The suitability of sterilization technique of microemulsions was estimated with bacteria colony counting. At the end of this time period of incubation no cloudy appearance or growth was determined on the agar medium and the developed microemulsions were determined as sterile.

The positive control plates with, *E.coli* colonies have a characteristic green sheen due to rapid fermentation of lactose & production of strong acids, caused reduction in the pH of the EMB agar to form the green metallic sheen (Fig. 5).[35]

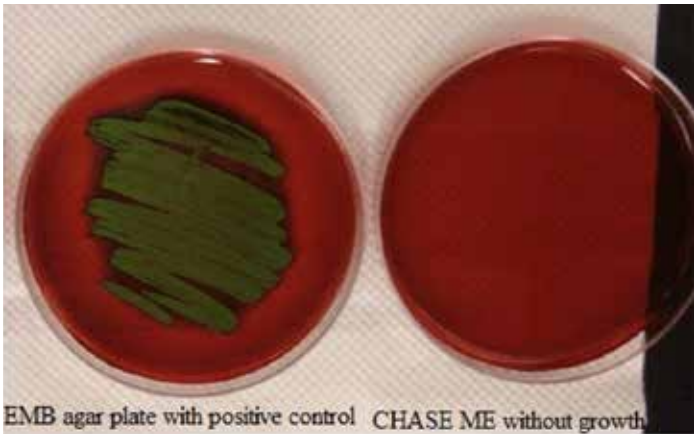

Figure 5. Sterility test on EMB agar indicates the developed microemulsions are sterile.

Thermodynamic Stability

As the two droplets coalesces with each other to form a single and larger size globule, it results in negative interfacial tension of the newly formed globule, and the system results in negative free-surface energy. To effect zero interfacial tension, the large globule increases its curvature spontaneously and results in two globules of the original size again. This process takes place in continuous manner as the bombardment of globules happens by molecules of dispersion medium. This is a dynamic equilibrium which maintains the microemulsion systems stable.[36] At the end of 30 min, the developed microemulsions did not show any signs of phase separation and drug precipitation after centrifugation at 3,500 rpm, to alter stability of the formulation.

Below freezing temperature, the ice crystals formation in an o/w type microemulsion may results in elongation and flattening of oil particles. In addition, the hydrophobic portion of the emulsifiers looses their mobility while the hydrophilic portions are "dehydrated" due to the freezing phenomenon of water. If the sample is thawed, release of takes place that migrate rapidly through the microemulsion system. In case of slower rate of re-dissolution of the ingredients, it may generate instability microemulsion system irrelevant to normal temperature processes.[13] The developed CHASE microemulsion formulations did not appeared with any instability evidences, the overall physical integrity of the formulation was remained unaltered throughout the cycle (Table 8).

Table 8. Different CHASE microemulsions with thermodynamic stability testing

Formulation code	Appearance	Centrifugation	Freeze-thaw cycle	Inference
ME1	Clear and transparent	P	P	Pass
ME2	Clear and transparent	P	P	Pass
ME3	Clear and transparent	P	P	Pass
ME4	Clear and transparent	P	P	Pass
ME5	Clear and transparent	P	P	Pass

P: Complies

Drug content estimation

The potency of CHASE as drug content in all formulations was found to be in range of 91.71–95.35%. The actual concentration of CHASE was calculated in range of 2.71 to 2.82 U/mL of microemulsion formulation (Table 9). These values overall correspond to 339.43 to 352.90 U/mg of CHASE enzyme. According to literatures, when CHASE having an enzymatic activity of 270 Units/mg protein or more is appreciated in the injectables to target proteoglycans and its appropriate degradation without affecting surrounding tissues.[37] Thus, the formulated microemulsion of CHASE would be the safe and effective system for drug delivery. The maximum drug content was estimated in CHASE ME4 as 2.82 U/mL with potency of 95.35% (w/v) among all formulations.

