ACTA PHARMACEUTICA SCIENCIA

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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The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part.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: 3. 2020
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Polymorphic Transformation of Ornidazole with Nicotinamide in Solid Dispersion to Enhance Solubility and Dissolution Rate

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ABSTRACT

The solubility study of Ornidazole with different concentration of Nicotinamide was carried out to construct phase solubility diagram. The solid dispersion was prepared and characterized for its solid-state properties by employing FTIR, DSC and powder XRD techniques. The morphology of prepared solid dispersion was evaluated by using SEM. The dissolution study was also carried out for Ornidazole, PM and SD. Phase solubility diagram indicated an Ap type of relationship for the Ornidazole and Nicotinamide. DSC result indicated the shifting of melting endothermic peak of Ornidazole from 91.2°C to 77.16 °C in SD, which could be attributed to polymorphic transformation of Ornidazole in Form II to Form I in presence of Nicotinamide and also confirmed by FT-IR and XRD studies. Hence, the present study showed the transformation of one polymorphic form of Ornidazole to another low melting point polymorphic form in presence of Nicotinamide lead to significant enhancement of their dissolution rate.

Keywords: Ornidazole, Nicotinamide, Solid dispersion, Polymorphic form, Phase solubility

INTRODUCTION

Ornidazole is a third generation nitroimidazole derivative, a potent antimicrobial drug and used to treat infections caused by protozoa and certain strains of anaerobic bacteria. It is used to treat infections of the stomach, intestine, urinary tract and genital area. This compound is white to pale yellow crystalline powder, freely soluble in methanol, very slightly soluble in ether and slightly

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soluble in water. It exhibits low dissolution rate due to which large variation appear in *in-vitro* dissolution testing and also affect its bioavailability.[1,2]

The problem of poor aqueous solubility and low dissolution rate of drugs has received extensive considerations and various techniques have been investigated to enhance their dissolution rate and bioavailability. The general methods include particle size reduction (formation of nanocrystals/ micronization etc.), inclusion complexation (using cyclodextrins), micellar solubilization, chemical derivatization (formation of polar prodrugs) and solid dispersion.[3,4,5,6,7,8,9] Solid dispersion is one of the most promising approaches to enhance the dissolution rate of drugs having poor aqueous solubility. Solid dispersion can easily be prepared with suitable carriers by different methods and used to devise amorphous or crystalline multicomponent systems. The widely used methods for preparing solid dispersions are fusion, solvent evaporation and fusion-solvent methods.[10,11] Some of the first carriers[11] employed in their preparations were crystalline carriers such as urea, sugars and Nicotinamide (Vitamin B_3). These types of carriers were able to create crystalline or partially crystalline solid molecular dispersions. They are more thermodynamically stable as compared to that of amorphous form. Therefore, solid dispersion offers potential benefit over the amorphous forms with respect to their stability. Previously, solubility of Ornidazole was found to be increased from 0.01g/mL to 0.0368g/mL and 0.0902g/mL in 10% w/v and 20% w/v Nicotinamide aqueous solution, respectively.[12] In marketed formulation (suspension), the dose is 125mg/5mL but the reported solubility of Ornidazole in water is 15mg/mL.[13] In this study, the solubility of Ornidazole was determined in higher concentration of Nicotinamide (30%w/v) which showed further increase in solubility of Ornidazole. Therefore, the main objective of the present work is to increase the solubility of Ornidazole by formation of solid dispersions with Nicotinamide as a carrier. The solid dispersion was prepared and characterized for their solid-state characteristics by employing differential scanning calorimetry, FT-IR, and powder XRD. Any chemical interaction of Ornidazole with Nicotinamide was also studied by using HPLC. Morphology of solid dispersion was studied by using Scanning Electron Microscope (SEM) to observe the difference from that of pure Ornidazole crystals. The dissolution study was carried out for Ornidazole, physical mixture and solid dispersion to assess the effect of solid dispersion on dissolution rate of Ornidazole.

METHODOLOGY

Ornidazole and Nicotinamide were procured from Yarrow Chem, and Qualigens Fine Chemicals, Mumbai, India. All other chemicals and solvents were used of analytical grade.

Phase Solubility studies[14]

The phase solubility studies were conducted as per the method reported by Higuchi and Connors (1965). Excess of Ornidazole was added separately into 25-mL stoppered conical flasks containing 10 mL of distilled water or aqueous solution (1–30% w/v) of Nicotinamide. The flasks were sealed and agitated on a rotary shaker at ambient temperature (25°C) for 24 h and equilibrated for 2 days. The whole material was centrifuged at 2000 g to separate out the excess drug. The supernatant was filtered through filter paper (Whatman Grade 41). The filtrate was appropriately diluted with methanol and the absorbance was measured at 311 nm [13] by UV spectrophotometer to determine drug concentration using regression equation y = 0.0419x + 0.0056. The apparent 1:1 stability constant, Ks, was calculated by the equation reported by Garg et al, 2010.[15]

Preparation of solid dispersions (SDs)[16]

For preparation of solid dispersions, Ornidazole and Nicotinamide (1:2, 1:4 and 1:6 in ratio) were transferred to 200 mL of Erlenmeyer flask. Firstly, Nicotinamide (6.6 g) was transferred with the addition of about 2-3 mL distilled water and kept at 60-65°C temperature to dissolve it. Afterwards, Ornidazole (3.3g) was added to it in portions and stirred well. The temperature of the whole mixture was allowed to come down. The whole mass was then transferred to watch glass and allowed to dry in an oven maintained at temperature 40°C±5°C. The dried mixture was scraped off. The whole mass was transferred to the pestle mortar to get a powder, again kept in oven for drying. After drying, the powder of solid dispersion was passed through sieve number 100 and kept in desiccators. Finally, the powdered mass was stored in air-tight container. Same procedure was followed to prepare SDs of 1:4 (Ornidazole 2.0g) and 1:6 (Ornidazole 1.4 g) ratios of Ornidazole and Nicotinamide as a carrier.

Physical mixture (PM)[17]

Only higher Drug: carrier ratio (1: 6) was used for preparation of PM. Ornidazole (1.4 g) and Nicotinamide (8.6 g) were accurately weighed and mixed for 10 min using glass pestle and mortar with trituration. Then, powder was passed through sieve number 100. There after the physical mixture was stored in air-tight glass bottles.

Determination of drug content in SD and PM

Solid dispersions (100 mg of each) and PM (100 mg) were accurately weighed and transferred to small beaker and dissolved in small volume of methanol. The content was quantitatively transferred to volumetric flask (100 mL) with the aid

of methanol using Whatman grade 41 filter paper. The volume was made upto the mark with methanol. Further dilutions were made to achieve the concentration in the range of standard calibration curve using methanol as a solvent and the absorbance of this solution was measured at 311 nm.[18] In each case, the experiments were carried out in triplicate and results were summarized in Table 1.

Table 1. Drug contents of physical mixtures and Solid dispersions (n = 3)

Drug: Hydrotropic Agent	Percent drug content (mean ± S.D.)	
	PM	SD
ONN 1 : 2	-	33.38±0.074
ONN 1 : 4	-	20.12±0.082
ONN 1 : 6	14.18±0.073	14.57±0.076

PM - Physical mixture; SD - Solid dispersion; S.D. – Standard deviation; ON – Ornidazole; ONN – Ornidazole Nicotinamide

FTIR Spectroscopy

About 1-5 mg of the sample was triturated with approximately 300 mg of dry, finely powdered potassium bromide (IR grade) in a pestle and mortar. The sample was scanned over wave number region of 4000 to 400 cm^{-1} at resolution of 4 cm^{-1}.

Differential scanning calorimetry (DSC)

A large number of solid dispersions have been characterized by DSC studies to assess the possibility of interactions between drugs and water-soluble carriers.[19,20,21] Attempts were made to assess the possibility of interaction of hydrotropic agent (Nicotinamide) with drug (Ornidazole). In order to obtain the DSC thermograph of Ornidazole, solid dispersion and physical mixture, DSC Q 20 (TA Instruments USA) was used. The samples (4 mg) were weighed accurately and sealed in aluminium pans. The sealed pans of both sample and reference were placed in heating cell and heated in range of 25°C-300°C at a rate of 10°C/min with purging of nitrogen.

Powder X-ray diffraction studies

The X-ray diffractions (XRD) of the Ornidazole, solid dispersion and physical mixture were studied on X'pert Pro (PANalytical, Netherland) using Ni-filter and CuKα1 radiation with Spinner PW3064. A voltage of 45 kV and 40 mA current were applied with a scintillation counter. The samples were scanned by the XRD instrument in a range of 5° to 80°.

HPLC analysis[22]

A High-performance liquid chromatography (HPLC) method was used to determine the chemical incompatibility between drug and hydrotropic agent. The HPLC system isocratic mode-Cyber lab LC 100 plus (USA) separation module having maximum pressure of 5000 psi, detector—LC-UV 100 Plus, UV detector was used. The mobile phase consists of acetonitrile: water, (50:50 v/v), 0.2% triethylamine, the pH was adjusted to 4 using ortho-phosphoric acid (The mobile phase was filtered using 0.45 µm membrane filters and degassed by ultrasonic vibrations for 30 minutes prior to use). The flow rate was kept at 1 mL min^{-1}. C18 Cosmosil packed column having 4.6 mm internal diameter and 250 mm length. The wavelength was selected at 312 nm. From SD (ratio 1:6), **1µg/mL solution was prepared in methanol and 10µL** was injected in HPLC.

Scanning electron microscopy (SEM)[23]

SEM analysis was performed to visualize the morphology of the pure crystal of Ornidazole, PM and SD. Sample powder was attached to a aluminum metal sample holder using double-sided adhesive tape and then made electrically conductive by coating with gold in a vacuum. The sample was observed by SEM (JEOL, JSM-6100, Japan) at various magnifications. The analysis was set at voltage was set at 5 kV and the current was 12 mA.

Dissolution rate studies[24]

Dissolution rates of pure crystal of Ornidazole, PM (ratio 1:6), and SD (ratio 1:2, 1:4 and 1:6) were studied using USP paddle type dissolution rate test apparatus. Drug (Ornidazole), PM, and SDs equivalent to 100 mg drug were taken in 900 mL of 0.1 M Hydrochloric acid as dissolution medium. The stirring rate of 50 rpm and temperature of 37±0.5°C was maintained throughout the dissolution study. Samples (5 ml) of dissolution medium were withdrawn at predetermined time intervals and replaced with same volume of fresh dissolution medium after each withdrawal. The samples were filtered through Whatman grade 41 filter paper and after suitable dilution with methanol analyzed for drug contents at 311 nm wavelength. The dissolution studies were performed in triplicate. The percentage dissolution efficiency (%DE) is defined as the ratio of area under the dissolution curve up to a definite time (t) to the area of the rectangle for 100% dissolution in that time. It is calculated by the following equation:[25]

$$DE_T = \frac{\int_0^T y_t \cdot dt}{y_{100} \cdot T},$$

where yt is the percentage of drug dissolved at any time t, y100 denotes 100% dissolution, and the integral represents the area under dissolution curve between time 0 and T in minutes. The time T in this study was 90 min. Dissolution efficiency was calculated for pure drug, PM and SD by using above equation.

RESULTS AND DISCUSSION

The phase solubility diagram for Ornidazole as a function of Nicotinamide concentration in water is shown in Figure 1.

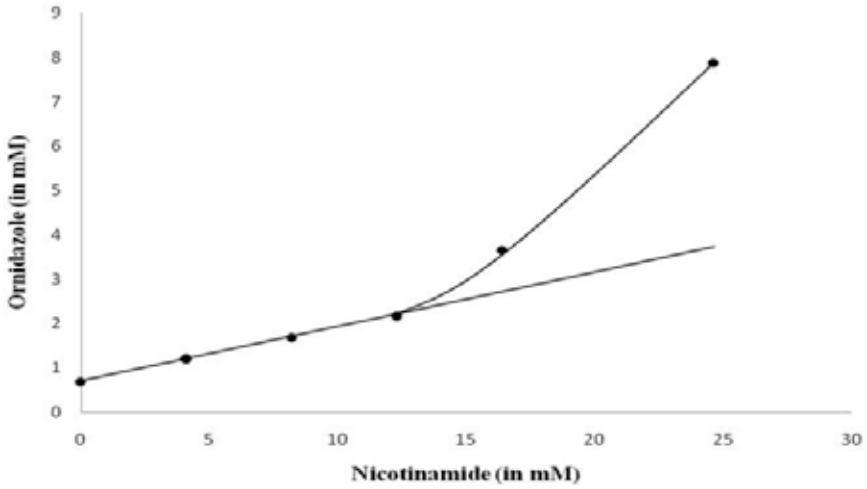

Figure 1. Phase Solubility Diagram

The phase solubility diagram clearly indicated that enhancement of solubility for Ornidazole in a linear relationship up to 12.29 mM of Nicotinamide. Further increase in the concentration of Nicotinamide up to 25 mM showed a huge increase in the aqueous solubility of Ornidazole. Hence; phase solubility diagram showed a divergence from linearity, demonstrated an A_p type of phase solubility diagram.[26] The apparent stability constant was calculated from the linear portion of the phase solubility diagram assuming the formation of 1:1 complex in the linear portion of the phase solubility diagram.[15] The value of apparent stability constant was found to be 5108 M^{-1}. This value of apparent stability constant is adequate to improve the solubility of Ornidazole in presence of Nicotinamide.[27] The solubility of Ornidazole was found to be increased by 11 times at 25 mM of Nicotinamide while only 3 times at 12.3 mM of Nicotinamide. The positive deviation in the solubility of Ornidazole at higher concentration of Nicotinamide was observed which could be attributed to formation of higher order complexes or to the polymorphic transformation of drug. Previ-

ously, solid dispersion of Nicotinamide with flurbiprofen lead to transform the polymorph form of flurbiprofen from high melting polymorph (112°C) to low melting polymorph (97°C) and their solubility phase diagram also indicated Ap type. Thus, it can be hypothesized that high concentration of Nicotinamide induce the transformation of one polymorph to another polymorph.[28]

Solid State Characterization of Ornidazole Nicotinamide Solid dispersion

DSC thermogram (Figure 2) of pure Ornidazole showed a single sharp endothermic peak at 91.2°C which corresponds to the melting point of Ornidazole.[29] DSC thermograms of physical mixture and solid dispersion of Ornidazole and Nicotinamide in ratio of 1:6 showed first endothermic peak at 80.26 °C and 77.16°C, respectively (Figure 3a and 3b). Three different polymorphic forms of Ornidazole were reported in literature.[30] The melting point of Form I, Form II and Form III was reported to be in the range of 76-78°C, 89.69°C and 86.9°C, respectively. Hence, the shifting of endothermic peak could be attributed to polymorphic transformation of Ornidazole Form II to Form I in presence of Nicotinamide. Second endotherm peak was observed at 127-128°C which indicated the melting of Nicotinamide (Figure 3c) in physical mixture as well as in solid dispersion.[23] Form I has the lowest melting point, hence expected to provide higher solubility as compared to that of Form II. The A_p type phase solubility diagram for Ornidazole and Nicotinamide was obtained in the present study which could be attributed to this polymorphic transformation in presence of Nicotinamide.

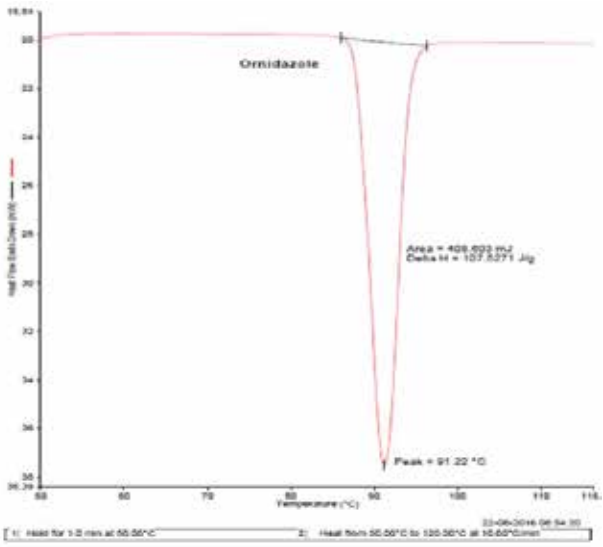

Figure 2. DSC Thermogram of Ornidazole

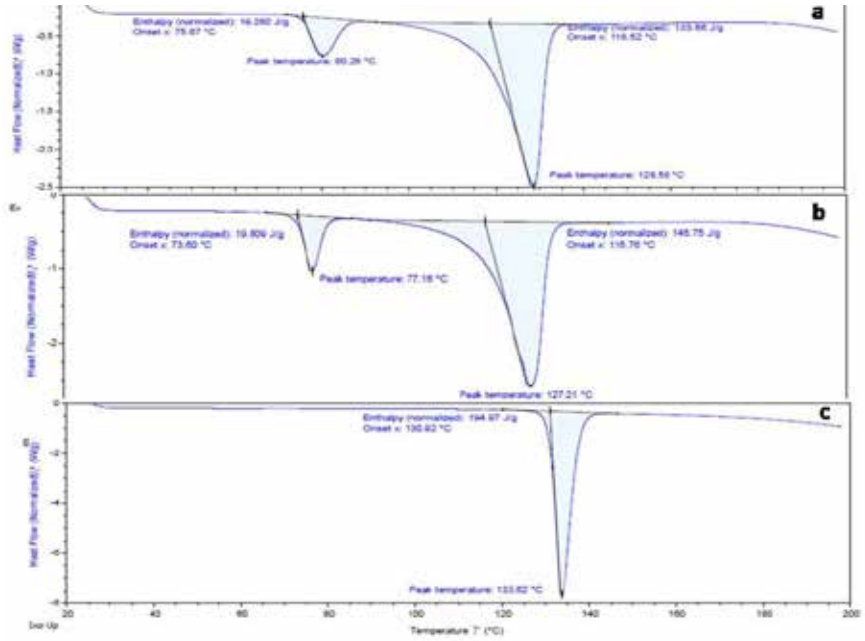

Figure 3. DSC Thermograms (a) PM, (b) Solid dispersion and (c) Nicotinamide

The Infra-Red spectra (Figure 4a) of Ornidazole exhibited peaks at 3174 cm^{-1} (O-H str), 3113 & 3089 cm^{-1} (C-H str), 1537 cm^{-1} (asymmetric NO_2 str), and 1361 & 1269 cm^{-1} (symmetric NO2 str).[1] The peak at 3174 cm-1 was found to be shifted to 3161 cm^{-1} and 3153 cm^{-1} in physical mixture (Figure 4b) and solid dispersion (Figure 4c), respectively indicating the change in O-H stretching of Ornidazole. The asymmetric peak of NO_2 at 1537 cm^{-1} was found to be diminished and observed at 1535 cm^{-1} in SD which could be attributed to involvement of oxygen of nitro group of Ornidazole in formation of weak hydrogen bonding with amide group of Nicotinamide. This weak hydrogen bonding between Nicotinamide and Ornidazole lead to change in the spatial arrangement of Ornidazole and transform to polymorph of low melting point as shown by DSC.

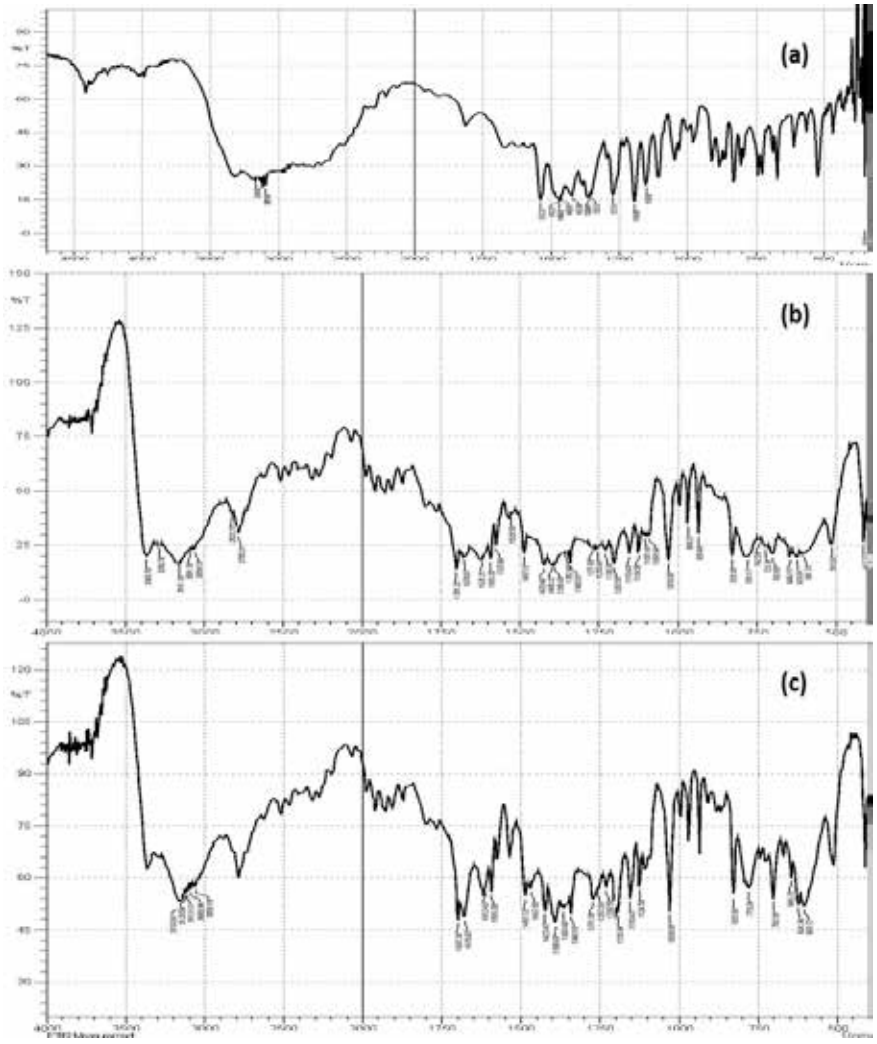

Figure 4. FTIR Spectra of a) Ornidazole, (b) PM and (c) Solid dispersion

The powder XRD pattern for Ornidazole, Physical mixture and solid dispersion of Ornidazole and Nicotinamide were depicted in Figure 5a-c. The diffraction pattern of Ornidazole showed that the solid drug is highly crystalline powder, with sharp diffraction peaks at 2θ of 13.33, 15.88, 20.83, 27.43 and 30.48.[1] Peak at 20.8 showed the 100% relative intensity in pure drug while in physical mixture (Figure 5b) and solid dispersion (Figure 5c), the relative intensity was found to be decreased significantly which indicated change in the crystalline nature of Ornidazole. Similarly, peak at 2θ of 30.48 was disappeared in physical mixture and solid dispersion, hence showed the change in the crystalline nature (i.e transformation of one form to another form or less crystalline form)

of Ornidazole in the presence of Nicotinamide. The characteristic peak of Nicotinamide was found to be at 2θ of 25.8 in physical mixture which is similar to the peak reported in literature.[31] However; the intensity of this characteristic peak was significantly reduced in the solid dispersion. Therefore, indicating the solid-state interaction with Ornidazole during formation of solid dispersion. The results of XRD can be well correlated to that of DSC.