Table 9. Concentration of CHASE in different microemulsion as drug content with its potency

Formulation code	CHASE concentration (U/mL)	CHASE activity (U/mg)	CHASE potency (% w/v)
ME1	2.71± 0.004	339.43± 0.54	91.71±0.15
ME2	2.74± 0.005	343.01± 0.61	92.68±0.17
ME3	2.77± 0.003	346.45± 0.35	93.61±0.10
ME4	2.82± 0.003	352.90± 0.35	95.35±0.09
ME5	2.79± 0.003	348.88± 0.41	94.27±0.11

In vitro skin permeation study

Compared to aqueous CHASE solution, the microemulsion formulations was significantly enhanced CHASE skin penetration in time course of 6 and 12 h after injection. The time-course of the in vitro CHASE skin penetration showed

that when CHASE was injected in the microemulsion form, its concentration in E+D membrane was about 60-85% while concentration in SC was found 15-20%. However, the CHASE ME1 shows behaviour almost similar to that aqueous CHASE solution it may be caused due to lower content of permeation enhancer and high aqueous media. The maximal concentrations of CHASE in 6 h was 22.86 and 34.29 ng/cm^2 while in 12 h 11.43 and 34.29 in SC and E+D respectively by CHASE ME1 similar to aqueous CHASE solution. The behaviour of ME2 and ME3 was found almost equal with 53.33 and 40 ng/cm^2 in (E+D) compartment at 6 and 12 hr respectively. On the other hand, no CHASE was detected in SC compartment and 66.67ng/cm2 after 12 h application by ME4 and ME5 almost 2-fold higher than CHASE aqueous solution. The formulation ME4 and ME5 was found in the different compartments were in almost equal concentration as shown (Fig. 6 A and B).

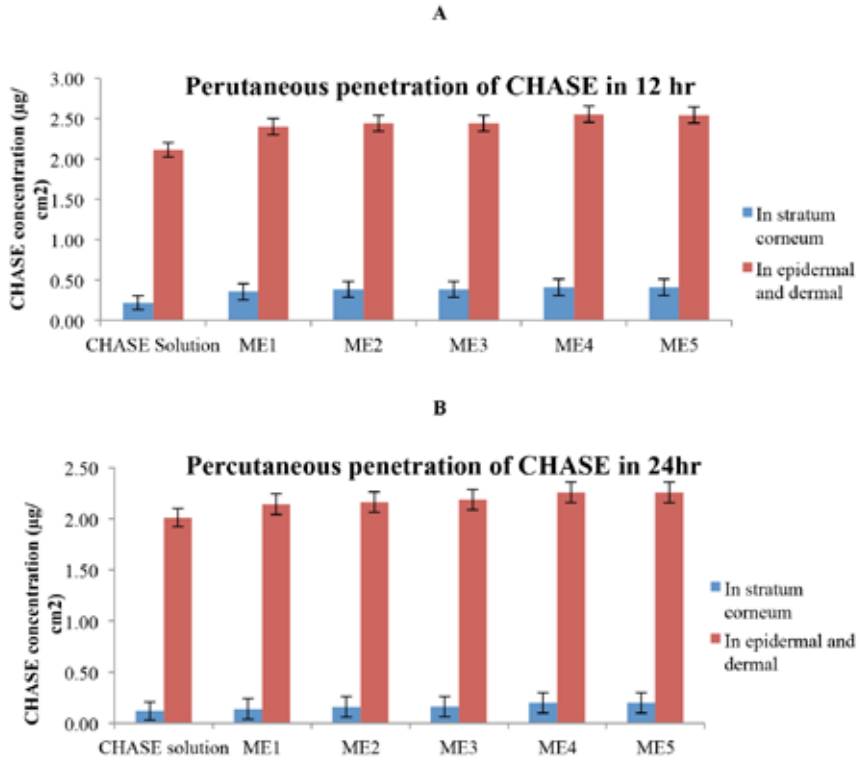

Figure 6. Time-course of the in vitro skin permeation of CHASE incorporated in different o/w type microemulsions in 6h (A) and 12h (B).

Stability studies

All CHASE microemulsion formulations, ME1 to ME5 exhibited transparency clarity and no drug precipitation, phase separation or colour change when it was subjected to stability study at 5±1°C in the refrigerator and 25±2°C at room temperature for 2 months. The physical properties like appearance, refractive index, viscosity and pH were also observed and no significant change was found in these characters. There was no significant change observed in globule size, zeta potential, PDI and drug content at refrigerated condition in first month while slight changes observed in drug content at the end of 2 months. The slight decrease in enzyme potency was evident at the end of 2 months in case of formulations kept at room temperature it is probably due to free fatty acid presence in oleic acid and also responsible in lowering of pH.[38] The mean globule size however was not affected during the storage. Thus, the o/w structure of microemulsion and suitable range from 5.8 to 7.0 provided suitable circumstance for avoiding the hydrolysis of drug. The results of stability studies are detailed in Table 10.

Table 10. Stability testing of CHASE ME4 after 1- and 2-month interval at various temperatures of 5ºC±3ºC and 25°C ± 2°C/60 ± 5% RH.

Temperature (ºC)	Time interval (month)	Viscosity	pH	Globule size	Zeta potential	CHASE concentration (U/mL)	CHASE activity (U/mg)	CHASE potency (% w/v)
5ºC±3ºC	1	10.7914±0.007	5.93±0.009	119.61±1.67	-0.116±0.004	2.81± 0.002	352.18± 0.20	95.16±0.06
	2	10.7851±0.006	5.95±0.014	122.28±0.69	-0.112±0.002	2.79± 0.003	349.60± 0.41	94.46±0.11
25°C ± 2°C/60 ± 5% RH	1	10.7963±0.009	5.95±0.022	122.27±1.08	-0.113±0.003	2.77± 0.003	346.45± 0.35	93.61±0.10
	2	10.7782±0.007	5.93±0.005	123.53±0.88	-0.118±0.002	2.75± 0.002	344.16± 0.20	93.33±0.42

In summary, we have formulated the microemulsions for the effective intralesional delivery of chondroitinase. The formulations displayed high permeability for dermal delivery through the *stratum corneum* membrane of skin evidenced by in vitro penetration skin study. The present study can open up a window for dermal application of proteins and enzymes in microemulsion form in treatment of excessive glycosaminoglycans and collagen in ECM responsible for lesions and keloids in deeper membrane of dermis and epidermis. Since the drugs via intralesional route are delivered in similar passion to that of intradermal delivery, it was more fruitful to investigate in vitro permeability studies using porcine ear skin tissue instead of in vivo permeability determination. Moreover, drug release kinetics data is irrational as the enzyme is

hardly absorbed in systemic circulation. Thus, a better alternative is provided over conventional lyophilised chondroitinase for constitution in terms of efficacy and stability.

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Ethanolic Whole Plant Extract of *Farsetia Jacquemontii* Showed Antipyretic & Analgesic Potential in Mice

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ABSTRACT

To evaluate the analgesic and antipyretic activities of ethanolic whole plant extract of *Farsetia jacquemontii* (EWFJ) in mice. The plant is the habitat of Rajasthan & Northwestern parts of India. In folk system of medicine, it is known as "Farid-booti", used traditionally in rheumatism, constipation, piles & abdominal pain. Antipyretic activity was assessed against Brewer's yeast induced pyrexia in mice using paracetamol (100 mg/kg) as standard. Analgesic activity was determined against hot plate & acetic acid induced writhing method in mice using diclofenac sodium (10 mg/kg) as standard. EWFJ (400 mg/kg) showed significant antipyretic effect after 2 hours of treatment when compared with standard paracetamol (100 mg/kg) in Brewer's yeast induced pyrexia model. EWFJ (400 mg/kg) reduced reaction time after 3 hours of treatment in the same manner as with standard diclofenac sodium (10 mg/kg) against hot plate method. EWFJ (400 mg/kg) showed the effect in similar manner as diclofenac sodium (10 mg/kg) against acetic acid induced writhing test. The study concluded antipyretic & analgesic effect of *Farsetia jacquemontii*.