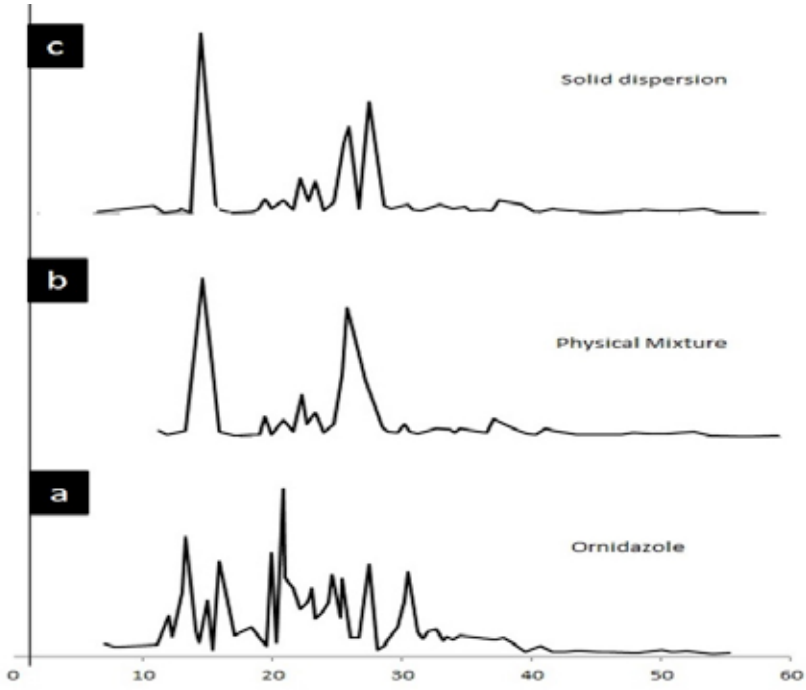

Figure 5. PXRD diffraction pattern of a) Ornidazole, (b) PM and (c) Solid dispersion

HPLC analysis of solid dispersion of Ornidazole was performed and chromatogram was obtained (Figure 6). The Ornidazole peak in the chromatogram was observed as a single peak at 4.14 min. The tailing factor and height equivalent theoretical plate were found to be 1.09 and 9489, respectively. The data observed from the HPLC showed no other peak was merged on the retention time of drug. All the parameters were found within the limit therefore results indicated no chemical incompatibility between Ornidazole and hydrotropic agent (Nicotinamide).

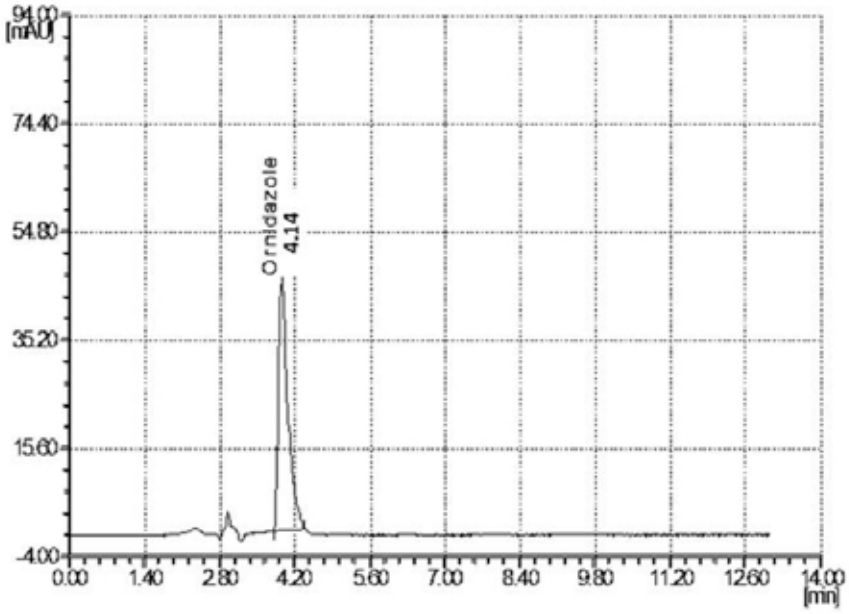

Figure 6. Chromatogram of Ornidazole in Solid dispersion

The photomicrographs of SEM for drug (Ornidazole), PM and SD were obtained as Figure 7a, 7b and 7c respectively. Figure 7a showed rectangular, thick plate like morphology of the drug.[30] In case of PM and SD, Figure 7b-c showed the change in morphology of drug (the rectangular crystals were transformed to some extent in round shape and increase in size). The original morphology of the drug was disappeared in PM and SD. It may assume that hydrotropic agent (Nicotinamide) cover-up the surface of the drug particles but still sharp edge of the particles can be observed, indicating that the crystalline nature of the drug was not completely reduced.

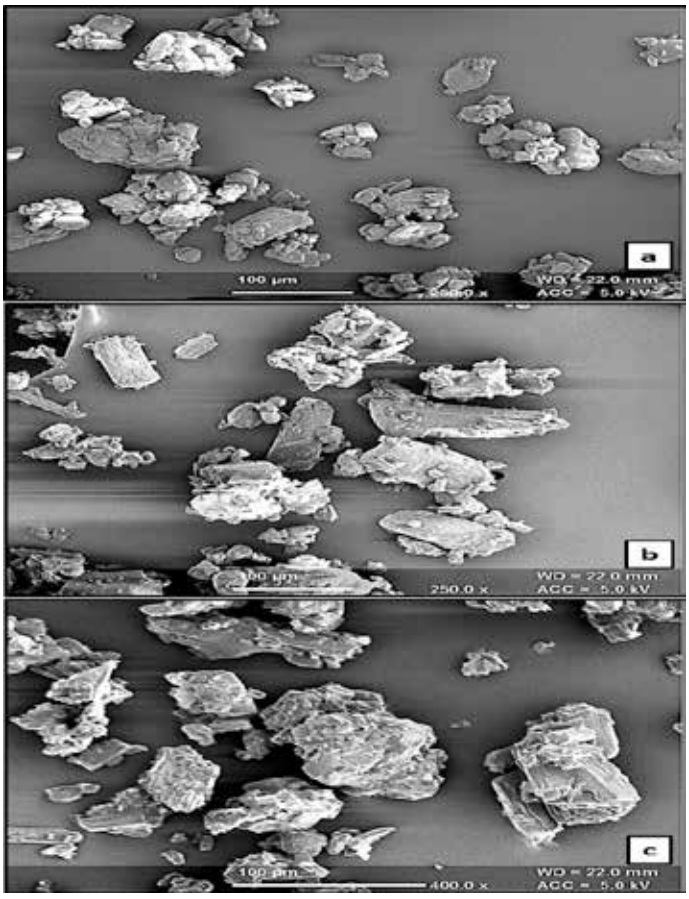

Figure 7. SEM Photomicrographs of a) Ornidazole, (b) PM and (c) Solid dispersion

^{1}H-NMR technique has been used as important tool for investigating the group involved in interaction and mechanism of complexation. Ornidazole (Figure 8a), Nicotinamide (Figure 8b), PM (Figure 8c) and solid dispersion of Ornidazole (Figure 8d) [1:6 ratio] were analyzed for ^{1}H-NMR. The absence of new peak in the spectra of solid dispersion suggested that the absence of any chemical interaction involving covalent bond. The spectra revealed that under the present condition only changes in chemical shift occurred. Downfield displacements of the protons indicate that they are close to an electronegative atom, like oxygen. The proton of amide moiety (H_5) experienced a pronounced chemical shift variation from 9.16 in pure Nicotinamide to 8.99 ppm in solid dispersion, hence confirmed that hydrogen of amide moiety was involved in the hydrogen bonding with Ornidazole. Results of FT-IR can be well correlated with the results of ^{1}H-NMR.[23]

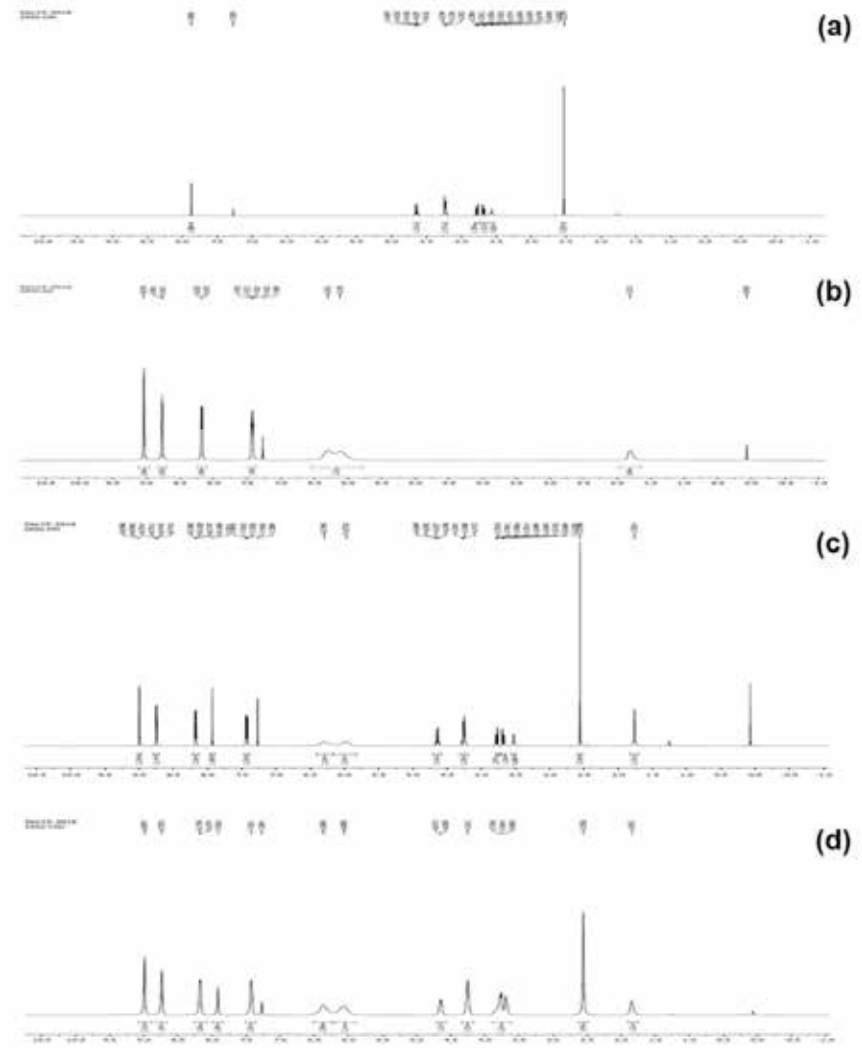

Figure 8. [1]H-NMR Spectra of a) Ornidazole, (b) Nicotinamide, (c) PM and (d) Solid dispersion

[13]C NMR was also performed for Ornidazole and solid dispersion of Ornidazole with Nicotinamide. In solid dispersion of Ornidazole and Nicotinamide, the characteristic peaks of both Ornidazole and Nicotinamide were observed in spectra.[31] The [13]C NMR spectra showed no changes in the characteristic peak of Ornidazole and Nicotinamide in solid dispersion hence confirmed the absence of any covalent bonding between them.

The results of dissolution rate studies were shown in Figure 9. Time to release 80% of Ornidazole was calculated from the graph and compared for pure Orni-

dazole, physical mixture and prepared solid dispersions. The $t_{80\%}$ was found to 30 min for pure Ornidazole. Physical mixture of Ornidazole with Nicotinamide in higher ratio decreased the $t_{80\%}$ from 30 min to 18 min. $t_{80\%}$ for solid dispersion of Ornidazole and Nicotinamide in ratio of 1:2 was found to be 13 min. Upon increasing the ratio of Nicotinamide in solid dispersion (1:4 and 1:6) lead to significant decrease in the t80% value (8 min and 4 min for 1:4 and 1:6 ratio, respectively).

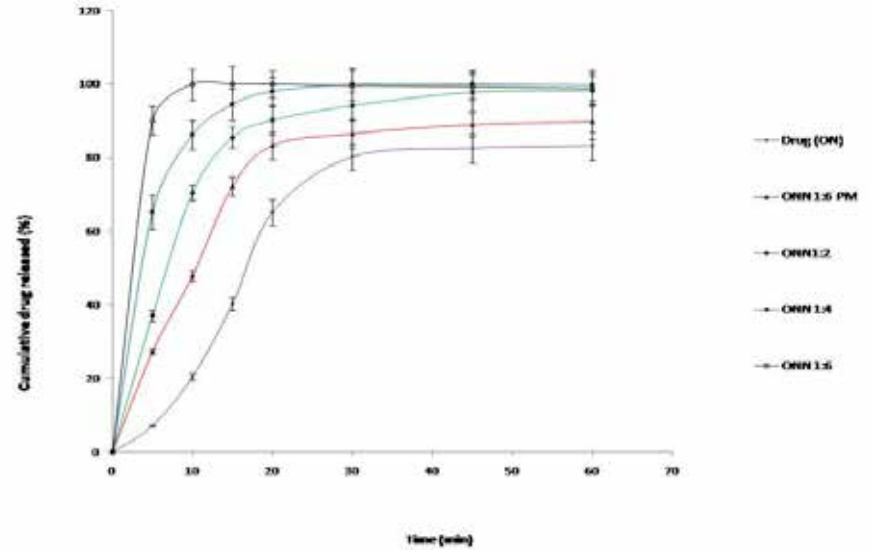

Figure 9. Dissolution profile of pure drug (ON), ONN 1:6 PM, ONN 1:2, ONN 1:4 and ONN 1:6 HSD in 0.1N HCl (n=3)

The percentage dissolution efficiency (%DE) at 30 min was calculated to compare the relative performance of pure drug, PM and solid dispersion. In literature, it was proved to be a better parameter than drug percentage released for comparison as it includes both rate and extent of release. The $\%DE_{30}$ of pure Ornidazole, PM and solid dispersion 1:2, 1:4 and 1:6 was found to be 40.95%, 59.82%, 70.39%, 82.17% and 89.71% respectively. The dissolution efficiency of solid dispersion at 30 min was found to be twice as compared to that of pure drug.

Hence, the present study showed that the transformation of polymorphic form of Ornidazole to another low melting point polymorphic form of Ornidazole in presence of Nicotinamide lead to significant enhancement of their dissolution rate.

The solubility phase diagram showed a significant linear increase in the aqueous solubility of Ornidazole with increasing concentration of Nicotinamide upto

12.3 mM while a non-linear increase in the solubility at higher concentration of Nicotinamide was observed. The solubility of Ornidazole was found to be increased by 11 times at 25 mM Nicotinamide. The study concluded polymorphic transformation of Ornidazole in the presence of higher amount of Nicotinamide as confirmed by the results of DSC, FTIR and P-XRD studies. The solid dispersion of Ornidazole with Nicotinamide can be used for enhancing the dissolution rate which in turn decreases the variability in bioavailability. Further, this solid dispersion could also be utilized to prepare a "Dry Syrup" for pediatric use. In future, dry syrup of Ornidazole will be prepared by using solid dispersion with Nicotinamide.

DECLARATION OF INTERESTS

Authors declare no conflict of interest.

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Effect of *Salvia Officinalis* on Neuromodulating and Oxidative Stress Status in Brain of Male Albino Wistar Rats Intoxicated with Aluminium

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ABSTRACT

The present study reflects the effect of plant extracts of *Salvia officinalis*on neuromodulating and oxidative stress status of Male Albino Wistar rats intoxicated with Aluminium chloride.

Rats were divided into7 groups of 6 in each. Apart from normal control, toxicant and standard, rats also received 250mg/kg and 500mg/kg doses of aqueous and ethanolic extracts of *Salvia officinalis* for20 days. Behavioral parameters, along with acetylcholinesterase enzyme levels, antioxidant markers and histopathology of brain tissues were determined.

Salvia officinalis improved behavioral parameters and reversed the reduced Acetylcholinesterase content thereby increased SOD and decreased MDA and NO when compared to $AlCl_3$ induced rats.

The study demonstrated the beneficial effects of *Salvia officinalis* in Alzheimer's disease by showing antioxidant, AchE inhibiting activity and by improving memory and cognitive functions.

Keywords: Alzheimer's disease, Aluminium chloride, Acetylcholinesterase, *Salvia officinalis*.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative, multifactorial, complex mental illness, and a form of dementia causing memory loss and neuronal death throughout the brain. It causes progressive behavioral (i.e., depression, agitation and psychosis), and neurological changes involving functional impairment,

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loss of independency, frustration, forgetting names, mood swings irritability and hostility, emotional problems, characterized by worsening of cognition and memory. [1] AD is mostly diagnosed in individuals above 65 years of age. Currently, it affects nearly 5% population of 65-year old, rising to 20% of those over 80 years and over 30% of 85-year old. Globally more than 27 million people are suffering with AD in the world and mostly in developed nations. [2]

Multiple pathogenic factors causing AD include aggregated extracellular ß-amyloid plaques, the formation of neurofibrillary tangles (NFTs) (highly phosphorylated tau proteins), cholinergic dysfunction and oxidative stress. [3] Oxidative cell damage occurs with an increase in level of free radicals which are usually held in balance by the body antioxidant system. Accumulation of intracellular reactive oxygen species (ROS) leads to oxidation of protein, lipids and DNA causing cellular damage. Elevated ROS levelsare also associated with amyloid-ß deposition which is an early feature of AD. [4]

Aluminum is a well-known neurotoxin. It causes neurodegeneration resulting in neurological changes in the hippocampus, cerebrum and also promotes biochemical changes. Literature shows that Aluminium induces neurotoxicity through production of free radicals resulting oxidative stress. It has a greater affinity to bio-membrane promoting the formation and aggregation of insoluble ß-amyloidal plaques which is vital characteristic of Alzheimer's disease. [5]

Medicinal plants have been used since ancient time to cure diseases, their progression and development. Medicinal plants with antioxidant properties have been used in the treatment of human diseases like cardiovascular disorders, cancer and neurological diseases such as AD.

So drugs for complete cure of Alzheimer's disease are not available clinically and greatly needed. Pharmacological activity and antioxidant property of phytoconstituents obtained from crude extract of medicinal plants are found its importance in various degenerative disorders.[6]

Salvia officinalis belongs to the family Lamiaceae is a native plant of East Mediterranean region which has been used as a traditional medicine by Middle Eastern and Asian countries to treat many disorders. *Salvia officinalis* (sage) has dual cholinergic activity. It is active against both Acetylcholine esterase and butyrylcholine esterase enzymes. Besides the cholinergic activity, it also have potent activity for CNS disorders, antioxidant activity, anti-inflammatory properties, nicotinic activity, glutamergic activities, and memory-enhancing effect. Its high antioxidant potential is due to its high phenolic contents isolated from this herb such as hydroxyl benzoicacid derivatives, ferulic acid, flavonoid de-

rivatives; luteolin and quercetin, caffeic acid derivatives (e.g., rosmarinic acid).[7] Hence in the present investigation we have attempted to demonstrate the anti-Alzheimer's property of *Salvia officinalis*.

METHODOLOGY

Collection of drugs

The whole plant of *Salvia officinalis* belonging to family Lamiaceae was collected, identified and authenticated by the botanist Dr. K. Madhavachetty, HOD, department of Botany in Sri Venkateshwara University, Tirupati, A.P. India.A voucher specimen (Voucher number: 1279) has been deposited in the department.

Chemicals:

a. Aluminium chloride - 300mg/kg b.w

Aluminium chloride anhydrous LR (granular)- SD fine-chem limited, industrial estate, 248, Worli road,

Mumbai-30. Batch No: A17A/0216/3108/13

MFD JAN 2017, Expiry Date DEC 2021

b. Donepezil – 0.75mg/kg b.w.

donepezil hydrochloride syrup – Donep syrup 5mg, Alkem laboratories Ltd, Thana Baddi, Himachal Pradesh-173205, India. Batch No: DNS 6002GB

MFD OCT 2016, Expiry date SEP 2018.

Preparation of plant extract:

The dried grounded powder of whole plant was subjected to ethanolic extraction using soxhalation technique[8] and aqueous extraction by decoction method.

Toxicity Studies

Extracts were tested for acute toxicity studies using 3 healthy male Albino Wistar rats weighing 150-180gms. Animals were fasted overnight priorto the experiment. Fixed dose acute toxicity studies were carried out according to the OECD guideline no.423.[9] The animals were given a dose of 2000mg/kg body weight of *Salvia officinalis* extracts and observed for any signs of mortality at 30minutes, 4hrs and thereby for next 24-hour post treatment. The animals were alsoexamined visuallyfor changes in behavior, skin color, and furfor 14 days. Dose was selected for the main study as per the oral acute toxicity results.

Experimental Design

The experiments were conducted with guidelines of Institutional animal ethical committee (IAEC), having approval number IAEC -01/SES/2018/101, governed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.Male Albino Wistar rats weighing between 180-200g were obtained from the animal house of Sainath agencies, Uppal, Hyderabad (282/PO/Bt/S/2000 CPCSEA).Rats were divided into 7 groups of six each **(Table 1)** and maintained at conditions of temperature (22 ±2°), humidity (50±5%) and 12-12 hour's light-dark cycles. All the animals were acclimatized for 7days before the study and provided with free access to food and water *ad libitum*.[10] During the experimental study rats were fed with pellets obtained from (Pranav Agro Industries limited, rat feed, India). The rats received humane care according to the criteria outlined in CPCSEA guidelines 2003, Government of India.

Table 1. Grouping of Animals with doses

SL.NO	GROUPS	AGE OF ANIMALS	TREARMENT
1.	Normal control	12 weeks	Normal saline (0.5ml p.o)
2.	Toxicant control	12 weeks	Aluminium chloride (300mg/kg, p.o)
3.	Standard control	12 weeks	$AlCl_3$ (300mg/kg, p.o) + Donepezil (0.75mg/kg, i.p).
4.	Aqueous extract (low dose)	12 weeks	$AlCl_3$ (300mg/kg, p.o) + aq extract (250mg/kg p.o)
5.	Ethanolic extract (low dose)	12 weeks	$AlCl_3$ (300mg/kg, p.o) + ethanolic extract (250mg/kg p.o)
6.	Aqueous extract (high dose)	12 weeks	$AlCl_3$ (300mg/kg, p.o) + aqueous extract (500mg/kg p.o)
7.	Ethanolic extract (high dose)	12 weeks	$AlCl_3$ (300mg/kg, p.o) + ethanolic extract of plant (500mg/kg p.o)

The above dosing schedule was continued for 20 days and behavioural parameters like locomotor activity, Conditioned avoidance response test, spatial long-term memory assessment, and motor coordination were determined.