Keywords: Antipyretic, Analgesic, *Farsetia jacquemontii*

INTRODUCTION

The importance of the reliability of medicinal plants had previously been focused on the treatment rather than the prevention of ailments. Despite that more than 90% of traditional medicinal remedies or formulations encompasses medicinal plants. Available literature also signified the implication of medicinal moieties from herbal resources in various preventive measures during adopted strategies for disease control.[1]

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In India, there are so many considerations involved giving rise to the use of medicinal plants in traditional system of medicines. Population escalation, insufficient supply towards the demand of drugs, unaffordable cost of therapies, adverse effects of various drugs of synthetic origins as well as the unavoidable development of resistance to the existing antimicrobials have directed to amplify the prominence for constituents from plant resources of their medicinal value against the widespread diversity of disease in human beings. [2]

Medicinal plants are measured as a rich source of constituents used in development of newer drugs either non- pharmacopoeial, pharmacopoeial or synthetic drugs. Furthermore, some plants are regarded as significant resource of nourishment which results in recommendation of their uses in therapeutic systems. Some of such plants comprise aloe, ginger, walnuts, green tea, turmeric & pepper etc. [2]

Exhaustive available literature establishes the medicinal uses of plant sources of wide & diverse origin in almost all aerial ailments of human body. However, there are so many plants still lacking to be scientifically proven in support of their traditional uses. Amongst those *Farsetia jacquemontii* is one which is widely used in eradication of pain, fever, inflammation, rheumatism constipation, piles, abdominal pain traditionally by native peoples of Rajasthan and Northwestern parts of India. [3,5] But its significance in these diseased conditions are still to be measured. Hence, this study encompasses the evaluation of antipyretic potential of *Farsetia jacquemontii* in mice.

Farsetia jacquemontii belonging to the family *Cruciferae,* is the habitat of Rajasthan and Northwestern parts of India. In folk system of medicine, it is known as "Farid-booti". [4]

METHODOLOGY

Collection & Authentication

The whole plant material was collected from native places of Jaipur (Rajasthan), in the month of January of 2018 which was authenticated by Prof. Alka Singh, Botanical department of Hindu college located at Moradabad (Uttar Pradesh), India wide letter no. 150118/B/09.

Preparation of extract

All aerial parts of *Farsetia jacquemontii* were dried in shade and powdered to a coarse form. The coarse powder was successively extracted according to their increasing polarity i.e. petroleum ether<chloroform<ethyl acetate<ethanol using continuous hot Soxhlet extractor at 60°C. The extracts were then concen-

trated under reduced pressure and preserved at low temperature for further evaluation of antipyretic & analgesic activity. [6,7] After extraction, individual extracts were introduced to preliminary phytochemical screening. Ethanolic extract selected for the further analgesic & antipyretic activities due to the presence of polyphenols with ethanolic extract that might be responsible for such pharmacological effects. Moreover, ethanolic extract produced high yield amongst all the selective solvent system and suggested to be safer for human consumption.

Chemicals and instruments

All chemicals used in this study were of analytical grade purchased from sigma Aldrich. Reference standard paracetamol & diclofenac sodium was received as gift sample from AKUMS, Haridwar, UK, India.

Animals

Healthy & adult mice of swiss albino strain weighing from 20 g to 30 g of either sex was used for antipyretic activity. All mice were housed under standard laboratory conditions and were fed with standard animal feed with freely access to water. All adopted standard experimental protocols were approved from the IAEC (Institutional Animal Ethical Committee) with CPCSEA registration number 1205/PO/Re/08/CPCSEA.

The mice were divided in six groups. Each group contained 6 mice (n=6) in each group. All mice in each group were overnight fasted except free access to water before performing experimentation.

Groups were designed as follow:

Group I (Control group): received only saline.

Group II (Standard group) administered with paracetamol (100 mg/kg) for antipyretic activity whereas with diclofenac sodium (10 mg/kg) for analgesic activity.

Group III (Test 1) received 50 mg/kg of EWFJ.

Group IV (Test 2) received 100 mg/kg EWFJ.

Group V (Test 3) received 200 mg/kg EWFJ.

Group VI (Test 4) received 400 mg/kg EWFJ.

Acute oral toxicity study

OECD guidelines 423 were adopted for oral acute toxicity studies. [7]

The ethanolic extract was devoid of any toxicity in mice using oral route. Hence 50, 100, 200 and 400 mg/kg doses of extract were selected for the study.

Antipyretic potential (pyrexia induced with Brewer's yeast)

Antipyretic potential of EWFJ was determined against pyrexia induced with Brewer's yeast in mice. A digital thermometer was utilized to record the normal temperature. Then the pyrexia was induced with s.c. injection of a 20% aqueous suspension of Brewer's yeast prepared as 10 mL/kg. Rectal temperature after eighteen hours was recorded and corresponding groups were served as per treatment with saline, standard & different doses of EWFJ respectively. Rectal temperature, at the interval of 0.5, 1, 2, 3- & 4-hours post drugs administration, were measured.[8]

Analgesic potential

Analgesic potency of EWFJ was evaluated adopting following methods.

Eddy's hot-plate method

Mice were placed on hotplate individually which was thermally constant at the temperature of 55°C. The time at which mice responded was noted down. Paw licking or the jumping (whichever appeared first) were the stimulus of pain perceived by mice after their respective treatments. A maximum of 15 seconds was kept as cut off time to avoid any injury to paws of mice. Percent increase in reaction time was noted (as index of analgesia) at each time interval (0.5, 1, 2, 3 hours). [9]

Writhing response induced with acetic acid

Intraperitoneal injection of 1% (v/v) acetic acid at a dose of 2.3 ml/kg induced abdominal constriction in mice. Animals were pre-treated with their respective treatments, 30 min before acetic acid administration. The number of abdominal constrictions was cumulatively counted for twenty minutes. The percentage inhibition was calculated by using below given formula to record analgesic potential: - [10, 11]

Percentage Inhibition (Analgesic activity) =

[Mean of writhing count {Control group- Treated group} / Mean of writhing count of control group] x 100

Statistical Analysis

Obtained data was illustrated in form of mean & SEM (Standard Error Mean). The data was further analyzed statistically adopting student's t-test whereas in case of unpaired data, p-values were determined.

Results were assumed to be of significance if p-values were less than 0.05 whereas if p-values were found to be lesser than 0.005, then the results were expressed as highly significant.

RESULTS AND DISCUSSION

Antipyretic potential (pyrexia induced with Brewer's yeast)

The obtained results are shown in **Table 1** & **Fig.1**

Table 1. Antipyretic activity revealed by different groups against pyrexia induced with Brewer's yeast

Groups	Rectal-Temperature (°C)						
	Initial before yeast injection	18 Hrs. after Yeast injection	Time after drug administration (hrs)				
			0.5 hrs	1 hr	2 hrs	3 hrs	4 hrs
Control	37.01±0.056	39.00±0.066	39.01±0.062	39.14±0.08	39.15±0.05	38.88±0.041	38.45±0.054
Paracetamol (100 mg/kg)	37.12±0.052	38.99±0.085	38.66±0.094[d]	37.88±0.077[d]	37.9±0.082[d]	37.23±0.084[d]	37.02±0.061[d]
Ethanolic Extract (50 mg/kg)	36.99±0.101	39.11±0.055	38.80±0.042	38.45±0.071[d]	38.66±0.079[d]	38.42±0.051[d]	38.12±0.031[d]
Ethanolic Extract (100 mg/kg)	37.10±0.081	39.1±0.072	38.45±0.055[c]	38.66±0.056[d]	38.22±0.072[d]	37.99±0.034[d]	38.10±0.051[d]
Ethanolic Extract (200 mg/kg)	37.06±0.068	39.90±0.062	39.1±0.055[d]	38.22±0.066[d]	38.01±0.087[d]	37.76±0.034[d]	37.33±0.045[d]
Ethanolic Extract (400 mg/kg)	37.12±0.045	38.99±0.064	38.45±0.049[d]	37.91±0.067[d]	37.45±0.045[d]	37.33±0.10[d]	37.12±0.081[d]