Behavioral Study

Locomotor activity

It is an index of wakefulness or mental alertness. Locomotor activity of animals was determined using digital photoactometer. When the light beam that falls on the photocells is cut off by the animal, account is recorded. The movements of animal were recorded for 5 min which can be expressed as counts for 5 min per animal. Assessment was done in control and experimental groups.[11]

Motor coordination

It was assessed by using Rota-rod apparatus. Animals were initially trained to hold the rotating rod at a certain slow speed for 3 min. Then afterwards the speed of rod was increased to 50 rpm. Motor function and coordination of the animal was assessed by the time latency or fall off time from the placement of rat on the rod until it falls off onto the plate below. Assessment was done in control and experimental groups. [12]

Conditioned avoidance response test

This is done by using Pole climbing apparatus to evaluate memory and cognitive function. Animals were placed in the chamber individually and allowed to move for 1 min. After that, a warning sound was introduced for 3 seconds followed by an electric shock. If the rat did not climb the pole to escape from electric shock, it was noted as none. If the rat escaped the shock by climbing the pole, this was noted as escape, and if the rat avoided the shock by climbing the pole within the warning period, before warning sound is ceased, then it is termed as avoid response.[13]

Spatial long-term memory assessment

Spatial long-term memory assessment was performed by using elevated plus maze. The parameters used for the assessment were frequency of entries of animal into the open and closed arms and transfer latency (TL). Transfer latency is defined as the time taken by an animal to move from open arm to closed arm. Assessment was done in control and experimental groups.[14]

Blood Sampling and Brain Isolation

At the end of the experiment (after 20 days), animals were kept fasted overnight. After overnight fasting, animals were kept in desiccation chamber for the inhalation of carbon dioxide. Blood samples were collected through retro-orbital puncture by using capillary tubes. Blood samples of all animals were subjected to centrifugation at 1000rpm for 15 minutes to obtain serum. After taking blood samples, the animals were sacrificed. The whole brain of each animal was rapidly dissected by opening the skull carefully, and washed thoroughly with saline, dried and weighed. Each brain sample was fixed in 10% formalin solution for histopathological investigations. [15]

Biochemical Analysis

Blood samples were collected, and serum was separated and analyzed for biochemical parameters. Acetylcholine esterase (AchE) activity was determined in serum colorimetrically by referring *Dietz et al.* [16] Antioxidant parameters like

superoxide dismutase (SOD), malondialdehyde (MDA) and nitric oxide (NO) were estimated in serum. Superoxide dismutase (SOD) levels in serum were measured by using the method Kono et al., 1978.[17] MDA was determined by the method Okhawa et al., 1979.[18] Nitric oxide (NO) levels were estimated in serum by the method described by Berkel et al., 2004.[19]

Histopathological Study

The isolated brains from the sacrificed animals were kept immediately in 10% formalin solution for a period of 24 hours. Washed with distilled water and dehydrated using serial dilutions of alcohol (methyl, ethyl and absolute ethyl). Xylene was used to clean the specimens and then embedded in paraffin at 56^{o}C in hot air oven and kept for 24 hours. Paraffin bees wax Tissue blocks were prepared by sectioning at 4 microns by microtome. The resulting tissue sections were kept on glass slides and subjected to removal of paraffin (deparaffinized). Hematoxylin and eosin stains were used for staining of tissue for histopathological examination using the light microscope. [20]

Statistical Analysis

The outcomes were expressed as the Mean ± SEM. Statistical evaluation (data) was carried out by one-way analysis of variance (ANOVA), followed by Dunnet 't' test using Graphpad Prism 5 software, version 5.3 La Jolla, San Diego, California, USA to compare significance between groups. $p<0.05$ was considered to be significant. [21]

RESULTS AND DISCUSSION

Results of Acute Toxicity Study

Both extracts were administeredup to a dose 5gm/kg body weight and it was found that none of the two extracts produced any mortality thus indicating their practically nontoxic nature. The dose was calculated as1/8thand 1/10th dose of maximum tested (5gm/kg) of both extracts and selected for the main experiment.

Aqueous Extract - 250 mg/kg, b.w and 500mg/kg, b.w

Ethanolic Extract - 250 mg/kg, b.w and 500mg/kg, b.w

Results of Behavioral Study

Locomotor Activity

From the **Table 2,** it is observed that locomotor activity of rats treated with $Alcl_3$ is reduced compared to the control group. Treatment with low and high doses of extracts of *Salvia officinalis* found to be efficient in improving the locomotor activity in group 4-7 with maximum improvement in high dose (500mg/kg) of ethanolic extract.

Table 2. No. of counts/5 min in Actophotometer

Group	Treatment	Locomotor activity (No. of counts/5 min)
Group 1	**Normal control** Saline, 0.5ml, p.o	583.8 ± 38.18
Group 2	**Toxicant control** $Alcl_3$ (300mg/kg, p.o)	214.7 ± 15.71@
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	466.3 ± 17.06#
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	354.3 ± 14.16@
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	359.3 ± 17.19@
Group 6	**Aqueous extract (high dose)** $Alcl_3$ +aqueous extract (500mg/kg p.o)	422.7 ± 15.09@
Group 7	**Ethanolic extract (high dose)** $Alcl_3$ + ethanolic extract (500mg/kg p.o)	495.8 ± 12.17$

All values are expressed as mean± SEM. @-p<0.001 compared to normal control, # p<0.01 compared to normal control, $- p<0.05 compared to normal control

Motor coordination

From the **Table 3,** it is inferred that animals treated with $Alcl_3$ show significant decrease in the fall off time and decreased motor coordination compared to the control group while, rats treated with donepezil, aqueous and ethanolic extracts proved to be enhancing the motor coordination in extract and standard treated groups compared with toxicant group.

Table 3. Fall off time in seconds using Rota rod test for motor coordination

Group	Treatment	Rota rod test (fall off time in sec)
Group 1	**Normal control** Saline, 0.5ml, p.o	68 ± 9.73
Group 2	**Toxicant control** $AlCl_3$ (300mg/kg, p.o)	25.17 ± 2.04$^{\#}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	59 ± 5.41^{ns}
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	45.17 ± 1.81$^{@}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	45.83 ± 2.52$^{\$}$
Group 6	**Aqueous extract (high dose)** $AlCl_3$ +aqueous extract (500mg/kg p.o)	58.67 ± 3.38^{ns}
Group 7	**Ethanolic extract (high dose)** $AlCl_3$ + ethanolic extract (500mg/kg p.o)	63 ± 3.44$^{@}$

All values are expressed as mean± SEM. #- p<0.0001 compared to normal group, ns- nonsignificant compared to normal, @- p<0.01 compared to normal, $- p<0.05 compared to normal

Conditioned Avoidance response test

The **table 4** showed reduction in time taken to climb the pole in standard and extract treated groups when compared to the toxicant group. Animals treated with high dose (500mg/kg) of aqueous and ethanolic extracts show "Avoidance" response which means that they avoided the shock by climbing the pole within the warning sound period. The animals treated with low dose of extracts show "Escape" as they climb the pole after the warning sound by escaping the shock. No response is taken as "none".

Table 4. Time taken to climb pole in seconds using pole climbing apparatus

Group	Treatment	Time taken to climb pole in sec
Group 1	**Normal control** Saline, 0.5ml, p.o	0
Group 2	**Toxicant control** $AlCl_3$ (300mg/kg, p.o)	157.3 ± 4.45@
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	112.8 ± 4.9@
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	125.7 ± 2.5@
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	126.2 ± 2.99@
Group 6	**Aqueous extract (high dose)** $AlCl_3$ +aqueous extract (500mg/kg p.o)	118.2 ± 4.96@
Group 7	**Ethanolic extract (high dose)** $AlCl_3$ + ethanolic extract (500mg/kg p.o)	116.2 ± 4.13@

All values are expressed as mean± SEM. @- p<0.0001 compared to control group.

Spatial long-term memory assessment

There is an improvement in memory in the extract treated group with maximum effect in high dose (500mg/kg) aqueous extract treated group. Transfer of latency is reduced in the extract treated groups compared to the $AlCl_3$ treated group and the frequency of entries in the closed arm and the open arms is increased in standard and extract treated group compared to toxicant as shown in **table 5.**

Table 5. Transfer of latency in seconds

Group	Treatment	Transfer of latency in sec
Group 1	**Normal control** Saline, 0.5ml, p.o	22.83 ± 1.54
Group 2	**Toxicant control** $Alcl_3$ (300mg/kg, p.o)	50.17 ±2.61$^{!}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	27.33 ± 1.08^{ns}
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	32.17 ± 1.39$^{@}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	31 ± 1.72$^{@}$
Group 6	**Aqueous extract (high dose)** $Alcl_3$ +aqueous extract (500mg/kg p.o)	28.33 ± 0.94$^{@}$
Group 7	**Ethanolic extract (high dose)** $Alcl_3$ + ethanolic extract (500mg/kg p.o)	26.67 ± 1.83^{ns}

All values are expressed as mean± SEM. !- p<0.0001 compared to normal, ns- nonsignificant compared to normal, @-p< 0.01 compared to normal group.

Results Acetylcholinesterase activities on AD-induced and treated groups:

AchE levels in plasma were determined in control, toxicant, standard, low and high doses of aqueous and ethanol extract treated groups.

The result in the **table 6** showed significant increases in AchE in $Alcl_3$ treated group when compared to normal control, standard and extracts treated group. This indicates cholinergic reduction in AD-induced rats. Treatment of AD-induced rats with donepezil showed significant reduction in AchE enzyme levels and treatment with extracts of *Salvia* showed reduction in AchE level compared to the $Alcl_3$ treated group as show in the **figure 1**. Effect of high doses both extract of *Salvia* showed almost similar effects in AD-induced rats by facilitating elevation of Ach level, by significantly reducing AchE enzyme levels. AchE enzyme levels were measure in units/liter.

Table 6. Effect of treatment on AchE levels in plasma

Group	Treatment	AchE (U/L)
Group 1	**Normal control** Saline, 0.5ml, p.o	268.9 ± 28.58
Group 2	**Toxicant control** $AlCl_3$ (300mg/kg, p.o)	604.5 ± 15.63$^{\#}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	257.1 ± 17.29^{ns}
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	492.4±8.58$^{\#,\$}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	481.3±12.89$^{\#,@}$
Group 6	**Aqueous extract (high dose)** $AlCl_3$ +aqueous extract (500mg/kg p.o)	417.1±7.28$^{\#,@}$
Group 7	**Ethanolic extract (high dose)** $AlCl_3$ + ethanolic extract (500mg/kg p.o)	427.3±38.22$^{\#,@}$

All values are expressed as mean± SEM. #-p<0.001 compared to normal control, ns-nonsignificant compared to normal control, $-p<0.01 compared to toxicant control, @-p<0.001 compared to toxicant control

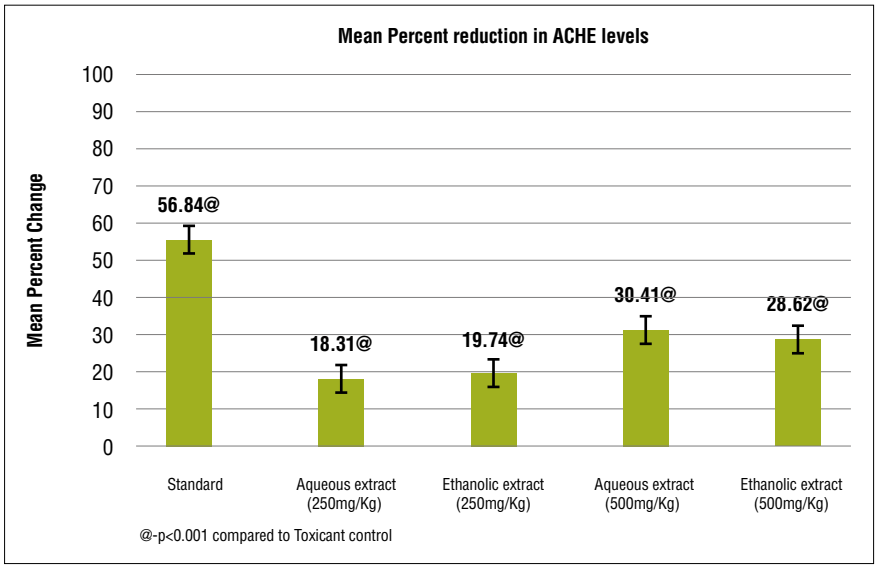


Figure 1. Effect of treatment on AchE levels

Results of Histopathology

Group 1-Treated with normal saline: Group 1 animals were given only saline. They show normal cerebral cortex and hippocampus and no enlarged ventricles are seen in **figure 2.**

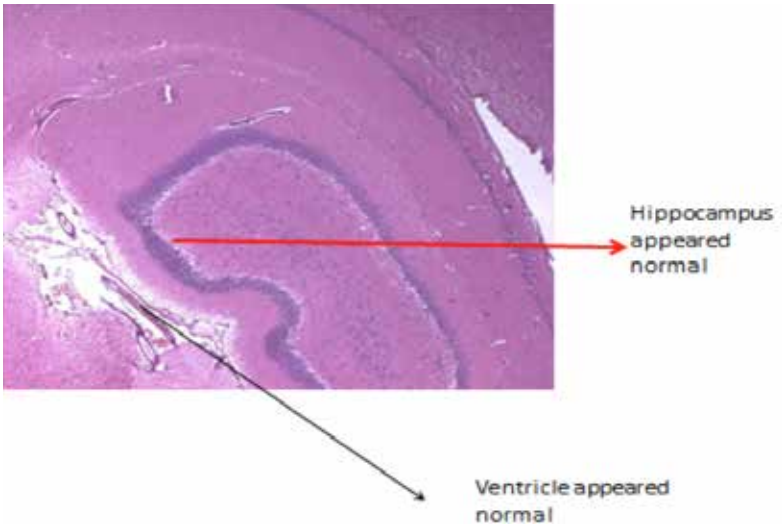


Figure 2. Brain section of control group rats showing normal structure of hippocampus.

Group 2-Treated with Aluminium chloride: Microscopic investigation of brain of $Alcl_3$ treated rats show neurodegeneration, enlarged ventricles and amyloidal plaques in hippocampus and brain atrophy when compared with the histological structure of brain of control group **(Figure 3).**

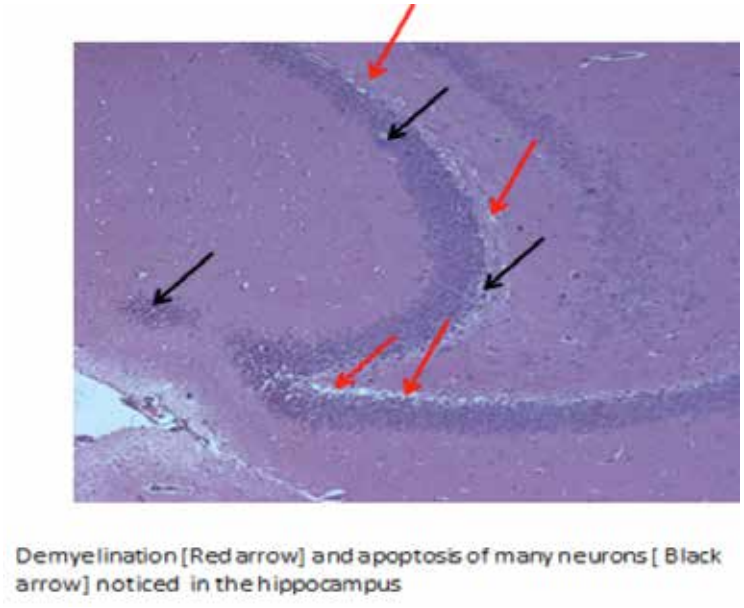


Figure 3. Micrograph of brain section of Alcl3 treated rats showing apoptosis of neurons and amyloidal plaques in hippocampus.

Group 3-Treated with Donepezil:

Group3 treated with donepezil revealed the disappearance of amyloid plaques formed due to $Alcl_3$ and normal histological structure of cortex and hippocampus compared to $Alcl_3$ treated which can be observed in **figure 4**.

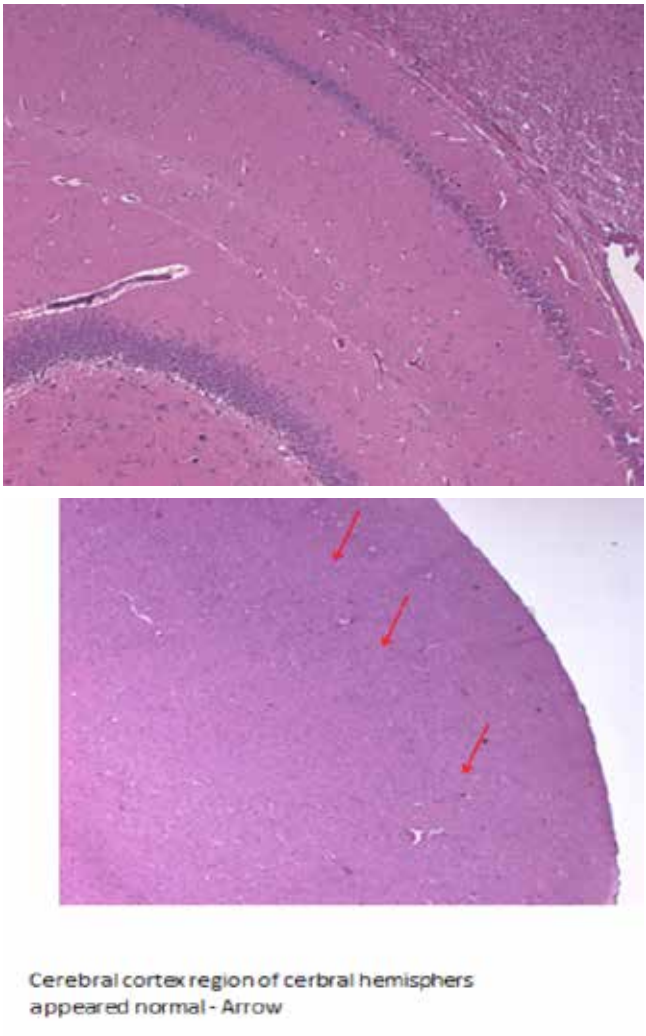


Figure 4. Brain section of AD-induced rats treated with donepezil showing normal cerebral cortex and hippocampus.

Group 4-Treated with aqueous extract (low dose) of *Salvia officinalis*:

Group 4 rats treated with low dose (250mg/kg, b.w) of aqueous extract shows mild neurodegeneration in the hippocampus region compared to $Alcl_3$ treated rats and disappearance of few amyloidal plaques formed due to treatment of $Alcl_3$ in **figure 5**.

Figure 5. Histology of brain section of AD-induced rats treated with low dose of aqueous extract shows disappearance of amyloid plaques and mild neurodegeneration compared to Alcl3 treated rats.

Group 5- Treated with ethanolic extract (low dose) of *Salvia officinalis*:

Group 5 rats treated with low dose (250mg/kg, b.w) of ethanolic extract shows mild neurodegeneration in the hippocampus region compared to $Alcl_3$ treated rats and disappearance of few amyloidal plaques formed due to treatment of $Alcl_3$ in **figure 6.**

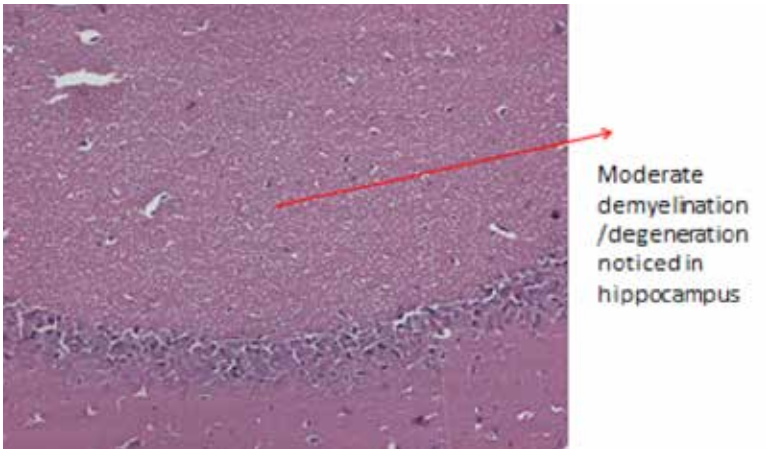
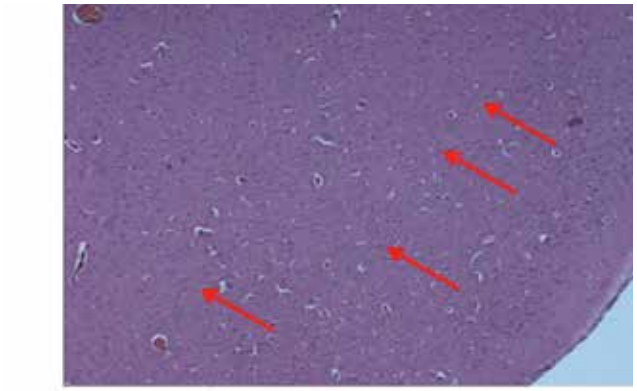


Figure 6. Micrograph of brain section of AD-induced rats treated with low dose of ethanolic extract shows disappearance of amyloid plaques and mild neurodegeneration compared to Alcl3 treated rats.

Group 6-Treated with high dose of aqueous extract of *Salvia officinalis*:

Group 6 rats treated with high dose (500mg/kg, b.w) of aqueous extract shows normal histological structure of hippocampus and cerebral cortex compared to $Alcl_3$ treated rats and disappearance of amyloidal plaques formed due to treatment of $Alcl_3$ **(figure 7).**It is inferred that high dose shows more potent effect with few dislocation of hippocampal cells.



Frontal cortex- cerebral hemisphere appeared normal–
Arrow. No necrosi s or inflammation noticed

Figure 7. Micrographic picture of brain section of AD-induced rats treated with high dose of aqueous extract shows normal histological structure of cerebral cortex and hippocampus with dislocation of few hippocampus cells and disappearance of amyloid plaques compared to Alcl3 treated rats.

Group 5 - Treated with high dose of ethanolic extract of *Salvia officinalis*:

Group 7 rats treated with high dose (500mg/kg, b.w) of ethanolic extract shows normal histological structure of hippocampus and cerebral cortex compared to $Alcl_3$ treated rats and disappearance of amyloidal plaques formed due to treatment of $Alcl_3$ **(figure 8).**

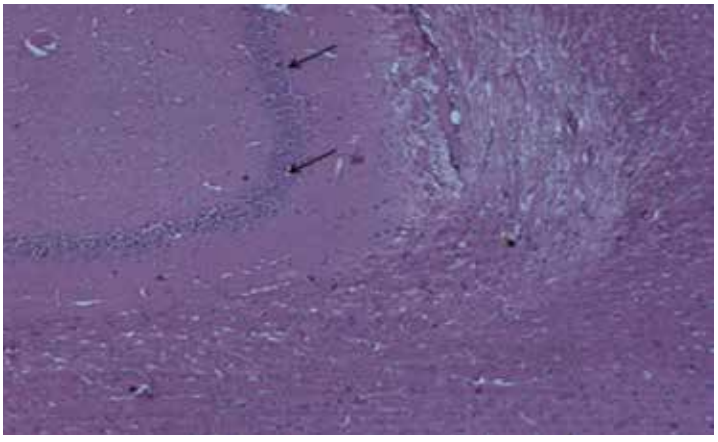


Figure 8. Brain section of AD-induced rats treated with high dose of ethanolic extract shows normal structure of cortex and hippocampus and disappearance of amyloid plaques compared to Alcl3 treated rats.

5. Results of Antioxidant Activity

Results of Malondialdehyde (MDA):

From **Table 7** it is inferred that toxicant control group treated with $Alcl_3$ shows elevated levels of MDA compared to normal due to oxidative stress. There is a reduction in the MDA level in groups treated with standard, drug extracts as per dose **(figure 9).**

Table 7. Effect of treatment on serum MDA levels.

Group	Treatment	MDA (nmol/mg protein)
Group 1	**Normal control** Saline, 0.5ml, p.o	6.58 ± 0.13
Group 2	**Toxicant control** $Alcl_3$ (300mg/kg, p.o)	$10.43 \pm 0.17^{@}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	6.97 ± 0.12^{ns}
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	$8.6 \pm 0.24^{@\#}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	$8.8 \pm 0.27^{@\#}$
Group 6	**Aqueous extract (high dose)** $Alcl_3$ +aqueous extract (500mg/kg p.o)	$7.9 \pm 0.13^{@\#}$
Group 7	**Ethanolic extract (high dose)** $Alcl_3$ + ethanolic extract (500mg/kg p.o)	$7.8 \pm 0.17^{@\#}$

All values are expressed as mean± SEM.@- $p<0.0001$ compared to normal group, ns-nonsignificant to normal, #-$p<0.0001$ compared to AlCl3 treated group.

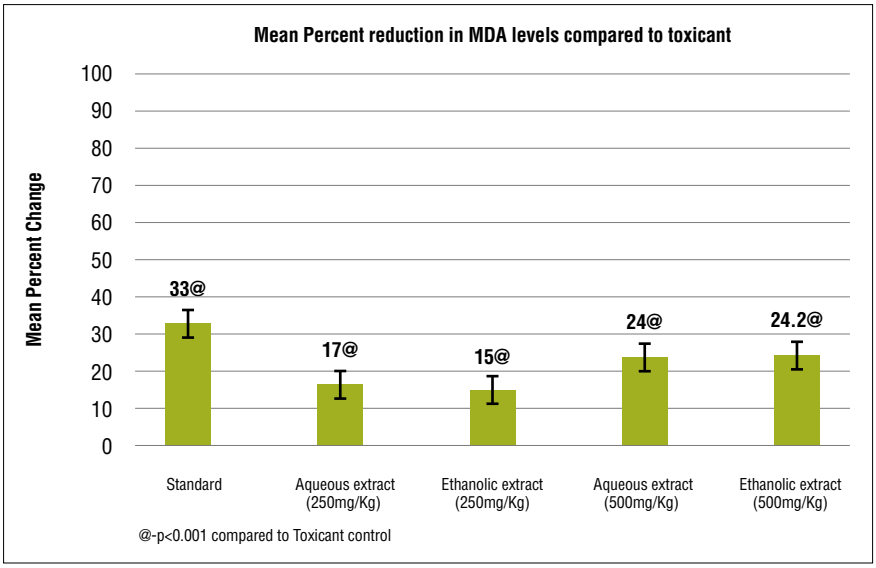


Figure 9. Mean percent reduction of MDA levels in serum

Results of Nitric oxide (NO):

Table 8 inferred that the elevated level of NO indicates oxidative stress in toxicant group treated with $Alcl_3$, whereas in groups treated with standard and extracts, the levels of NO is reduced compared to the toxicant group **(figure 10).**

Table 8. Effect of treatment on serum NO levels.

Group	Treatment	NO (µ/mg protein)
Group 1	**Normal control** Saline, 0.5ml, p.o	4.6 ±0.16
Group 2	**Toxicant control** $Alcl_3$ (300mg/kg, p.o)	10.46 ±0.12$^{@}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	6.11 ±0.23$^{@}$
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	8.12 ±0.1$^{@\#}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	8.09 ±0.11$^{@\#}$
Group 6	**Aqueous extract (high dose)** $Alcl_3$ +aqueous extract (500mg/kg p.o)	7.94 ±0.15$^{@\#}$
Group 7	**Ethanolic extract (high dose)** $Alcl_3$ + ethanolic extract (500mg/kg p.o)	7.68 ±0.12$^{@\#}$

All values are expressed as mean± SEM. @-p<0.0001 compared to normal control, #-p<0.0001 compared to AlCl3 treated group.

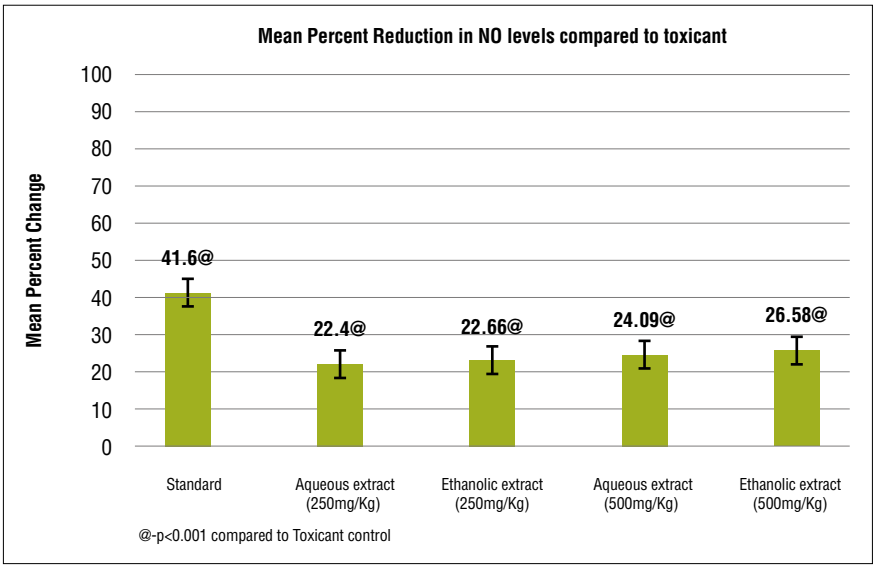


Figure 10. Mean percent reduction in NO levels in serum

Results of Superoxide Dismutase:

SOD level is less in animals treated with $Alcl_3$ when compared to normal group which indicates oxidative stress **(table 9).** SOD levels increased in group treated with standard, drug extracts when compared to the AD-induced rats treated with $AlCl_3$ **(figure 11).**

Table 9. Effect of treatment on serum SOD levels.

Group	Treatment	SOD (U/mg protein)
Group 1	**Normal control** Saline, 0.5ml, p.o	2.71 ±0.11
Group 2	**Toxicant control** $AlCl_3$ (300mg/kg, p.o)	1.21 ±0.09$^{@}$
Group 3	**Standard control** Alcl3 + Donepezil (0.75mg/kg, i.p).	2.43 ±0.13^{ns}
Group 4	**Aqueous extract (low dose)** Alcl3 + aqueous extract (250mg/kg p.o)	2.20 ±0.09$^{\#*}$
Group 5	**Ethanolic extract (low dose)** Alcl3 + ethanolic extract (250mg/kg p.o)	2.29 ± 0.08$^{\$*}$
Group 6	**Aqueous extract (high dose)** $AlCl_3$ +aqueous extract (500mg/kg p.o)	2.34 ±0.12$^{\#*}$
Group 7	**Ethanolic extract (high dose)** $AlCl_3$ + ethanolic extract (500mg/kg p.o)	2.36 ±0.10^{ns*}

All values are expressed as mean± SEM.@-p<0.0001 compared to control group, ns-nonsignificant to control group, #-p<0.01 compared to control group, $-p<0.05 compared to control group, *-p<0.0001 compared to AlCl3 group.

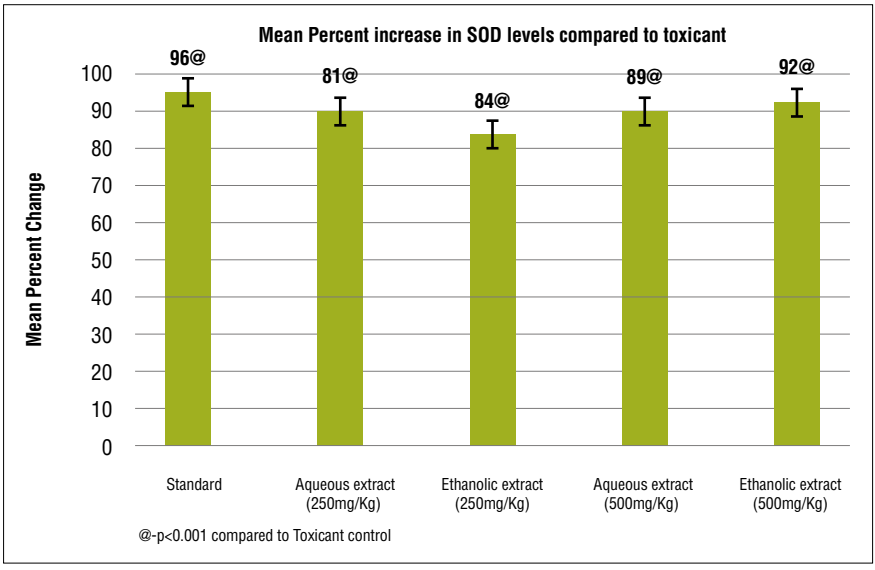


Figure 11. Mean percent increase in SOD levels in serum.

RESULTS AND DISCUSSION

Alzheimer's disease rises continually all over the world. It becomes a challenge for the modern health care to develop a treatment for the neurodegenerative diseases like Alzheimer. It's a complex, multifactor, progressive neurodegenerative disorder causing atrophy of brain. Pathogenic factors of AD include aggregated extracellular ß-amyloid plaques, the formation of neurofibrillary tangles (NFTs) (highly phosphorylated tau proteins), cholinergic dysfunction and oxidative stress. [22]

Aluminum is a well-known neurotoxin which causes neurodegeneration. Aluminium alters blood-brain barrier (BBB) and gets deposited in the cortex and hippocampus region causing brain toxicity. It promotes the formation and aggregation of insoluble ß-amyloidal plaques characteristics of Alzheimer's disease. It also cause disturbance in the enzyme activity of acetylcholinesterase involved in acetylcholine metabolism and leads to cognitive dysfunction.[23,24] Similarly in our study, we have observed that aluminum intoxicated rats (toxicant control group) showed significant elevation in AchE level compared to the normal, and this was supported by histopathological study, which showed the presence of amyloid plaques and neural damage in the brain tissues. Such results are in harmony with those obtained by Kaizeret al.[25]

From this study we found that the Acetylcholine esterase enzymes levels in the brain increases in Alzheimer induced rats due to aluminium chloride. This elevated level of AchE was found to be reduced with the treatment of extracts of *Salvia* and the standard drug. When compared to the toxicant control there is a reduction of 58% of AchE enzyme in group treated with donepezil, 28% reduction in high dose (500mg/kg) of ethanolic extract and 30% reduction in aqueous extract. Aqueous extract of *Salvia* showed potent effect in reduction of AchE enzyme level, thereby increasing Ach level. Hence the cholinergic activity in the extract treated groups was observed to be improved in the animals treated with ethanolic and aqueous extracts. These results are coincided with Perry et al, [26] who stated that the relevant component of *Salvia* can cross the blood-brain barrier and increase cholinergic transmission via inhibition of cholinesterase enzyme.

It has been well documented that Aluminium induces neurotoxicity through free radical production causing oxidative stress. According to Dickstein et al,[27] oxidative stress play an important role in the pathogenesis of AD. Accumulation of ROS takes place as a consequence of oxidative stress. If this ROS level exceeds the cellular protective mechanism, oxidative damage occur leading to cell death. However, the increased Al concentration deleteriously affects the

neurons, causing depletion of antioxidants which exhaust the SOD capacity to neutralize the free radical processes. This results in decreased activity of SOD, and increased activity of MDA and NO. Therefore, substances having antioxidant potential which can reduce oxidative stress are selected as the potential drug for treatment of AD. [28, 29]

The present study showed that oxidative stress was found in the group of animals treated with $Alcl_3$ which was analyzed by the high levels of MDA and NO which are the parameters of oxidative stress. And also, the low levels of SOD (antioxidant parameter) due to oxidative stress. These results are coincided with Gustaw-Rothenberg at al [30].This oxidative stress was recorded to be reduced in the groups treated with extracts of *Salvia* which was estimated by the reduced levels of MDA and NO and increased levels of SOD in the extract treated group.[31] The aqueous and ethanolic extracts higher doses i.e., 500mg/kg showed more potent anti-oxidant activity. This antioxidant activity of *Salvia* is due to its high phenolic content such as rosamarinic acid, caffeic acid, sage coumarin etc.

AchE inhibitors are the drugs approved by FDA for the treatment of AD. Acetylcholinesterase inhibitors (AchE-Is) prevent the metabolism of the Ach in the brain and found to improve cognition in patients with AD. AchE-Is are used currently for the symptomatic treatment of AD to improve and maintain central cholinergic function. Acetylcholine esterase inhibitors like Donepezil, rivastigmine, galantamineare currently used as a symptomatic treatment to improve and maintain central cholinergic function. In the present work, we used *Donepezil*as a standard drug for the comparison of drug extracts. This was done in accordance with Cutuli et al. [32]

Salvia officinalis (sage) is considered as a medicinal plant since ancient times. It has dual cholinergic activity. It has both Acetylcholine esterase and butyrylcholine esterase inhibiting activity. Besides the cholinergic activity, it has potent activity for CNS disorders, antioxidant activity, anti-inflammatory properties, nicotinic activity, glutamergic activities, and memory-enhancing effect. The plant is known to improve the mental functions according to Howes et al [33]. Sage extracts have been shown to possess antioxidant, anti-inflammatory, anticancer and antimicrobial activities. Its high antioxidant activity is due to its high phenolic contents isolated from this herb such as hyd-roxybenzoic acid derivatives, ferulic acid, flavonoid derivatives; luteolin and quercetin, caffeic acid derivatives (e.g., rosmarinic acid).[34] *Salvia* act as acetylcholinesterase inhibitor in comparison with standard drug Donepezil by inhibiting the enzyme activity and increasing Ach levels.

Behavioral study reveals the improvement of motor coordination, memory, functional ability, learning with the treatment of *Salvia* extracts when compared to the toxicant animals. High dose (500mg/kg) of aqueous and ethanolic extracts shows significant improvement in behavioral parameters. Such results are in harmony with those obtained by Somasekar et al, [1]who reported that *Salvia* plant extract having maximum antioxidant activity which may be due to the presence of high amount of flavonoids and phenols showed improvement of behavioral parameters like motor coordination, locomotor activity, functional ability and memory. Hasanein et al, in their study has explained that the protective effect of hydroalcoholic extract of *Salvia* against diabetes induced memory and learning deficit could be due to the presence of antioxidants such as rosimarinic acid as main flavonoid constituent. Mirrodi et al in their clinical investigation proved the beneficial effect of *Salvia* on cognitive functions in both healthy and patients with deficient cognition. Similar results were published by Moss et al, which indicated the aroma from essential oil of *Salvia* can improve learning and memory in healthy volunteers. Likewise, Scholey et al have demonstrated the preventive effect of ethanolic extract of *Salvia* on cognizance in healthy elderly subjects. [35]

Histological study of $Alcl_3$ treated group revealed neuronal damage, enlarged ventricles and amyloidal plaques in the brain. The amyloidal plaques formed due to induced AD were disappeared in the groups treated with low and high doses of *Salvia* extracts. Histological structure of brain of rats treated with low dose of extracts showed mild neurodegeneration whereas high dose showed more potent effect with normal histological structure of brain. Aqueous and ethanolic extracts showed overlapping results in treating the neurodegeneration with a lesser and higher effects as per low and high dose. High doses were proved to be more effective than the low doses of *Salvia* extracts. [36, 37]

The whole investigation concludes that the treatment of Alzheimer's disease with extracts of *Salvia officinalis*, and Donepezil (standard drug) were significantly reduced the oxidative stress and improves neurodegeneration of brain in Male Albino Wistar rats. Neurological and behavioral functions like memory, learning, physical activity, motor functions were enhanced with the treatment of Aqueous and ethanolic extracts. High dose (500mg/kg, b.w) of both the extracts of *Salvia* showed more potent effect on AD induced rats then the lower dose (250mg/kg, b.w). Biochemical Analysis revealed the improvement of cholinergic functions by the inhibition activity of AchE enzyme resulting in Ach elevations. Oxidative stress markers MDA and NO were decreased, and the antioxidant biomarker SOD was increased in the extract treated groups compared

to Alcl3 treated. Histopathological investigations proved that there was disappearance of amyloidal plaques, neuronal damage characteristic of AD with the extracts treatment compared to the AD-induced rats with Aluminium chloride. The results of the study give rise to the potent effect of *Salvia officinalis* extracts on the progressive disease of Alzheimer with improvement in oxidative stress. The possible mechanism by which the *Salvia* offcinalis extracts improve learning and memory functions could be due to its plausible involvement with cholinergic network. Further studies are warranted in clinical set up for elucidating the molecular mechanisms involved in *Salvia officinalis* which are responsible for producing favourable action in Alzeimers.

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Exploring Irritant Activity of Some of the Phytochemical Components from Wild *Sonchus arvensis* (L.) ssp *arvensis* (D.C.) Kirp herb

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ABSTRACT

Irritant activeness of sesquiterpene lactone glycosides, isolated from *Sonchus arvensis* (L.) ssp *arvensis* (D.C.) Kirp herb were investigated. These compound 1 to compound 7 were identified as sonchuside-E, sonchuside-F, sonchuside-G, sonchuside-H, sonchuside-I sonchuside-A and pirciside C. Such activity was appraised by open mouse ear assay, estimated by ID_{50}. These sesquiterpene lactones exhibited well-defined irritant responses compared with euphorbium, used as reference compound. Compound-1 (sonchuside-E) appeared to be the most potent and persistent irritant with least ID_{50} whose reaction lasted for more than 48 hours. Compound-2 (sonchuside-F), Compound-3 (sonchuside-G), compound-5 (sonchuside-I) and compound-6 (sonchuside-A), revealed an irritant reactions of medium intensity while compound-4 (sonchuside-H) and compound-7 (pirciside C) displayed the least irritant and least persistent reaction. The likely mechanisms of their action were discussed.

Keywords: Irritant activity, Sesquiterpene lactone glycosides, *Sonchus arvensis*.

INTRODUCTION

Sonchus arvensis (L.) ssp *arvensis* (D.C.) Kirp (Asteraceae or Compositae) also convoked 'milk thistle' or 'moist sow-thistle', is an insalubrious perennial tumid herb with intensified horizontal root system, 1 to 6 feet eminent excavated stems and produce an acrimonious milky latex[1-3]. The plant acquires indiscreet yellow flowers[1,2,3]. It is broadly distributed as weed in the Punjab plains of Paki-

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stan, very common along roadsides and in the vicinity of cultivated wheat and paddy fields. [4,5]

Among the phytochemical ingredients from different species of genus *Sonchus*, alkaloids, saponins, cardiac glycosides, tannins, ascorbic acid and polyphenols[6-8,21], anthraquinones, cyanogenic glycosides, leucoanthocyanins[8-10], flavonoids and flavonoid glycosides such as coumarins and hydroxy-coumarins[8-19,21], mono-acyl-galactosyl-glycerol[16], isocynaroside[17,18], phyto-sterols, triterpenes from nonsaponi-fiable lipid fraction[19] have been isolated and characterized from their different parts. Miyase and Fukushima (1987) on the other hand, quarantined nine sesquiterpene glycosides from the elevated parts of *Sonchus oleraceus* L.[20]. These were named as sonchusides-A, -B, -C and -D along with five others namely glucozaluzanin-C, macrocliniside-A, crepidiaside-A, picrisides-B and -C whose structures were established on the bases of chemical and spectral data[20]. Tapan (2016), appraised the presence of ascorbic acid, free phenolic acids, catechin, rutin, quercetin, myrecetin, apigenin and kaempferol in four solvent extracts of leaves of wild *S. arvensis*[7]. Heavy metals such as Pb, Fe, Cu, Zn, Ni and Ag in *Sonchus asper* were also assessed[22].

The whole herb is known as bitter, diuretic, sedative, hypnotic, diaphoretic, antiseptic, expectorant and employed for chronic fever, useful in cooling, cough, bronchitis, asthma and in pulmonary tuberculosis[30,32,33]. Young elevated parts of this herb are boiled, then fried in a pan or cooked and fabricated in tomato sauce are exploited for these purposes[24]. Its leaves are employed by the indigenous inhabits as sedative[34,36] while its tea is said to have placid impressions on nerves[34,35]. Its root are also utilized for asthma, coughs and in different chest ailments[36,37]. Constraining consequences of *S. arvensis* extract against bladder stones formation, have also been reported[38]. It was further intimated that the aerial parts of *S. arvensis* herb also restrained bioactive antioxidative phenolic compounds with strong radical scavenging capabilities and reducing power[8,39].

During Dispatching of *S. arvensis* weed from the cultivated wheat and paddy fields by indigenous people, erythema, itching and rashes, were ascertained on abaxial sides of proletarian's hands. The inauspicious impressions of *S. arvensis* on human beings or on animals have not encountered any attention in Pakistan. No endeavors has been flourished to sequestrate and appraise its harmful constituents. In the present communication, we depicted some of its irritant constituents extracted from methanol. The irritant effect of crude methanol was appraised on albino mice, succeeded by fractionation, to sequestrate and characterize its active compounds, whose effectivity were further evaluated by ID_{50}.