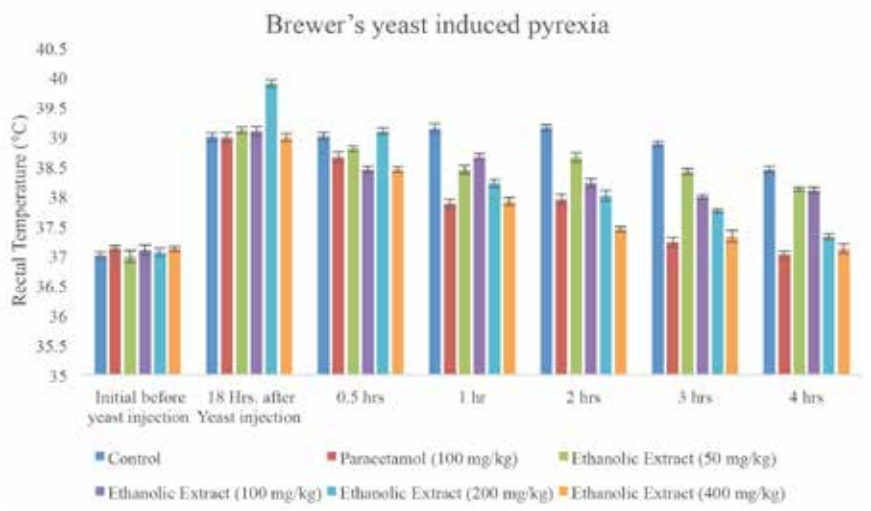

Figure 1. Antipyretic activity revealed by different groups against pyrexia induced with Brewer's yeast

Values are expressed as mean ± SEM (n=06): Significance at $p<0.001^d$ as compared to control

As revealed from obtained results, EWFJ (400 mg/kg) showed significant antipyretic effect after 2 hours of treatment when compared with standard paracetamol (100 mg/kg) in Brewer's yeast induced pyrexia model.

The study affirmed the antipyretic potential of EWFJ (400 mg/kg).

Analgesic Activity

Analgesic activity of EWFJ was evaluated against Eddy's hot-plate test & writhing response induced with acetic acid.

Eddy's Hot-Plate Test

Obtained results from Eddy's hot-plate test are shown in **Table 2** & **Fig.2**.

As depicted in the figure, EWFJ (400 mg/kg) reduced reaction time after 3 hours of treatment in the same manner as with standard diclofenac sodium (10 mg/kg) when explored to hot plate method.

The study evidenced the existence of analgesic effect of EWFJ at the dose of 400 mg/kg.

Table 2. Reaction time (seconds) shown by different groups at different time intervals.