METHODOLOGY

General Experimental Procedures

Unless otherwise declared, all the chemicals used were of analytical grades. Melting points were uncorrected. Optical rotation measurements were calculated with Perkin Elmer 241 digital polarimeter. HR-FABMS and FABMS spectra were compiled on JEOL JMS-700 spectrometer in glycerol. UV spectra were quantified on Hitachi 270-30 spectrophotometer in MeOH, while IR spectra were procured by KBr discs or as thin film on NaCl discs on a Pye-Unicam SP 8-400 spectrophotometer. ^{1}H NMR spectra were acquired in DMSO-d_6 solvent at 270 MHz using tetramethylsilane (TMS) as an internal standard. ^{13}C NMR spectra were taken at 75 MH_z on Bruker AM-300 NMR spectrometers at 26 ± 2.5°C with 0.2-0.5 mM / ml concentrations of the samples, using 10 mm tubes. Chemical shifts were calculated in the δ (ppm), using tetramethylsilane (TMS) as an internal reference. Column chromatography was carried out on silica gel G_{60} (70–230 mesh from E. Merck Germany). Analytical TLC was performed on silica gel HF_{254} (from E. Merck Germany) with 0.25 mm thickness. Spots were visualized by exposure to UV light (at 254/365 nm), or with I_2 vapors, or with vanillin/H_2SO_4 reagent or with anisaldehyde/ H_2SO_4 reagent[39,40]. Preparatory TLC of the pooled column fractions were also executed on 0.75 mm thick silica gel HF_{254} TLC glass plates where the samples were applied as narrow bands. The separated bands were scraped off and eluted with a suitable solvent.

Plant materials

Whole of the *Sonchus arvensis* (L.) ssp *arvensis* (D.C.) Kirp herb (about 8.76 kg) were accumulated from wet atrophied areas and around the paddy fields from the vicinity of Shakhupura near Lahore (i.e., central plain areas of Punjab) in July/August 2018. These herbs were authenticated by Prof. Dr. Zaheer-ud-Khan, in-charge herbarium, Department of Botany, Government College University, Lahore. A voucher specimen of the sample (No. **P-cog.0154**) was deposited in the Herbarium of Pharmacognosy Section, Faculty of Pharmacy, University of Central Punjab, Lahore for further reference. Aerial parts of the herbs were detached from roots, air dried in shade and pulverized to a fine powder.

Extraction and isolation

Air dried aerial parts of *Sonchus arvensis* (4.90 kg) were extracted three times with MeOH in a soxhlet apparatus. The extract was concentrated under reduced pressure and residue was suspended in water. This suspension was then extracted with Et_2O. Water layer was passed through an Amberlite XAD-2 column and MeOH eluate was concentrated under reduced pressure that succumbed

80.23 g of residue. A portion of residue (46.50 g) was rechromatographed on silica gel column (4×250 cm). The elution of column was carried out with solvent system of $CHCl_3$ and $CHCl_3$-MeOH, while increasing the amount of latter solvent gradually. The fractions containing similar compounds were pooled together after monitoring by analytical TLC. The pooled fractions were evaporated to dryness under reduced pressure. Preparative thin layer (0.75 mm thick) chromatography of the pooled column fractions were executed on silica gel by applying the samples as uniform bands and using suitable solvent system. The separated bands were scraped off and eluted with methanol.

Compound 1

Compound 1 was eluted from column with $CHCl_3$/MeOH (95:5) and from preparative TLC (with solvent system $CHCl_3$/MeOH 90:10). It appeared as amorphous powder and after re-crystallization with hot EtOH, 490 mg (yield = 1.054%) of acicular crystals were procured. HR-FABMS m/z: 426.18897[M^+]; (Found C = 56.71; H = 7.20. $C_{21}H_{30}O_9.H_2O$ requires C = 56.75; H = 7.26%). $[\alpha]_D^{26}$ -27.8° (H_2O; $_C$ 0.49) CD (H_2O;$_C$ 0.49). UV λ_{max}^{MeOH} nm (log ε) 252 (3.70). IRν_{max} (thin film) cm^{-1}: 3512 (hydroxyl groups), 3082, 2930, 1780 ((γ-lactone ring), 1720, 1640 (double bonds), 1212, 1020. ^{1}HNMR, *δ*(ppm): 0.94 (3H, *s*, H_3-14), 1.83 (1H, *t*, *J* = 11H_z, H-5), 3.71 (1H, *dd*, *J* = 11.6 H_z, H-1), 3.80 (1H, *t*, *J* = 11H_z, H-6), 4.87 (1H, *d*, *J* = 6H_z, anomeric proton), 5.28 (1H, *d*, *J* = 3.2H_z, H-13a), 6.10 (1H, *d*, *J* = 3.6H_z, H-13b), 9.71 (1H, *d*, *J* = 4H_z, H-15). ^{13}CNMR, *δ*(ppm): Aglycone moiety indicated at 83.5(C-1), 25.7(C-2), 24.9(C-3), 48.9(C-4), 50.4(C-5), 82.2(C-6), 49.6(C-7), 21.8(C-8), 36.9(C-9), 41.1(C-10), 140.5(C-11), 169.9(C-12), 117.5 (C-13), 12.9(C-14), 203.2(C-15); sugar moiety demonstrated 102.3(C-1), 75.3(C-2), 78.4(C-3), 72.4(C-4), 78.8(C-5), 63.7(C-6). The compound 1 exhibited eudesmanolide-type skeleton and was identified by comparing its spectral data with reported data[41-43] and with CAS ID = C00013017 as sonchuside E (Fig. 1).

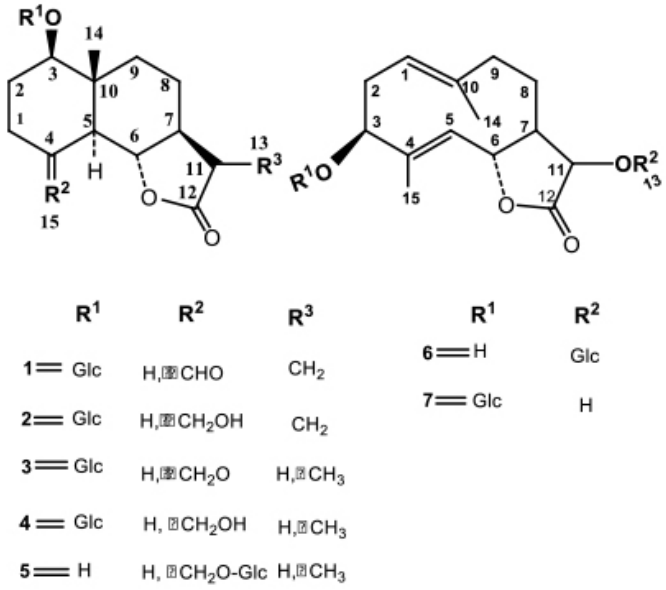

Fig 1. Compounds isolated from Dried *Sonchus arvensis* herb

Compound 2

Compound 2 was eluted from column with $CHCl_3$/MeOH (90:10) and from preparative TLC (with solvent system $CHCl_3$/MeOH 80:20). It was visualized as an amorphous light-yellow powder and after re-crystallization with hot Et_2O/EtOH (50:50 mixture), 134 mg (yield = 0.288%) of light yellow colored purified compound was obtained. HR-FABMS m/z: 428.20471[M^+]. (Found C = 56.41, H = 7.66. $C_{21}H_{32}O_9$ H_2O requires C = 56.49, H = 7.68%).$[\alpha]_D^{26}$ -5.9° (MeOH; $_C$ 0.64); CD (MeOH; $_C$ 0.34). UV λ_{max}^{MeOH} nm (log ε) 252 (2.70). IRν_{max} (thin film) cm^{-1}: 3502 (hydroxyl groups), 2902, 1763 (γ-lactone ring), 1717, 1652 (double bonds), 1232, 1024. ^{1}HNMR, δ(ppm): 0.95 (3H, *s*, H_3-14) tertiary methyl signal, 4.84 (1H, *d*, *J* = 8H_z, anomeric proton), 5.24 (1H, *d*, *J* = 3.2 H_z, H-13a) and 6.16 (1H, *d*, *J* = 3.6H_z, H-13b), two olefinic proton signals, which are characteristic of an exocyclic-α-methylene-γ-lactone. ^{13}CNMR, δ(ppm): Aglycone moiety indicated by 84.8(C-1), 28.9(C-2), 26.7(C-3), 40.2(C-4), 50.9(C-5), 84.2(C-6), 50.6(C-7), 21.9(C-8), 37.6(C-9), 42.4 (C-10), 141.8(C-11), 171.6(C-12), 115.8(C-13), 13.7(C-14), 66.6(C-15); sugar moiety showed at 102.8(C-1), 75.6(C-2), 78.6(C-3), 72.6(C-4), 78.7(C-5), 63.6(C-6). The compound 2 was identified by comparing its spectral data with reported data[41-43] and with CAS ID = C00013013 as sonchuside F (Fig. 1).

Compound 3

Compound 3 was eluted from column with $CHCl_3$/MeOH (80:20) and from preparative TLC (with solvent system $CHCl_3$/MeOH 70:25). It came out as an amorphous colorless powder and after re-crystallization with hot EtOH, 106 mg (yield = 0.228%) of colorless purified compound was obtained. HR-FABMS m/z: 428.20463[M^+]. (Found C = 53.28, H = 7.87. $C_{21}H_{32}O_9$. H_2O requires C = 53.29, H = 7.88%). $[\alpha]_D^{26}$ -37.9° (H_2O; $_C$ 1.82). UV λ_{max}^{MeOH} 252 nm. IRν_{max} (thin film) cm^{-1}: 3422 (hydroxyl groups), 3082, 2932, 1771 (γ-lactone ring), 1726, 1652 (double bonds), 1224, 1028. ^{1}HNMR, δ(ppm): 0.94 (3H, *s*, H_3-14) tertiary methyl signal, 1.14 (3H, *d*, *J* = 7H_z, H-13), 4.81 (1H, *d*, *J* = 8 H_z, anomeric proton), 9.68 (1H, *d*, *J* = 4 H_z, H-15). ^{13}CNMR, δ(ppm): Aglycone moiety indicated at 83.4(C-1), 25.8(C-2), 24.6(C-3), 49.2(C-4), 49.4(C-5), 81.9(C-6), 53.3(C-7), 23.6(C-8), 37.6(C-9), 42.2(C-10), 41.6(C-11), 177.6(C-12), 12.8(C-13), 12.4(C-14), 24.2(C-15); sugar moiety showed at 102.4(C-1), 75.5(C-2), 78.6(C-3), 72.3(C-4), 78.7(C-5), 63.4(C-6). The compound 3 was recognized by comparing its spectral data with reported data [41-43] and with CAS ID = C00013018 as sonchuside G (Fig. 1).

Compound 4

Compound 4 was eluted from column with $CHCl_3$/MeOH (70:25) and from preparative TLC (with solvent system $CHCl_3$/MeOH 70:20). It came out as an amorphous colorless powder and after re-crystallization with hot $CHCl_3$/EtOH (50:50 mixture), 79 mg (yield = 0.172%) of colorless purified compound was obtained. HR-FABMS m/z: 430.22030[M^+]. (Found C = 56.09, H = 8.10. $C_{21}H_{34}O_9$ H_2O requires C = 56.12, H = 8.15%). $[\alpha]_D^{26}$ -38.2° (MeOH; $_C$ 0.23). UV λ_{max}^{MeOH} 252 nm. IRν_{max} (thin film) cm^{-1} 3414 (hydroxyl groups), 2924, 1782 (γ-lactone ring), 1643 (double bonds), 1158, 1026, 992. ^{1}HNMR δ(ppm): 0.96 (3H, *s*, H_3-14) tertiary methyl signal, 1.09 (3H, *d*, *J* = 7H_z, H-13) a doublet methyl signal, 4.88 (1H, *d*, *J* = 8H_z, anomeric proton). ^{13}CNMR δ(ppm): Aglycone moiety indicated by 84.6(C-1), 28.8(C-2), 26.9(C-3), 39.4(C-4), 50.3(C-5), 82.6(C-6), 52.6(C-7), 22.6(C-8), 37.8(C-9), 41.9(C-10), 41.2(C-11), 180.6(C-12), 12.9(C-13), 13.7(C-14), 74.5(C-15); sugar moiety showed by 102.6(C-1), 75.4(C-2), 78.6(C-3), 72.3(C-4), 78.4(C-5), 63.3C-6). The compound 4 was agnized by comparing its spectral data with reported data[41-43] and with CAS ID = C00013014 as sonchuside H (Fig. 1).

Compound 5

Compound 5 was eluted from column with $CHCl_3$/MeOH (70:30) and with preparative TLC (with solvent system $CHCl_3$/MeOH 60:30). It came out as an

amorphous yellowish white powder and after re-crystallization with hot $CHCl_3$/EtOH (40:60 mixture), 38 mg (yield = 0.082%) of colorless purified compound was obtained. HR-FABMS m/z: 430.22029[M+H]$^+$. FABMS m/z: (rel. int.): 430 [M+H]$^+$(76), 415(11), 369(15), 269(100). MF = $C_{21}H_{34}O_9$. $[\alpha]_D^{26}$ -31.6^o (MeOH; $_C$ 0.53). UV λ_{max}^{MeOH} 253 nm. IRν_{max} (KBr)cm^{-1}: 3512 (hydroxyl groups), 2884, 1762 (γ-lactone ring), 1714, 1642 (double bonds), 1222, 1022. ^{1}HNMR δ(ppm): 1.03 (3H, *s*, H_3-14) tertiary methyl signal, 1.16(3H, *d*, *J* = 7H_z, H-13) a doublet methyl signal, 3.58(1H, *dd*, *J* = 10.6 H_z, H-1), 4.96(1H, *d*, *J* = 8H_z anomeric proton). ^{13}CNMR δ(ppm): Aglycone moiety indicated 78.6(C-1), 31.8(C-2), 28.9(C-3), 38.0(C-4), 49.9(C-5), 83.2(C-6), 55.5(C-7), 23.7(C-8), 37.9(C-9), 42.8(C-10), 40.9(C-11), 178.6(C-12), 12.8(C-13), 13.2(C-14), 73.8(C-15); sugar moiety showed 103.6(C-1), 75.6 (C-2), 78.4(C-3), 72.2(C-4), 78.4(C-5), 63.3(C-6). The compound 5 was accredited by comparing its spectral data with the reported data[41-43] and with CAS ID = C00013015 as sonchuside I (Fig. 1).

Compound 6

Compound 6 was eluted from column with $CHCl_3$/MeOH (70:35) and with preparative TLC (with solvent system $CHCl_3$/MeOH 60:40). It came out as a very light yellowish white powder and after re-crystallization with hot $(CH_3)_2O$/EtOH (75:25), 32 mg (yield = 0.069%) of light yellowish white fine acerose purified compound was procured. HR-FABMS m/z: 412.2098[M+H]$^+$. FABMS m/z (rel. int.) 412 [M+H]$^+$ (22), 397 (21), 387 (15), 329(26), 307(37), 269(64). MF = $C_{21}H_{32}O_8$. $[\alpha]_D^{26}$ -43.2^o (MeOH; $_C$ 0.60). UV λ_{max} nm(log ε): 279 (3.75). IRν_{max} (KBr)cm^{-1}: 3492 (hydroxyl groups), 2942, 1766 (γ-lactone ring), 1722, 1600 (double bonds), 1222, 1026. ^{1}H-NMR, δ(ppm): 1.02 and 1.04 (each 3H, *s*, H_3-11 H_3-12) due to methyl group, 2.09 (3H, *d*, *J* = 6H_z, H-13), 2.27 (3H, *s*, H-10), 4.12 (1H, *dt*, *J* = 12.4 H_z, H-3), 4.36 (1H, *d*, *J* = 4H_z, H-4), 4.96 (1H, *d*, *J* = 8H_z, anomeric proton), 6.08 (1H, *d*, *J* = 18H_z, H-8), 7.19 (1H, *d*, *J* = 16H_z, H-7). ^{13}CNMR, δ(ppm): Aglycone moiety indicated 37.6(C-1), 43.4(C-2), 65.4(C-3), 84.4(C-4), 130.9(C-5), 139.7(C-6), 142.0(C-7), 133.6(C-8), 197.7(C-9), 27.7(C-10), 27.8(C-11), 30.2(C-12), 19.9(C-13); sugar moiety showed 106.9(C-1), 75.3(C-2), 78.6(C-3), 71.7(C-4), 78.6(C-5), 62.8(C-6). The compound 6 was identified by comparing its spectral data with reported data[44-47] and with CAS ID = C00032170 as sonchuside A (Fig. 1).

Compound 7

Compound 7 was eluted from column with $CHCl_3$/MeOH (60:40) and with preparative TLC (with solvent system $CHCl_3$/MeOH 55:45). It came out as a fine lustrous powder and after re-crystallization with hot $CHCl_3$/EtOH (30:70 mixture), 26 mg (yield = 0.0602%) of white fine crystalline mass of purified com-

pound was obtained. HR-FABMS m/z: 410.19386[M+H]+. FABMS m/z (rel. int.) 410 $[M]^+$ (60), 354 (17), 340 (21), 270 (18). MF= $C_{21}H_{30}O_8$. $[\alpha]_D^{26}$ -63.5° (MeOH; $_C$ 0.49). UV λ_{max} nm (log ε): 278 (3.62). IRν_{max} (thin film) cm^{-1}: 3454 (hydroxyl groups), 2922, 1764 (ester group), 1666, 1552, (double bonds)1452, 1408, 1290, 1248, 1146, 1082, 972, 938. ^{1}HNMR, δ(ppm): 1.34 (3H, br s, H3-14), 1.98 (3H, br s, H3-15), 5.57 (1H, d, J=3.1Hz, H-13a), 6.26(1H, d, J = 16Hz, H-8), 6.41 (1H, d, *J*=3.4 Hz, H-13b), 7.29(1H, d, J = 16Hz, H-7). ^{13}CNMR, δ(ppm): 12.8(C-15), 16.5(C-14), 28.6(C-8), 33.8(C-2), 41.5(C-9), 50.3(C-7), 63.2(C-6'), 71.9(C-4'), 75.5(C-2'), 78.6, 78.8(C-3'/C-5'), 81.4(C-6), 83.5(C-3), 102.7(C-1'), 119.6(C-13), 125.5(C-1), 127.3(C-5), 137.9(C-10), 140.7(C-4), 142.3(C-11), 170.4(C-12). The compound 7 was identified by comparing its spectral data with the reported data[44,45,48] and with CAS ID = C00012186 as pirciside C (Fig. 1).

Animals

Albino mice weighing 10 to 15 g were procured from the Drug Testing Laboratory, Jail Road Lahore, Pakistan. The animals were housed in plastic cages on wood shavings in an animal house in PCSIR Laboratories, Ferozepure Road Lahore. Six mice were managed per cage in a laminar air flow room maintained under a temperature 28±2.5°C and relative humidity 35±4.2%. Palette food and de-ionized water were available *ad libitum*.

Irritant activity

10 mg of the test compound was dissolved in 10 ml of acetone to prepare 10 mg/10 ml (w/v) solution. It was further diluted according to the method of Evans and Schmidt[49] and Kinghorn and Evans[50]. Ten dilutions were disposed for the main assay. The main procedure for appraising the irritancy on mouse ears was also espoused from Evans and Schmidt[49] and Kinghorn and Evans[50]. For the main assay, a group of 12 animals were used for each dilution. 5µl of one of the dilutions under test was employed to the inner surface of one of the mouse ears using Drummond Microcaps (Drummond Scientific Co. USA), while other ear was regarded as negative control which was without any testing material. Other nine consecutive dilutions were employed substitutable for other groups of animals. 10mg/10ml of euphorbium (a resin from *Euphorbia helioscopia*)[49,50] in acetone was employed as a positive control group. Euphorbium was chromatographically purified by column chromatography, prior to its use. Each of the ears was probed for redness after 30 minutes, then after every 15-minute intervals until two observations displayed that further inflammation would not be anticipated. Time of maximum erythema was detected. Number of ears educing the degree of redness agreed with at least ++ intensity on Hecker's scale at peak irritancy[51] were observed and expressed in µg/5µl per ear. Ears of the animals

were also examined after 24, 48 and 72 hours, to find out any assiduous irritant effectuate of the test compounds. Number of red ears with at least ++ strength (denoted by IU — Irritant units on Hecker scale)[51] were commemorated after these times. If no redness was detected, the process was reiterated with higher concen-trations of the test solution on ears of another group of animals. Total number of red ears per dilution were tabulated. ID_{50} (Irritant doses in 50 % individuals) were calculated by probit analysis[52], using a computer program[53]. Number of inflamed mice ears induced by the seven isolated compounds and euphorbium (positive control), their ID_{50}, χ^2, time of ++ irritant reaction, upper and lower confident limits have been outlined in Table 1

Table 1. Mice with positive irritant responses after testing with compounds 1-7

Dose levels (µg/5µl)	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Comp. 7	Euphor-bium
10	——		——	9/12	10/12	11/12	——	12/12
5.0	12/12	11*/12†	9/12	8/12	9/12	9/12	9/12	12/12
2.50	11/12	10/12	9/12	7/12	9/12	8/12	6/12	12/12
1.25	9/12	9/12	8/12	6/12	8/12	6/12	4/12	12/12
0.625	9/12	7/12	7/12	5/12	6/12	5/12	3/12	9/12
0.3125	8/12	5/12	7/12	3/12	5/12	4/12	2/12	8/12
0.15625	7/12	3/12	6/12	2/12	4/12	4/12	1/12	7/12
0.078125	6/12	2/12	5/12	1/12	3/12	2/12	0/12	6/12
0.0390625	3/12	1/12	3/12	0/12	2/12	1/12	——	3/12
0.01953121	2/12	1/12	0/12	——	2/12	0/12	——	2/12
0.00976562	1/12	0/12	——	——	1/12	——	——	2/12
		——						
ID_{50} µg/5µl	0.135	0.857	0.318	1.571	0.515	0.782	2.054	0.099
S. D.	0.099	0.099	0.187	0.139	0.149	0.106	0.204	0.094
x^2	2.102	0.738	3.732	0.931	0.531	3.286	0.713	2.943
t	1.0 h	2.5h	1.5h	4.0h	1.5h	2.5h	4.5h	30 min
U.C.L.	0.235	1.498	0.72	3.475	1.142	1.453	5.047	0.165
L.C.L.	0.073	0.513	0.141	0.858	0.241	0.442	1.195	0.056
IU after 24 h	1.26	2.5	1.25	5	2.5	2.5	5	1.25
IU after 48 h	2.5	5	2.5	>10	5	5	>10	2.5

Where:

* Number of animal ears reacted to irritant compound. † Total number of animals used.

Comp. **1** = sonchuside E; Comp. **2** = sonchuside F; Comp. **3** = sonchuside G;

Comp. **4** = sonchuside H; Comp. **5** = sonchuside I; Comp. **6** = sonchuside A;

Comp. **7** = pirciside C.. ID_{50} = Irritant dose in 50 % individuals;

S.D. = Standard deviation; χ^2 = Chi square; t = Time of maximum irritant reaction;

IU = Irritant units; U.C.L. = Upper confident limits; L.C.L. = Lower confident

Limits. h = hours after application.