Groups	Reaction Time (Seconds)				
	Time after drug administration (Hrs)				
	Initial	0.5 hrs	1 hr	2 hrs	3 hrs
Control	6.12 ± 0.066	6.22 ± 0.068	6.18 ± 0.077	6.18 ± 0.06	6.20 ± 0.087
Diclofenac Sodium (10 mg/kg)	6.31 ± 0.056	7.58 ± 0.054[d]	8.60 ± 0.067[d]	9.41 ± 0.062[d]	9.23 ± 0.1092[d]
Ethanolic Extract (50 mg/kg)	6.12 ± 0.0479	6.25 ± 0.022[a]	6.71 ± 0.042[d]	7.10 ± 0.102[d]	7.23 ± 0.066[d]
Ethanolic Extract (100 mg/kg)	6.17 ± 0.073	7.11 ± 0.067[d]	7.25 ± 0.049[d]	7.90 ± 0.067[d]	7.85 ± 0.045[d]
Ethanolic Extract (200 mg/kg)	6.15 ± 0.036	7.45 ± 0.062[d]	7. 73 ± 0.045[d]	8.21 ± 0.063[d]	8.20 ± 0.034[d]
Ethanolic Extract (400 mg/kg)	6.15 ± 0.066	7.78 ± 0.063[d]	7.78 ± 0.067[d]	8.81 ± 0.098[d]	8.85 ± 0.066[d]

Values are expressed as mean ± SEM (n=06): Significance at $p<0.001^{d}$ as compared to control

Writhing Response induced with acetic acid

Obtained results are summarized with the help of **Table 3** & **Fig.3**.

As shown in the figure, EWFJ (400 mg/kg) showed the effect in similar manner as diclofenac sodium (10 mg/kg) against acetic acid induced writhing test.

Table 3. Number of writhing & Percentage inhibition shown by different groups

S. No.	Groups	No. of Writhing (Mean ± SEM)	% Inhibition
1	Control	37.83	
2	Diclofenac Sodium (10 mg/kg)	7.83	79.3
3	Ethanolic Extract (50 mg/kg)	24.66	34.81
4	Ethanolic Extract (100 mg/kg)	15.16	59.92
5	Ethanolic Extract (200 mg/kg)	10.16	73.14
6	Ethanolic Extract (400 mg/kg)	9.16	75.79

Values are expressed as mean ± SEM (n=06): Significance at p<0.001[d] as compared to control

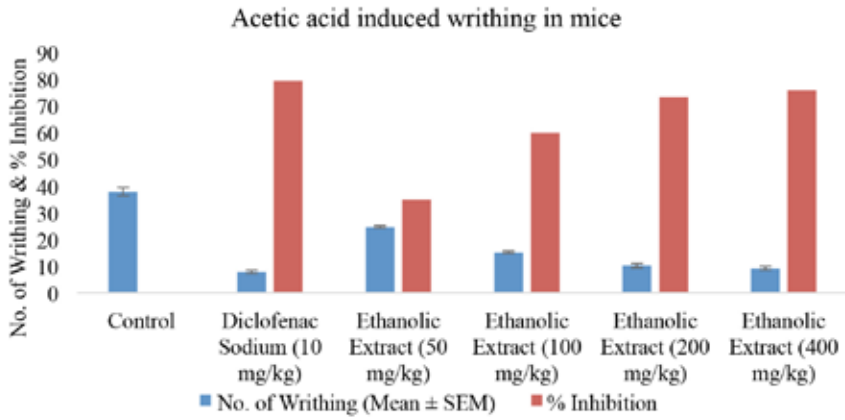

Figure 3. Number of writhing & Percentage inhibition shown by different groups

The study revealed the analgesic potential of EWFJ at the dose of. 400 mg/kg.

Present study concluded the confirmation of antipyretic & analgesic potenctial of *Farsetia jacquemontii*. Antipyretic potential of ethanolic whole plant extract of *Farsetia jacquemontii* was measured against pyrexia induced in mice with Brewer's yeast, using paracetamol at the dose of 100 mg/kg as standard and results demonstrated moderately significant (p<0.001) after 2 hours of pretreatment when compared to standard paracetamol. Analgesic activity of same was determined against hot plate method & writhing induced with acetic acid in mice using diclofenac sodium at the dose of 10 mg/kg as standard. The

results revealed EWFJ being significant ($p<0.001$) confirming to antipyretic as well as analgesic effects of the plant.

The study will be beneficial for further researchers in progress to isolate as well as identify the compounds responsible for such activities.

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