RESULTS AND DISCUSSION

Methanol extract of the dried *S. arvensis* herb succumbed crude fraction that mortified sesquiterpene lactone glycosides. First endeavour of direct chromatographic analysis of this mixture into pure components was not successful, it was further fractionated into water and ether soluble fractions. Aqueous fraction after passing through an Amberlite XAD-2 column was eluded with MeOH and re-chro-matographed on silica gel column and preparative thin layers, which afforded seven sesquiterpene lactone glycosides (compound **1** to **7**). All these compounds were identified by comparison of their chromatographic and spectroscopic data with the published values[41-48]. It is known that sesquiterpene lactones present in various species of family Asteraceae are pledged in a number of functions. A panoptic spectrum of their biological activeness have been reported[19,20,26,27,32-34,37,38]. The structure-activity relationships (SAR) of these lactones ascertained that the mien of an α-methylene exocyclic to a γ-lactone ring is substantive for most of their stringy toxic pharmacological and biological activeness, especially when such compounds are prosecuted on the human and animal's skins[49,50,54-56]. This is staunch for such active group, when an inflammatory reaction due to irritancy, allergenicity or cross-sensitivity are pertained. It has also been investigated that C = C grouping conjugated to a lactone, is an immunological prerequisite[49,50,54-56]. Plant extracts are frequently a complex mixture of phytochemical compounds that prosecute on skin by dissimilar mechanisms, with different effectivity and continuance of action[55,56]. Mouse ear test is known to be invaluable for screening various plant extracts for inflammatory reaction[49-51]. For comparability of the irritant activities of *S. arvensis*'s sesquiterpene lactones, the total number of mice, indicating inflammatory reaction were enumerated at the time of peak irritancy. The data was analyzed by computer program[52,53] which enable us to draw a comparison between the potentialities by means of ID_{50} that afford plenteous assurance, because limits were located on upper and lower confident levels, along with the standard deviation. Standard deviation also manoeuver to quantify the slop of probit regression line which intimated the overall 'shape' of Gaussian distribution of tolerance curve[52]. The intention of χ^2 test, computed by the probit program,

was to ascertain whether the results of the assay after transformation, were appropriately symbolized by the probit regression line[52]. All the abstracted seven sesquiterpene lactone glycosides paraded irritant effectuates on the mice's ears after comparison with euphorbium reaction. In all the cases, the reaction first came out as red inflamed area (erythema). The profound response then turned to a scale formation, followed by oedema. Redness of the mouse's ear appeared between twenty minutes to about two hours after assiduity and in some cases, it reached to a maximum level of ++++ intensity in about four hours. Later on, oedema of the ears was espoused by a little transudation of watery fluid from the notorious skin. Compound 1(sonchuside-E) revealed more escalated irritant reaction than all the other six compounds, with minimum ID_{50} (Table 1.). Compounds 2 (sonchuside-F), -3(sonchuside-G), -5(sonchuside-I) and -6(sonchuside-A) imparted an irritant reactions of medium vividness, while compounds -4(sonchuside-H) and -7(pirciside C) revealed the least irritant chroma on the mice ears, when compared with the euphorbium reaction (Table 1.). The results further betoken that the irritant reactions of these sesquiterpene lactones litigated up to 24 or 48 hours. The inauspicious reaction of compounds-1 (sonchuside-E) was continued even languished more than 48 hours, in a similar way as the reaction revealed by euphorbium (Table 1.). The presence of α-methylene group exocyclic to γ-lactone ring in these sesquiterpene lactone glycosides from *S. arvensis* was plausibly the main causation of scaly dermatitis of irritant type[49-51], within a short time that was lasted up to 48 hours or even more as in case of compound-1 (Table 1., Fig. 1). The pioneering of erythema, scales and oedema was credibly due to the presence of exocyclic oxygen and double bonds present in their molecules (Fig.1) which perhaps constituted some kind of adduct with the skin proteins and as a consequence inflammation and scaling of mouse skin was anticipated. Since compound-1 possessed an exocyclic α-methylene-γ-lactone signals at δ 5.28 and 6.10, an aldehyde proton signal at δ 9.71 and an anomeric proton signal at δ 4.87, the stronger irritation of this compound was probably due to such moieties (Fig. 1). Medium irritant response of compound-3 compared to others due to the absence of exocyclic methylene proton signals at C-13 and the appearance of a doublet methyl signal at δ 1.14. The situation in case of compound-7, that have least irritation, might be due to some double bonds present in its molecule (Fig. 1).

Although the definite mechanism of this activity has not been elucidated, these phenomena might be considered to occur, due to the differences in binding affinities of these sesquiterpene lactones on the active sites of proteins or receptors present in animal's skin. We had exploited only that much quantities of these compounds which at least gave ++ reaction on Hecker's scale[51]. The doses

of the compounds utilized in this work, only caused erythema, scaling and oedema, while in case of compound-1, exudation of water from the implemented area was also perceived, possibly due to some biological damage of skin cells. In all these reactions, hyper-pigmentation was not ascertained. This was likely that due to less quantities of these lactones were able to be penetrated in animal skin and thus were not spendable for hyper-pigmentation.

We concluded that *S. arvensis* herb constrained irritant sesquiterpene lactone glycosides, which could be injurious not only to the animals, but also to the human beings. The despicable and reiterated doses of these compounds, with controlled clinical conditions may be pursued as an anti-inflammatory and sedative agent, as it was arrogated in the folk medicines[5,6,20-25]. Oral presidency of these sesquiterpene lactone glycosides in low and ingeminated doses may be a safer route than topical application for anti-inflammatory and sedative purposes, particularly in case where erythema is of diffused type and whole of the body is involved. On the other hands, the topical application of higher dosages of these compounds might commove the normal pattern of skin cells. These perturbations often induce neoplasmic growth in skin cells, including skin carcinomas. These possibilities necessitate furthermore investigation. Moreover, further substantial work is indispensable to inflate these properties, through the grooming of their derivatives that would possible lead to the structure-activity relationship of such important molecules of natural origin.

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Omega 3 Fatty Acid and Vitamin A Ameliorate Carrageenan-induced Joint Inflammation in Wistar Rats

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ABSTRACT

This study investigates the effect of omega 3 and Vitamin A on joint inflammation in Wistar rats. Joint inflammation was induced by carrageenan and the animals were treated with omega 3, Vitamin A or a combination of both for 10 days. Changes in Knee diameter (KnD), Tumor Necrosis Factor-α (TNF-α) level and C-Reactive protein (CRP) level were assessed to determine treatment efficacy. Carrageenan caused significant increase in KnD, TNF-α and CRP compared to the control. Treatments with Omega 3 alone or in combination with Vitamin A significantly reduced the elevated KnD, TNF-α and CRP. Vitamin A alone produced similar effect on KnD and TNF-α but had no effect on CRP. It is thus concluded that though both treatments decreased knee diameter at the 10th week, Omega 3 alone and in combination with Vitamin A showed a better outcome with a higher decrease in TNF-α and CRP than Vitamin A alone.

Keywords: Joint inflammation, Omega 3 fatty acids, Vitamin A, knee diameter, TNF-α, C-reactive protein

INTRODUCTION

Inflammation of the joints is a known feature of arthritis disease affecting one or more joints[1]; it is strongly associated with age, joint trauma, altered biomechanics, and obesity[2,3] particularly osteoarthritis characterized by joint pain

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with loss of joint form and function secondary to articular degeneration[3,4]. The structural changes during arthritis was conclusively shown to be preceded by inflammation/synovitis[5,6] and prominent inflammatory mediators such as cytokines, Nitric Oxide, Reactive Oxygen Species and matrix degrading enzymes produced by chondrocytes and synoviocytes have been identified[7]. These mediators are activated by exogenous materials or autologous antigens in the case of autoimmune diseases like rheumatoid arthritis[8] while in the case of non-autoimmune arthritis like osteoarthritis, it could be activated by obesity, ageing or trauma[2,3]. As the joint is continuously exposed to these factors, degradation of collagen and proteoglycans in cartilage leads to fibrillation, erosion and cracking in the superficial cartilage layer which could later spread deeper.

A high prevalence of arthritis has been identified in the low- and medium-income countries[9] where 90 % of the global disease burden are found yet contributing 12 % in global spending on health[10]. In adults above 60 years of age, 9.6 % of men and 18 % of women in the world suffers varied degree of osteoarthritis with a 10-fold increased risk caused by farming for more than 10 years[11]. The multiplying effect of farming on osteoarthritis may account for a higher prevalence in the low-income country were obesity might not be rampant as obtained in high income countries. The United Nation's projection indicates that by the year 2050, 20 % of the world population will be above 60 years of age, 15 % of which would have symptomatic osteoarthritis with one third of them severely disabled. By this projection, when 130 million suffers osteoarthritis, 40 million of them will be disabled[12,13] in the year 2050. A clarion call was then made by WHO to intensify efforts at identifying a cost-effective, safe, and efficacious therapy for long-term management of osteoarthritis[13]. The current therapy ranges from biological (e.g. bone marrow transplant, gene therapy, nanotechnology etc) to chemical [e.g. non-steroidal anti-inflammatory drugs, corticosteroids, and Disease-modifying anti-rheumatic drugs (DMARDs)] which sometimes cause sustained remission with side effects that discourage long term usage yet they are expensive and the chances of infection are high[14].

Diet modification and food supplements have been documented to be beneficial in long-term prevention and treatment of chronic disease in the aging adults[15]. Omega 3 fatty acids are polyunsaturated fatty acids (PUFAs) which include α-linolenic acid (ALA) (found in plant oils), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) commonly found in marine oils. All are essential in the diet for normal metabolism, as there is no mechanism in humans for producing these fats from other substances[16,17]. Studies in humans and animals have shown a negative association between the ingestion of polyunsaturated

fatty acids and the incidence of cardiovascular diseases, diabetes, other autoimmune diseases and cancer; by decreasing the concentrations of proinflammatory cytokines, arachidonic acid derivatives and other inflammatory biomarkers[18]. Specifically, current evidences have shown that dietary intake of EPA and DHA as contained in omega 3 confers anti-inflammatory function by altering the production of proinflammatory markers prostaglandin E2, thromboxane B2, and leukotriene B4 toward a more anti-inflammatory profile in rheumatoid arthritis[19]. Also, omega 3 reduces the need for concomitant analgesic treatment when used as adjuvant to DMARD in rheumatoid arthritis patients[20].

Oxidative stress is also implicated in the aetiology of arthritis thus, use of antioxidants such as Vitamin A is also beneficial in relieving joint inflammation. Vitamin A acts as a first line defence against free radical attack and lipid peroxidation by its ability to stabilize highly reactive free radicals either independently or as a part of large enzyme system[21]. Vitamin A has been postulated to improve joint inflammation by protecting against oxidative damage, and modulating inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis[22]. Thus, both omega 3 fatty acids and Vitamin A possess anti-inflammatory activity that could be used as alternative medicine for the management of chronic joint inflammation, it is however not known if a combination of both could act synergistically to alleviate joint inflammation. This study was therefore designed to investigate the effect of omega 3 and Vitamin A on joint inflammation induced by carrageenan in female Wistar rats.

METHODOLOGY

Drugs

Omega-3 fish oil (containing EPA 180 mg and DHA 120 mg) and Vitamin A (10,000 IU [from fish liver oil and retinyl palmitate]) manufactured in USA for Mason Vitamins.

Animals

A total of 24 female Wistar rats weighing 150-200g were used for the study. The animals were obtained from Department of Human Physiology, Ahmadu Bello University Zaria and housed in cages under standard laboratory conditions and had access to food and water *ad libitum*.

Experimental design

The animals were randomly divided into 6 groups (n=4) and used for the ten weeks study. In the first 2 weeks, animals received CGN injection at their knee joint 3 times/week to induce inflammation (0.02ml of 1% CGN) as described

by Manole et al[23]. After establishment of inflammation, which was assessed by clinical signs such as swelling, redness, deformity and ankylosis in the knee joints, 200mg/kg omega 3 and Vitamin A (4000IU/kg) were orally administered daily, and treatment with CGN 3 times/week was continued for the remaining period. The experimental groups were as follows:

Group 1: Served as normal control and were given normal feed and water (NC);

Group 2: Served as injection control and were injected normal saline solution in their knee joint 3 times/week (NSi);

Group 3: Served as disease control and were injected 0.02 ml of 1% carrageenan solution in their knee joint 3 times/week (CGN);

Group 4: Oral administration of 200mg/kg omega 3 daily[24] + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+Omg3);

Group 5: Oral administration of 4000IU/kg Vitamin A daily[25] + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+VitA);

Group 6: Oral administration of 200mg/kg omega 3+ 4000IU/kg Vitamin A daily + 0.02 ml of 1% carrageenan solution injected in their knee joint 3 times/week for 8 weeks (CGN+Omg3+Vit A).

Induction of Joints inflammation and measurement of knee joint diameter

Female Wistar rats were subjected to knee joint inflammation by injecting 0.02ml of 1% CGN solution into the knee joint 3 times/week for 10 weeks[23]. Joint diameter was measured before first injection (at day 0), second week and thereafter, weekly for 8 weeks. Appearance of clinical symptoms such as swelling, redness, deformity and ankylosis in the knee joints indicates a sign of joint inflammation. Joint inflammation and its severity were measured weekly by measuring the diameter of the swelling at the knee of the hind limbs using veneer calliper[26,27].

Blood collection

At the end of the 10th week, the animals were anesthetized with 0.15ml/kg ketamine hydrochloride injection[28] and blood samples were collected by cardiac puncture into plane sample bottles for serum determination of tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP) level.

Determination of Tumor Necrosis Factor-α

The assay for TNF-α activity was carried out using Rat TNF-α ELISA kit purchased from Elabscience (Catalog No: E-EL-R0019 96T) USA.

The micro ELISA plate provided has been pre-coated with an antibody specific to Rat TNF-α and the kit uses sandwich-ELISA principle. Standards or samples were added to the micro ELISA plate well and combined with specific antibody. Then a biotinylated detection antibody specific for Rat TNF-α and Avidin-Horseradish peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain Rat TNF-α, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour. The enzyme-substrate reaction was terminated by the addition of stop solution and the colour turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450nm. The concentration of Rat TNF-α in the samples was calculated by comparing the OD of the samples to the standard curves.

Determination of C-Reactive Protein

The assay for CRP was carried out using CRP rapid latex slides (REF 514002: Spectrum bioscience). Spectrum CRP latex reagent is a suspension of polystyrene particles sensitized with anti-CRP.

All reagents and specimens were brought to room temperature. Serum to be titrated was serially diluted (1:2, 1:4, 1:8 etc) in 0.9 g/ml saline solution. One drop of positive control was placed on slide. Each serum dilution (50µl) was placed individually in successive circles on the slide and (50µl) of the positive control into separate circles on the glass slide. CRP latex reagent was shaken gently and one drop (45µl) was added on each circle next to the sample to be tested and control. It was well mixed using a disposable stirrer spreading the mixture over the whole test area and the slide was tilted gently. Agitated for about 2 minutes with hand and the presence or absence of agglutination was observed.

Presence or absence of agglutination indicate positive (indicating a CRP level of more than 6 mg/L) or negative result respectively. The serum CRP titre can be defined as the highest dilution showing a positive result. The approximate CRP level (mg/L) present in the sample can be obtained by the following formula:

CRP Titre (mg/L) = Highest dilution with positive reaction x Reagent sensitivity (6 mg/lL)

Statistical analysis

The results were expressed as Mean ± Standard Error of Mean (SEM) and values were analysed using mixed analysis of variance (ANOVA) for knee diameter, one-way ANOVA for TNF-α and CRP followed by Tukey's post hoc test. The data were analysed using SPSS for windows (Version 22). *P*-values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of omega 3 and/or Vitamin A on Knee diameter

The effects of omega 3 and/or Vitamin A on Knee diameter (KnD) at the 2nd and 10th week are shown in table 1. At week 2, KnD was significantly increased in CGN (5.61 ± 0.17 mm), CGN+Omg3 (5.25 ± 0.17 mm), CGN+Vit A (5.12 ± 0.20 mm) and CGN+Omg3+VitA (5.91 ± 0.07 mm) compared to NC and NSi (4.08 ± 0.04 mm and 4.26 ± 0.10 mm, respectively). Progression of inflammatory response was confirmed by steady rise in KnD of CGN (7.06 ±0.21 mm) compared to NC and NSi (4.30 ±0.06 and 4.59 ±0.05, respectively) at the 10th week. Treatment with Omega 3 alone (CGN+Omg3) or in combination with Vitamin A (CGN+Omg3+Vit A) significantly decreased KnD at the 10th week (4.51 ± 0.15 mm and 4.51 ± 0.07 mm, respectively) compared to their respective week 2 values and that of CGN at week 10. While KnD of the animals in CGN+VitA group at week 10 (5.20 ± 0.18 mm) was not different from its value at week 2 (5.12 ± 0.20 mm), it was however significantly lower than the week 10 KnD of CGN (7.06 ±0.21 mm).

Table 1. Effect of omega 3 and/or Vitamin A on Knee diameter at the 2nd and 10th week of carrageenan-induced joint inflammation in female Wistar rats.

Group	Treatment	Knee Diameter (mm)	
		WK 2	WK 10
1	NC	4.08 ± 0.04	4.30 ±0.06
2	NSi	4.26 ± 0.10	4.59 ±0.05
3	CGN	5.61 ± 0.17*	7.06 ±0.21*
4	Omg3+CGN	5.25 ± 0.17*	4.51 ±0.15#
5	Vit A+CGN	5.12 ± 0.20*	5.20 ±0.18#
6	Omg3+Vit A+CGN	5.91 ± 0.16*	4.51 ±0.07#

*P<0.05 compared with control, #P<0.05 compared with CGN

Effect of omega 3 and/or Vitamin A on Tumor Necrosis Factor-Alpha

As shown in figure 1, serum TNF-α was significantly increased (P<0.01) in the CGN group (228.81 ± 25.74 mg/L) compared to NC (51.27 ± 4.57 mg/L) and NSi (61.39 ± 8.45 mg/L), this was however reduced by treatment with omega 3 and/or Vitamin A in the CGN+Omg3 (27.13 ± 3.33 mg/L), CGN+VitA (123.99 ± 17.92mg/L) and CGN+Omg3+VitA (31.48 ± 1.55 mg/L). In fact, Omega 3 either alone or in combination with Vitamin A significantly reduced TNF-α to values below the control levels.

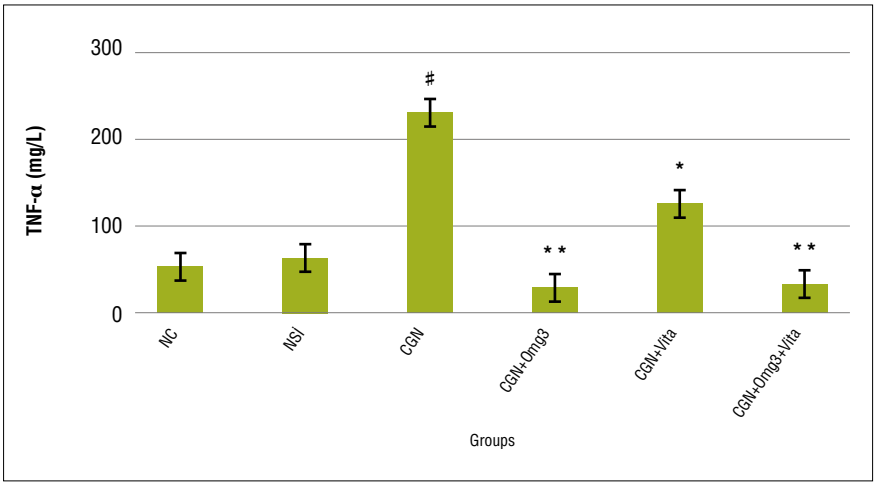

Figure 1. Effect of omega 3 and Vitamin A on Tumor Necrosis Factor-alpha following carrageenan-induced joint inflammation in female Wistar rats. # P<0.05 compared to control, *P<0.05, **P<0.01 compared to CGN.

Effect of omega 3 and/or Vitamin A on C-Reactive Protein

The serum CRP concentration was significantly increased (P<0.01) in CGN compared to NC and NSi, signifying an elevated inflammatory activity in response to carrageenan injection at the 10th week, this was however significantly reduced by omega treatment in the CGN+Omg3 and CGN+Omg3+VitA animals. Treatment with Vitamin A alone in the CGN+VitA animals produced no significant difference in the CRP compared to CGN animals (figure 2).

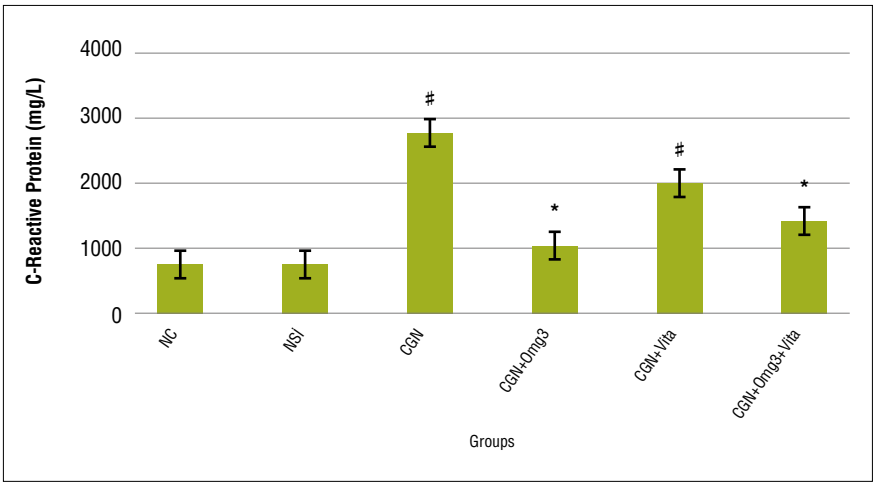

Figure 2. Effect of omega 3 and Vitamin A on C-Reactive Protein following carrageenan-induced joint inflammation in female Wistar rats. # P<0.05 compared to control, *P<0.05compared to CGN.

This study examined the influence of omega 3 fatty acids, Vitamin A and their coadministration on TNF-α and CRP following injection of carrageenan on knee joints of female Wistar rats using the method of Manole *et al* [23]. An elevated inflammatory activity in response to carrageenan injection was observed, which indicates a successful induction of sustained inflammation throughout the period of treatment[23,29]. Assanga *et al.*[30] reported an increase in rat paw oedema and CRP concentration after injection of (0.1ml/paw) of carrageenan solution.

The significant decrease in KnD presented within the groups treated with omega 3 FAs alone and its coadministration with Vitamin A indicate a high degree of anti-inflammatory activity, which was observed by suppression or reversion of inflammatory changes with duration of treatment. That is, decrease in levels of TNF-α and CRP. The effect observed in coadministration of these supplements is assumed to be mostly contributed by omega 3 FAs since Vitamin A alone could not show a similar outcome. Report by El-Seweidy *et al.*[31] supports the finding of the present study, that omega 3 FAs exerted a significant improvement in knee joint of osteoarthritic rats which may imply a significantly decreased pathological change in joint articular surface. A possible mechanism through which Omega 3 fatty acids reduced inflammation may be proposed as due to the fact that omega 3 reduces the formation of eicosanoids with inflammatory characteristics by competing with omega-6 fatty acids for the same enzymatic pathway that leads to the inhibition of TNF-α, IL-1, IL-6 synthesis, cartilage-degrading enzymes and reducing the intercellular adhesion molecule-1 (ICAM-1) expression[32,33].

The above report is in support of the present finding in animals treated with omega 3 FAs where they showed more effectiveness in lowering inflammatory activity by reverting the concentration of TNF-α to the lowest concentration. El-Seweidy *et al.*[34] who reported a significant decrease in TNF-α level following administration of omega 3 FAs in murine osteoarthritic rats. They also observed that omega 3 FAs can reduce serum soluble TNF-α receptor p55 and production of pro-inflammatory cytokines induced via the NFκB system. Nobre *et al.*[29] also reported a decrease in TNF-α along with a decreased paw swelling after treatment with omega 3 FAs in carrageenan-induced rat paw oedema. Omega 3 FAs was able to mediate its effect due to it being an important precursor for resolvins and protectins which are lipid-derived modulators of cell inflammatory processes. These lipid mediators have anti-inflammatory and inflammation resolving capabilities as they inhibit migration of neutrophils from capillaries and limit neutrophil infiltration at sites of inflammation with consequent inhibition of the production of TNF-α[35,36,37].

The positive outcome observed in coadministration of these supplements could be due to TNF-α reverting effect mainly mediated by omega 3 FAs. This implies that omega 3 FAs is more effective in decreasing TNF-α concentration than Vitamin A in CGN-induced joint inflammation and combination of these supplements did not show a better outcome than omega 3 FAs alone. This could be due to potential implication of retinoic acid in cytotoxicity induced NrF2 target genes at some concentrations; NrF2 participate in adaptive cellular defence against retinoid toxicity by enhancing transcription of antioxidant gene[38]. With chronic administration of Vitamin A this mechanism would have been depleted and retinoic acid toxicity outweighed the NrF2 activity. It could also be considered that anti-inflammatory and bone growth supporting effect of Vitamin A at the concentration used (4000IU/kg) is improved when co-administered with omega 3 FAs rather than Vitamin A alone. This could explain the reason why decrease in KnD was more pronounced in Omg3 and Omg3+Vit A than Vit A when compared to CGN, also, when considering response within groups, omega 3 FAs alone and in combination with Vitamin A had a linear decreasing effect across the weeks of experimental period.

When observing the findings of CRP concentration, it could be seen that omega 3 FAs and its coadministration with Vitamin A had a significant decrease compared to CGN control while Vitamin A did not show significant decrease. This confirms omega 3 FAs ability to suppress inflammatory response which is also supported with the decrease in KnD as well as a fall in TNF-α concentration that was earlier mentioned. Hepatic production of CRP could have been decreased

through a mechanism involving omega 3, by the protectin D1 pathway. Protectin D1 is a bioactive product of DHA generated from 17S-hydroperoxy DHA (a metabolic intermediate). It potently regulates critical events related to inflammation and its resolution which involves inhibition of polymorphonuclear cells (PMN) infiltration, T-cell migration and decreased TNF-α level. PD1 has also been shown to decrease COX-2 mRNA expression and block NFκB activation[39]. These could most likely lower the production of proinflammatory cytokines, subsequently decreasing hepatic production of CRP.

It could be suggested that Vitamin A can depress progression of inflammation without necessarily reverting the already formed inflammatory response. This shows a lesser anti-inflammatory activity in Vit A group with duration of treatment, indicating that Vitamin A could suppress but not revert inflammatory changes caused by CGN injection. This finding supports the work of Nagai *et al.*[40] who reported that Am80 (a newly synthesized retinol) significantly reduced the development of foot pad swelling and bone damage in collagen induced arthritis, implying that Vitamin A has short-term anti-inflammatory activity which could be through its ability to modulate inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis[41].

Treatment with Vitamin A showed lower anti-inflammatory activity by having a higher TNF-α concentration than that of omega 3 FAs alone and in combination, this coincide with the failure of Vitamin A to show significant decrease in KnD across experimental periods. This implies that Vitamin A could suppress, but not revert inflammatory activity following CGN injection. This contradicts the findings of Nagai *et al.*[40] who reported that Am80 (a synthetic derivative of retinoid) which exhibit specific biological activities of retinoic acid did not inhibit lipopolysaccharide induced TNF-α production in mice. The differences in the results may be due to the fact that in the present study, a different model of inflammation was used, there was a longer duration of treatment, accompanied with continuous feeding on standard nutritive diet. Also, differences in geographical location which could account for varying environmental condition can contribute to the contrasting outcome of these experiments. It could also be proposed that Vitamin A was able to decrease TNF-α through its ability to inhibit translocation of the transcription factor NFκB and interrupt the secretion of inflammatory cytokines[42].

The C-reactive protein concentration observed after treatment with Vitamin A could be the reason why the KnD of Vit A at week 10 could not show significant decrease when compared to week 2, signifying higher inflammatory activity

than omega 3 FAs and its combination, on knD. However, treatment with Vitamin A showed significant decrease in KnD when compared to CGN control; this at least shows a degree of anti-inflammatory activity which supports its lower CRP level compared to CGN. The finding that Vitamin A could not show significant decrease in CRP is in contrast with that of Cha *et al.*[25] who reported that 4000IU/kg of Vitamin A supplementation might be considered as adequate level that should show favourable effect in rats. In this study however, Vitamin A at 4000IU/kg showed a weak response to decrease CRP concentration; this could probably be due to difference in geographical as well as laboratory conditions or model of inflammation used. Nonetheless, Vitamin A has been reported likely to be toxic to cells even at levels considered safe affecting cell survival and function[43].

The measure of inflammatory activity through the knee diameter, inflammatory markers: TNF-α and CRP have been shown to be regulated at various level in response to the administration of omega 3 and Vitamin A either alone or in combination. From the result, these supplements show a promising outcome in reverting and suppressing carrageenan-induced joint inflammation in Wistar rats; but omega 3 alone or in combination with Vitamin A had a greater effect on knee diameter and other parameters investigated than Vitamin A alone.

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RP-UPLC Method Development and Validation for Simultaneous Estimation of Mometasone Furoate and Miconazole Nitrate in Semisolid Dosage Form

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ABSTRACT

An innovative, rapid and precise RP-UPLC method was developed and validated as per ICH guidelines for simultaneous estimation of Mometasone furoate (MF), and Miconazole nitrate (MN) in topical dosage form. Chromatographic separation was carried out using Agilent C_{18}(4.6mm×100mm, 5µm) column and mobile phase consists of 0.1% v/v triethylamine: methanol: acetonitrile (40:30:30 V/V/V; pH 3.5). The flow rate was 0.6mL/min and detection was set at 235 nm in UV detector. Retention time of MF and MN were 0.59 min and 1.13 min respectively. The method shows good linearity over the concentration range of 10-30 µg/mL MF and 200-600µg/ml MN. Recovery for both analytes was found to be 99.58% and 98.51% respectively. LOD and LOQ for MF and MN were found as 5.452 and 0.501µg/ml, 1.485 and 1.20µg/ml respectively. This newly developed RP-UPLC method can be successfully applied for simultaneous determination of MF and MN in topical dosage form.

Keywords: Mometasone furoate (MF), Miconazole nitrate (MN), RP-UPLC, LOD, LOQ

INTRODUCTION

Mometasone furoate (MF) is a topical glucocorticoid and chemically 9α, 21-dichloro-11β, 17dihydroxy-16α-methylpregna-1-4-diene-17yl furan-2-carboxylate (Figure1). It possesses anti-inflammatory and anti-proliferative activity. It is also used for treatment of skin diseases like dermatitis, psoriasis. It acts by the simulation of phospholipase A2 inhibitory protein and biosynthesis

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of potent mediators of inflammation such as prostaglandins, leukotrienes.[1] Miconazole nitrate (MN) is an antifungal agent and an imidazole synthetic derivative. It is chemically known as ((RS)-1-[2-(2,4-Dichloro-benzyloxy)-2-(2,4-dichloro-phenyl)-ethyl]-1H-imidazole (Figure 2). It is commonly applied to the skin and also in mucous membrane for the treatment of fungal infective disorder. It works by inhibiting the cytochrome P450 complex and bio synthesis of ergo sterol in fungal cell membrane. It has a powerful activity against candida albicans and dermatophytes as well as Gram-positive bacteria.[2]

The literature study reveals that there are numerous analytical methods reported for quantification of MF and MN. The study includesUVspectrophotometry[3-7], TLC[8], HPTLC [9-11,13]and HPLC[11-30]. However, no methods were reported in UPLC till now.

Ultra Performance Liquid Chromatography a special version of HPLC with the advantage of technological strides led to a very significant increase in resolution, sensitivity and efficiency with faster results. The intrinsic worth of the method in terms of very low solvent consumption, more robust method with greater confidence, substantial cost reduction makes the technology environment friendly. The aim of the present work is to develop a simple UPLC method with better resolution and to quantify the drug with a short retention time in the selected dosage form.

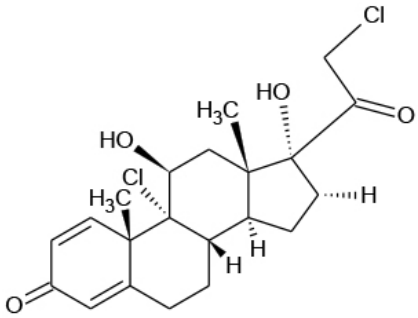

Figure 1. Structure of Mometasone furoate

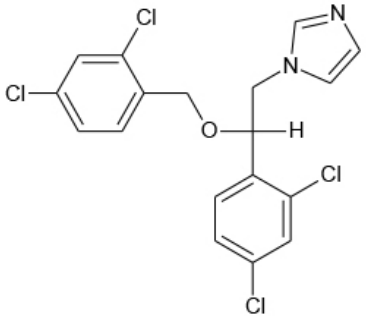

Figure 2. Structure of Miconazole nitrate

METHODOLOGY

Materials

Reference standard of Mometasone furoate and Miconazole nitrate gift sample provided from Synthiya research lab private limited, Pondicherry. Cream formulation (Each gm. of ELICA-M cream contains 0.1% of MF and 2% MN) were purchased from the local pharmacy in Chennai. HPLC grade acetonitrile, methanol, and triethylamine, ortho phosphoric acid and water were purchased from Merck.

UPLC instrumentation and chromatographic condition

Chromatographic separation was carried out in Agilent C_{18}(4.6mm×100mm, 5µm) column. Isocratic elution of mobile phase consists of buffer 0.1%v/v of triethylamine: Acetonitrile: methanol in the ratio of 40:30:30 (pH3.5) by ortho phosphoric acid. Data acquisition and processing was performed using open lab CHEMSTATION software in UPLC Agilent technology-1200 infinity series with high speed auto sampler. The flow rate was 0.6ml/min with injection volume of 5µl. The column temperature was maintained at ambient condition throughout the separation process. Mobile phase was freshly prepared and filtered through 0.45µ nylon filter.

Preparation of buffer

Buffer was prepared by dissolving 1 ml of triethylamine in 1000 mL distilled water. pH was adjusted to 3.5 with ortho phosphoric acid and solution was filtered through 0.45 µ nylon filter.

Preparation standard solution

Standard stock preparation

Stock was prepared by 20 mg of MF (400 µg/mL) transferred in 50 ml volumetric flask and dissolved in diluent (mobile phase).

Standard preparation

Weigh accurately about 40 mg MN transferred in 100 ml volumetric flask and add 50 ml of mobile phase sonication for 5 min and add 5ml standard stock preparation and volume make up with same. The final concentration was of 20µg/ml of MF and 400µg/ml of MN.

Sample preparation

Weigh accurately about 1g sample (1 mg of MFand20 mg of MN) transferred into 50 mL volumetric flask. About 30 mL of mobile phase was added to this volumetric flask and diluted to 50 mL and sonicated in an ultrasonic bath for 15 min. The solution was filtered through 0.45µm nylon syringe filter.

RESULTS AND DISCUSSION

Method development

Literature survey reveals that there are only three HPLC methods are reported for the simultaneous estimation of MF and MN in creams. Khushali Shah and co workers[15] reported the simultaneous determination by both RP-HPLC and HPTLC of MF and MN. The total runtime of the method was 14 min and also the retention time was too long (8.1, 4.2 min). In the same way Ramzia IE and co authors[14] indicated that the RP-HPLC a method which was comparatively lengthy (12 min) than the developed UPLC method. Also, the mobile phaseused 5% w/v aqueous ammonium acetate buffer, pH 7.6 and acetonitrile used doesn't showed good resolution. Similarly, the El-Bagary *et al.*,[13] also showed the simultaneous determination with the run time of 10 min and maximum retention time with 2.08, 5.7 min.

Hence, the present research work was intended to optimize chromatographic condition, for the proposed study. Various mobile phase composition and pH condition were altered during the trial studies. The mobile phase composition of phosphate buffer of pH 6.8 and methanol (60:40) was tried but this resulted in delayed elution of MN. Again, in the second trial (ammonium acetate buffer pH 4.5 acetonitrile (70:30)) the outcome was peak with tailing factor and resolution was poor for both analytes. After various combinations trials, finally we tried with mobile phase composition 0.1 % v/v triethylamine: Methanol: Acetonitrile (40:30:30) resulted good peak shape and better resolution. Moreover, it was observed during the study that the triethylamine reduced the tailing factor in the chromatogram. So, this combination was fixed as a mobile phase for the development of chromatogram.

Method validation

The method was validated as per ICH Q2 (R1)[31]and the following parameters were considered: system suitability, accuracy, precision, robustness, specificity, linearity, LOD and LOQ.

System suitability

System suitability was performed by six replicate injection of standard solution with the concentration of 20µg/mL of MF and 400µg/mL of MN was injected. The parameters like retention time, theoretical plate, resolution and peak area are shown in the Table 1 and Figure 3.

Specificity

Specificity is the ability to check clearly the analyte in the presence of components which may expect to be present. Typically, these might include impurities, degradant and matrix. There was no interference from excipient and other component with the drug peak. So, the developed method has been found to be specific (Figure 4).

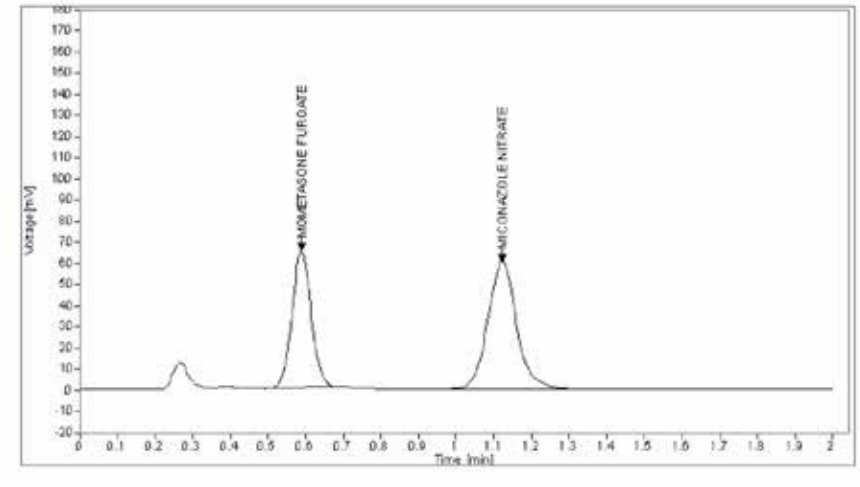

Figure 3. UPLC chromatogram of Mometasone furoate and Miconazole nitrate

Table 1. Results of system suitability

S.NO	Parameter	MF	MN
1	R_t	0.59	1.13
2	Theoretical plates	6003.22	10045.34
3	Tailing factors	1.07	1.05
4	SD	0.94	0.63
5	% RSD	0.42	0.23
6	Resolution	4.7836	

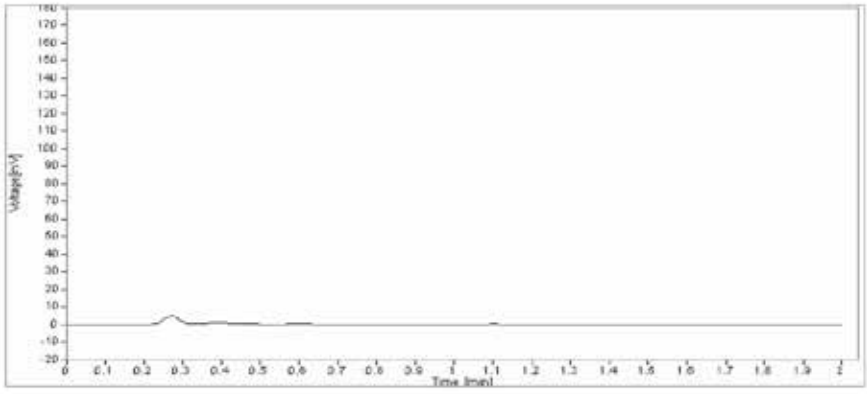

Figure 4. Specificity chromatogram of Mometasone furoate and Miconazole nitrate

Linearity

The linearity of the method was performed by preparing the concentration range of 9.95-29.84 µg/mL and 198.57-595.71µg/mL for MF, MN, from standard stock solution. Calibration curves were constructed by plotting concentration versus area of MF and MN. The results are shown in Figure 5 and 6.

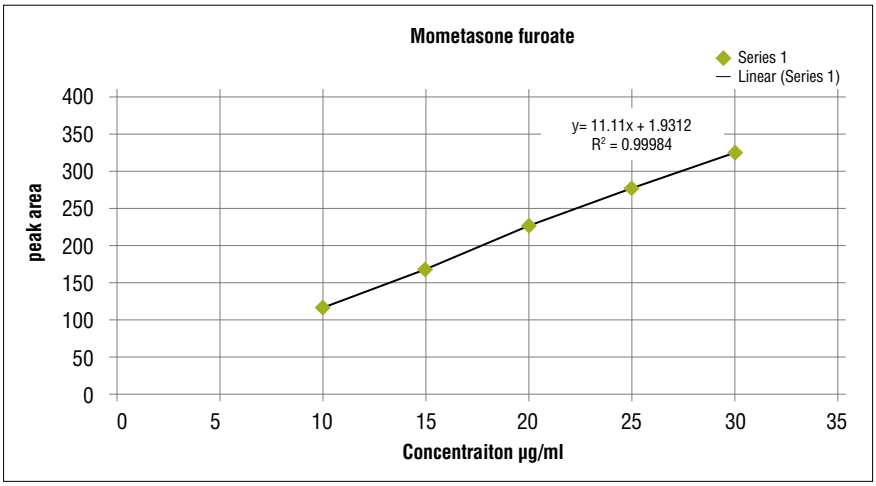

Figure 5. Calibration curve of Mometasone furoate.

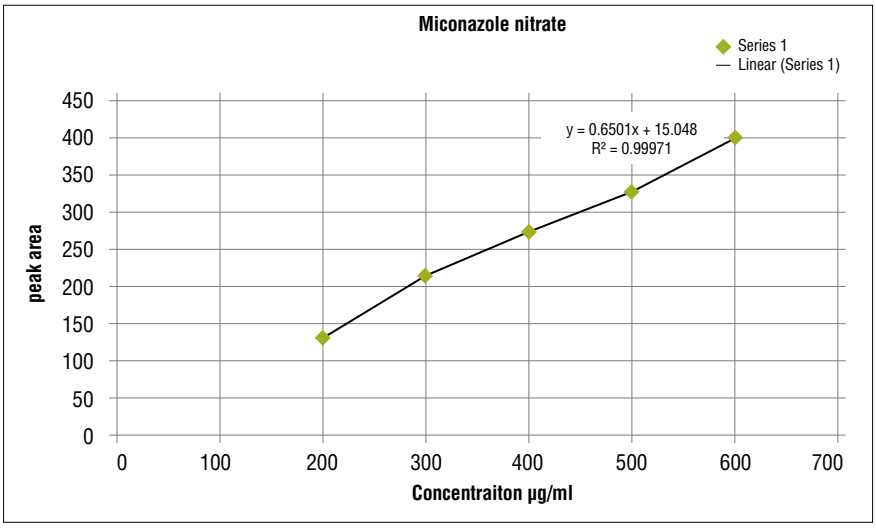

Figure 6. Calibration curve of Miconazole nitrate

Recovery

The concentration of standard solution of MF and MN comprising 0.107mg/ mL, 1.968mg /mL and0.212mg /mL, 3.888 mg /mL and 0.314 mg / mL, 5.748 mg/mL which represents 10%, 20%, 30% level) was injected to LC and recovery was measured to the pre analyzed sample solution.

The recovery mean percentage of MF and MN are 99.58 and 99.51 respectively and these results are within the reference limit of 90-110 %. The % RSD for MFand MN is 0.50, 0.33 respectively % RSD is within the reference limits ≤ 2. Hence proposed method is accurate.

Accuracy

The accuracy was calculated by the analysis of cream and standard at low, mediumand high concentration level. The accuracy was estimated from three replicate injections and calculated as the µg/mL drug recovered from the drug matrix. The method is found to be accurate and results are summarized in table 2.

Table 2. Accuracy data results of the UPLC method

S.NO	Sample ID	MF		MN	
		In mg	In %	In mg	In %
1	LOW-SPL-1	0.990	99.00	19.953	99.77
	LOW-SPL-2	0.990	99.00	19.948	99.74
	LOW-SPL-3	0.990	99.00	19.957	99.79
2	MID-SPL-1	1.001	100.10	19.944	99.72
	MID-SPL-2	1.000	100.00	19.919	99.60
	MID-SPL-3	1.000	100.00	19.926	99.63
3	HIGH-SPL-1	1.002	100.20	19.749	98.75
	HIGH-SPL-2	1.007	100.70	19.830	99.15
	HIGH-SPL-3	1.003	100.30	19.769	98.85
4	AVERAGE	1.00	99.81	19.888	99.44
5	SD	0.01	0.64	0.08	0.41
6	% RSD	1.00	0.64	0.42	0.41

Precision

The precision of the proposed assay method was assessed by analyzing standard and sample solution of 20 µg/mL of MF and 400 µg/mL of MN in six replicates in intraday and interday precision. The precision of test method results aredisplayed in Table 3.

Table 3. Data of Intraday precision and Interday precision

INTRADAY PRECISION					INTERDAY PRECISION			
MF			MN		MF		MN	
Injection	Peak area	Assay%	Peak area	Assay%	Peak area	Assay%	Peak area	Assay%
Injection - 1	223.56	100.30	294.665	99.64	232.292	99.70	300.904	99.11
Injection - 2	223.748	100.60	295.081	99.91	232.895	99.80	301.505	99.24
Injection - 3	223.752	100.60	295.010	99.87	232.839	99.80	301.505	99.22
Injection - 4	223.464	100.50	294.762	99.85	232.626	99.60	301.507	99.11
Injection - 5	223.803	101.00	295.107	100.16	233.868	99.80	302.403	99.27
Injection - 6	223.533	101.10	295.052	100.30	234.057	99.70	302.883	99.23
Avg	223.6433	100.38	294.9462	99.96	233.0962	99.73	301.7845	99.20
SD	0.13	0.310	0.17	0.240	0.64	0.080	0.66	0.070
% RSD	0.06	0.310	0.06	0.240	0.28	0.080	0.22	0.070

Robustness

The robustness of a method was analysed by changing experimental, chromatographic condition. Altering in flow rate (0.6±1 mL/min), changes in column oven temperature (40±5 °C), Changes in mobile phase buffer pH (3.5±0.2), changes in mobile phase composition and changes in wavelength allowable limits from actual chromatographic condition. It was noted that there was no recognizable change in mean RT and RSD and parameters fell within the limit of ≤ 2. The theoretical plate, tailing factor, resolution was found to be good of MF and MN. This method is robust with variability condition. The analytical condition results are shown in Table 4.

Table 4. Data of Robustness study

Drug name	Parameter	Chromatographic condition			
	Flow rate change±1%	**RT**	**AREA**	**Theoretical plate**	**Tailing factor**
Mometasone furoate	0.5ml/min	0.62	191.523	6005.45	1.09
	0.6ml/min	0.59	189.457	6003.22	1.07
	0.7ml/min	0.53	187.876	6007.56	1.05
	Wavelength change ±2%				
	234nm	0.59	189.543	6012.23	1.04
	235nm	0.59	191.735	6005.67	1.06
	236nm	0.59	192.567	6008.54	1.07
Miconazole nitrate	Flow rate change±1%				
	0.5ml/min	1.14	274.678	10057.76	1.10
	0.6ml/min	1.13	277.356	10045.34	1.05
	0.7ml/min	0.98	271.049	10010.58	1.03
	Wavelength change ±2%				
	234nm	1.12	271.812	10031.23	1.13
	235nm	1.13	276.635	10047.56	1.06
	236nm	1.12	268.487	10067.44	1.10

Solution stability

Stability of sample solution was confirmed by storing it at ambient temperature for 15hrs.The assay of MF and MN were analysed. It was found that percentage labeled amount of MF at 5,10 and 15 were 100.02, 100.07 and 100.12 respectively; Percentage labeled amount of MN were 5,10,15 were 99.64, 99.73, and 99.88 respectively.

Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ were estimated using equation LOD =3×s/S and LOQ =10×s/S where s= standard deviation of Y intercept S= average slope of calibration curve. The LOD can be expressed as the minimum level of analyte that produce a considerable reaction. And LOQ was analyzed as the lowest amount of anaytes that was quantified reproducibly. Based on the standard deviation of the response and slope results are presented in table 5.

Table 5. LOQ and LOD results of MF and MN

S. No	Parameter	Mometasone Furoate	Miconazole Nitrate
	LOQ (µg/ml)	1.485	1.20
	LOD (µg/ml)	5.452	0.501

The major supremacy of the UPLC method is significant saving in run time. Based on the study reports of the present research work, it is obvious that the developed method also had a very short noticeable reduction in the total run time i.e., only 2 min whereas the literature reported method[13, 14] is tedious which takes around 10-14 min of total run time. In addition, it is a very simple and a novel method in the midst of commercial applicability. The current developed method offers a lot of advantages over the others like speedy acquisition of results, remarkable savings in operational cost and short, sharp retention time with good resolution. Moreover, this UPLC method is found to be accurate and precise. The Validated data by ICH guidelines also confirms the effectiveness of the developed method.

The rapid and economic RP-UPLC method was developed for quantitative analysis of MF and MN in pharmaceutical dosage form which was found to be accurate. The present work done was also precise, linear, robust and specific. The validated results of the current study are additional supporting evidences of the method. This method reveals an admirable performance in terms of speed and sensitive.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this paper

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The Pharmaceutical and Antimicrobial Properties of Dermatological Formulations Containing *Hyptis Suaveolens* (L.) Poit (Lamiaceae) Aerial Extract

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ABSTRACT

The plant *Hyptis suaveolens* (L.) Poit (Lamiaceae) has received a lot of recognition because of its ethno-medicinal claims, some of which have been justified by scientific studies. The intention of this present work was to develop an antimicrobial dermatological dosage formulation. The powdered aerial extract was macerated in methanol for 72 h and the extract was formulated using vanishing cream base as vehicle at concentrations of 2.5, 5.0 and 7.5 % w/w respectively. The formulations were tested against selected microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa* (Clinical isolates and standard strains), *Trichophyton rubrum* and *Candida albicans*. The results showed that activities against bacterial clinical isolates were not as encouraging as that of standard strains, while exhibiting significant concentration dependent antifungal activity ($p<0.05$) against the microorganisms tested with. This therefore shows potential for development as a standardized dosage form for the treatment of skin infections where the interrogated microorganisms are implicated.

Keywords: *Hyptis suaveolens,* Aerial extract, Dermatological formulations, Antimicrobial properties.

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INTRODUCTION

Herbal preparations are believed to contain ingredients that are active alongside many other substances. Currently, medicines derived from plants are being developed by many drug industries due to a wide spread perception that they are more effective, less toxic, less expensive and more reliable than the synthetic ones[1]. World Health Organization has also indicated readiness in promoting the development and use of herbal medicines especially in third world countries. Works by scientists are on-going in search for ingredients that are active biologically and have low side effect profile, though natural products as antibacterial agents have long been evaluated[2]. Higher plants are rich in secondary metabolites and are very good sources of antimicrobial compounds. Pharmacological characters in these metabolites have been utilized to arrest and destroy disease-causing pathogens.

Currently, infectious diseases are still a major cause of mortality and disability globally. As infection is increasing, resistance against antibiotics is also increasing. Resistance to synthetic antibiotics is worrisome.

Hyptis suaveolens (L.) Poit is also known as pignut, bush mint, horehound, wild spikenard and a variety of names in French, Portuguese, Spanish and Hindi[3]. It is a plant which is usually known as a weed with a variety of medicinal and food uses cultivated in India and Mexico. It is a small erect plant usually about 2.5 m in height with woody, hairy stem[4]. It is a plant belonging to the family Lamiaceae. It is basically considered an obnoxious weed and widely distributed throughout the tropics and subtropics[4]. Almost every part of the plant has been reported to have medicinal properties. This plant is known for its strong smell which confers on it its insecticidal properties. It produces flowers and seeds in abundance annually which make it a good candidate for pollination[5]. It contains alkaloids, phenols, saponins and most importantly essential oils. The essential oil can be obtained from the leaves, shoots and roots; however, the composition of the oils will vary based on geographical location at any point in time[6].

The seeds of the plant are used as a drink or for food in some parts of the world while the shoots and leaves are used as flavoring and tea respectively[7]. Medicinally, the roots are used as an appetizer while the whole plant part is useful for dermatological infections, headaches, fevers and malaria[7]. Its essential oil finds application in infections and inflammatory conditions and as an insect repellent against mosquitoes[8]. The essential oil obtained from the leaves has shown potential as an antibacterial agent against gram positive and gram-negative bacteria most especially *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* etc. In addition, the essential oil from the leaves has

shown good antifungal activity compared to the standard antifungal, ketoconazole[6]. The anti-inflammatory activity of components of the essential oil, suaveolol and methyl suaveolate has also been studied and its effect was similar to ibuprofen, a standard anti-inflammatory agent. Interestingly, its antiplasmodial activity has been studied showing activity against chloroquine resistant and sensitive *Plasmodium falciparum*. The component responsible for this activity is dehydroabietinol[9].This present study involves the preparation and evaluation of an herbal skin care formulation for antimicrobial action against selected microorganisms which are associated with localized skin infections. The cream formulation consists of the methanol extract of *Hyptis suaveolens* (aerial) and this herb has been selected based on the scientific justification of its properties.

METHODOLOGY

Materials used were Methanol (Merck KGaA 64271 Darmstadt Germany), Stearic acid, Glycerin monostearate, Cetyl alcohol, Potassium hydroxide (Allied Chemicals Ltd, England), Methyl paraben, Propyl paraben (Clariant, UK), Distilled water (National Institute for Pharmaceutical Research and Development, Nigeria).

Plant collection

Hyptis suaveolens (aerial) was collected on 31st January 2019 from Idu industrial area of Abuja Municipal Area Council of Nigeria. It was identified and given a voucher number (NIPRD/H/7020) and air dried at room temperature for 5 days at the Department of Pharmaceutical Technology and Raw Materials Development, National Institute for Pharmaceutical Research and Development (NIPRD).

Preparation of crude extract

The dried aerial parts were pulverized using mortar and pestle. It was then sieved using sieve size 600 μm. An amount (500 g) of the sieved powder was poured into a bowl and 2.5 L of methanol was added to it, covered and allowed to stand for 72 h. It was then decanted and filtered using a Whatman No 1 filter paper and the filtrate was concentrated on a water bath at 40 ^{o}C. It was air dried, pulverized and the yield was calculated.

Formulation of creams

The oil phase was prepared by weighing 13 g of stearic acid, 1 g of glycerin monostearate and 1 g of cetyl alcohol into a beaker. The beaker was then heated on a water bath to 70 ^{o}C. The aqueous phase was prepared by weighing 10 g of glycerin and 0.9 g of potassium hydroxide into another beaker, 0.1 g of methyl paraben and 0.05 g of propyl paraben were also added as preservatives. Suffi-

cient amount of water (73.95 g) was added to make up to 100 g and the mixture heated to 75 ºC. The oily phase was added to the aqueous phase, vigorously stirred together until the pre-emulsion was formed. Thereafter, appropriate quantities of the extract were incorporated to make 0.0, 2.5, 5.0 and 7.5 %w/w concentrations. Later, the emulsion was transferred into a porcelain mortar and then triturated with a pestle for globule size reduction and smoother emulsion.

Physical evaluations

The appearance, colour, odour, texture, ease of removal, homogeneity and phase separation of the different concentrations of the creams prepared were observed and recorded.

Viscosity

The viscosity of the formulation was carried out using a Brookfield viscometer (VT 181, Karlsruhe, Germany) at 27 ± 2 ºC using spindle number seven and the rotational speed of 0.5 to 100 rpm. The shear stress was determined by calculation and appropriate plots were made.

pH determination

The pH of the prepared creams was determined using a pH meter (Jenway 3505) in triplicate and result expressed as mean ±SD.

Occlusion properties

Each cream formulation (200 mg) was carefully weighed on a filter paper and evenly spread out. This was then placed on a clean sample bottle having 2.3 cm diameter surface. The average perimeter of the filter paper was 22.9 cm. Distilled water (20 mL) was carefully transferred into each bottle and the filter paper containing the spreaded cream was placed on top to cover the entire mouth of the bottle. The water loss was determined at 24-144 h; controls with ordinary filter paper i.e. positive control (CP) and without any filter paper i.e. negative control (CN) were set up as well. Readings were taken every 24 h. Plots of percentage water loss against time were prepared. The occlusion factor (F) was calculated using the equation below:

$$F = \frac{A - B}{B} \; x\,100$$

where, B is the water loss from the test formulations, A is the water loss of the filter without a sample (blank reference). An occlusion factor of zero indicates no occlusive effect compared with the reference, and 100 was the maximum occlusion factor[10].

The globule sizes of the dermatological formulations

The globule sizes of the internal phase for the dermatological formulations were measured using an optical microscope (Olympus Light Microscope). The microscope was fitted with a camera and computer software-Motic MC 1000 (Motic China Group Co.Ltd.,) for image analysis transmitted on the monitor. The microscopic samples were prepared by spreading a very thin layer of the cream on a specimen slide, stained with crystal violet and spread evenly with the aid of a cover slip. One hundred globules were measured from each preparation and the photomicrographs also documented.

Evaluation of cream Stability

An accelerated stability study was carried out to check for changes in colour, phase separation and pH of the different concentrations of the creams at 4 ^{0}C, 25 ^{0}C and 40 ^{0}C for 3 months. Samples were placed in the refrigerator, room temperature and photo stability chamber (Model 1810, Vindon Scientific, Germany) set at 40 ^{0}C. Samples were withdrawn at monthly interval and checked for changes in colour, phase separation and pH at day 1, after 1 month, after 2 months and after 3 months.

Antimicrobial assessment

The cream formulations were screened for antibacterial activity on *Staphylococcus aureus* and *Pseudomonas aeruginosa* using clinical isolates obtained from the Department of Medical Microbiology of the University College Hospital, Ibadan. Standard microorganisms were obtained from the International Centre for Drug Research, Lucknow and used as well. The standard microorganisms used were *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Cream formulations were used in diluted (25, 50 and 100 mg/mL) and undiluted form (100 mg). Microorganisms were seeded in Mueller Hinton agar (Merck, Germany) and agar wells (6 mm) bored after setting. Sterile spatula was used to fill the holes with the cream formulations. Diffusion was allowed to occur for 45 minutes before incubating at 37 °C for 24 hours. Zones of growth inhibition were measured in millimetres. Control experiments were done using **distilled** sterile water and Betamethasone-neomycin cream. All experiments were done in triplicate and results presented as mean ± SD.

The antifungal activity of the cream formulations was assessed using *Candida albicans* and *Trichophyton rubrum* obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Ibadan. Cream formulations were used in diluted (25, 50 and 100 mg/mL) and undiluted form (100 mg). Previously sterilized saboraud dextrose agar (20 mL) was

cooled to 45 °C and poured into sterile petri dishes. After the agar has set, about 0.2 mL of a 24-hour old culture of the fungal isolates in nutrient broth was streaked unto the agar. Holes were bored with sterile cork borer of 6 mm and a sterile spatula was used to fill the holes with the cream formulations. Diffusion was allowed to occur for 45 minutes before incubating at 25-27 °C for a minimum of 48 hours. Zones of growth inhibition were measured in millimetres. Control experiments were set up using distilled sterile water and ketoconazole cream. All experiments were done in triplicate and results presented as mean ± SD.

RESULTS AND DISCUSSION

A limiting factor to most medicinal plants for managing disease conditions is the form in which they are presented[11]. In this study, extracts from the aerial part of *Hyptis suaveolens* was used to formulate various concentrations of oil-in-water emulsion cream that can be used in the treatment of various skin diseases (Table 1).

Organoleptic properties

The extract from *Hyptis* plant was dark green with a characteristic pungent and sharp smell. Creams formulated with various concentrations of *Hyptis suaveolens* aerial extract were army green in colour with degree of sharpness of odour increasing as concentration increases. Suitable colourant may enhance its elegance and acceptability. All the creams prepared were easily removed with water, this is an advantage because patients comply easily with non-greasy, non-occlusive, easily washable dermal preparations. This also means that the density of the particles in the formulations are proportionate to that of the cream base[12]. All creams formulated were non-irritating when applied to the skin after a period of 8 hours, with a soft and smooth feeling. There was no extract-vehicle incompatibility observed. Phase separation was not noticed in any of the formulations even after 3 months at 4, 25 and 40 °C respectively. This implies that the difference in density of the extract to that of the base is insignificant[13] and that the formulation will be able to withstand different storage conditions without losing their structural integrity.

pH

The pH of a medium, measures the acidity or alkalinity of a phase. A slight difference in pH of a product can affect how a formulation interacts with the skin[14]. The literature reports the pH of the skin to be between 5.4-5.9[14]. It is therefore necessary that the base chosen should have desirable characteristic including an acceptable pH. The pH of the creams formulated was in the range of 6-7 at

the point of preparation. After exposure to different storage conditions (4, 25 and 40 °C) for 3 months, changes in pH were not significant ($p>0.05$) as they were still within the range as that of baseline (Figure 1-3). Changes in pH may be a signal to chemical decomposition, most probably of a hydrolytic nature, and if detected, could be a reason to return a product[15].

Table 1. Composition of the *Hyptis suaveolens* herbal cream formulations

Ingredients	A1	B1	C1	D1
Stearic acid (g)	13.00	13.00	13.00	13.00
Glycerin monostearate (g)	1.00	1.00	1.00	1.00
Cetyl alcohol (g)	1.00	1.00	1.00	1.00
Glycerin (g)	10.00	10.00	10.00	10.00
Potassium hydroxide (g)	0.90	0.90	0.90	0.90
Methyl paraben (g)	0.10	0.10	0.10	0.10
Propyl paraben (g)	0.05	0.05	0.05	0.05
Extract (g)	0.00	2.50	5.00	7.50
Water (g)	73.95	71.45	68.95	66.45
Total weight (g)	100.00	100.00	100.00	100.00

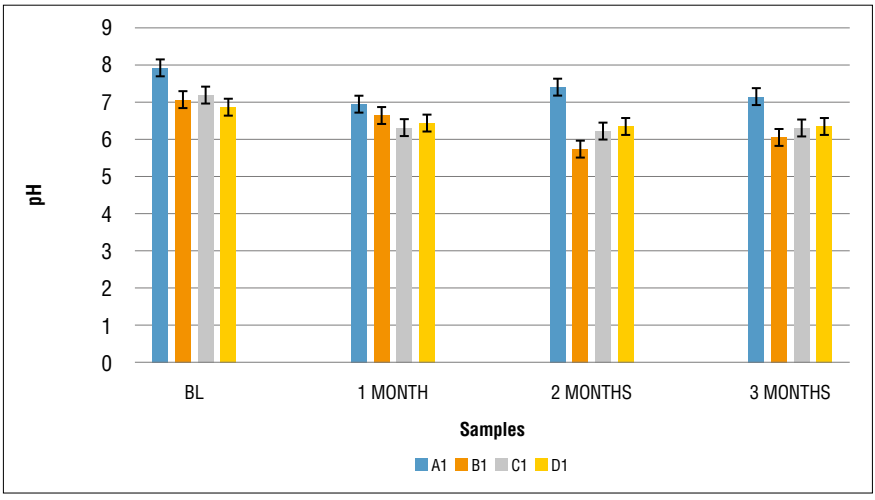
A1, B1, C1 and D1 are 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ respectively of *Hyptis suaveolens* dermatological formulations.

Table 2. Organoleptic properties of the extract and creams at baseline and after 1, 2 and 3 months at 4 ^{0}C, 25 ^{0}C and 40 ^{0}C.

	Colour	Odour	Texture	
Hyptis extract	Dark green	Pungent and sharp	Fine	
Properties of cream	**A1**	**B1**	**C1**	**D1**
Appearance	Smooth	Smooth	Smooth	Smooth
Colour	White	Army green	Army green	Army green
Odour	Odourless	Pungent	Pungent	Pungent
Texture	Smooth and soft	Smooth and soft	Smooth and soft	Smooth and soft
Ease of application	Easy to apply on rubbing	Easy to apply on rubbing	Easy to apply on rubbing	Easy to apply on rubbing
Skin irritation	Non irritant	Non irritant	Non irritant	Non irritant

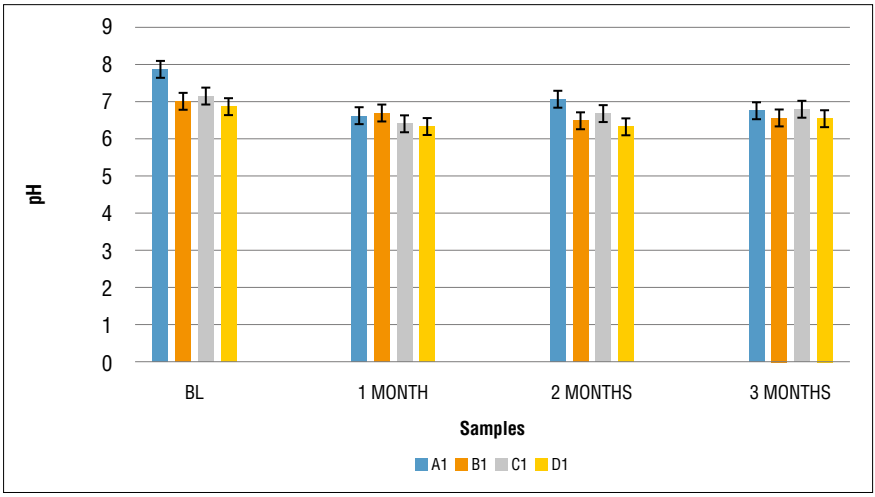
Table 3. The occlusion factor and globule sizes of *Hyptis suaveolens* dermatological formulations

Formulation code	Occlusion Factor, F (%)	Remark	Globule size (μm)
A1	41.18	Fairly occlusive	25.60 ± 23.12
B1	41.18	Fairly occlusive	23.79 ± 9.93
C1	41.18	Fairly occlusive	31.64 ± 14.16
D1	70.59	Moderately	33.24 ± 10.52



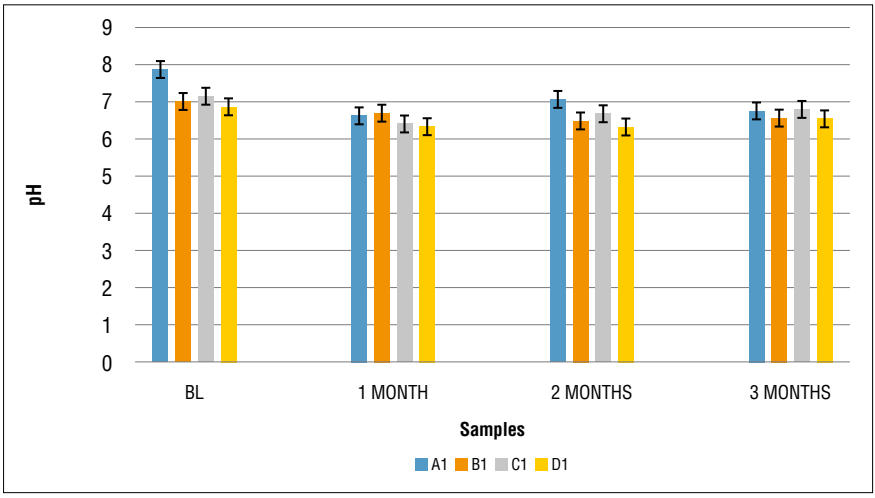
A1, B1, C1 and D1 are 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ respectively of *Hyptis suaveolens* herbal cream. formulations.

Figure 1. pH of the creams at baseline (BL), after 1, 2 and 3 months at 4 ^{0}C.



A1, B1, C1 and D1 are 0.0, 2.5, 5.0, and 7.5 % $^{w}/_{w}$ of *Hyptis suaveolens* aerial extract cream respectively

Figure 2. pH of the creams at Baseline (BL), after 1, 2 and 3 months at 25 ^{0}C



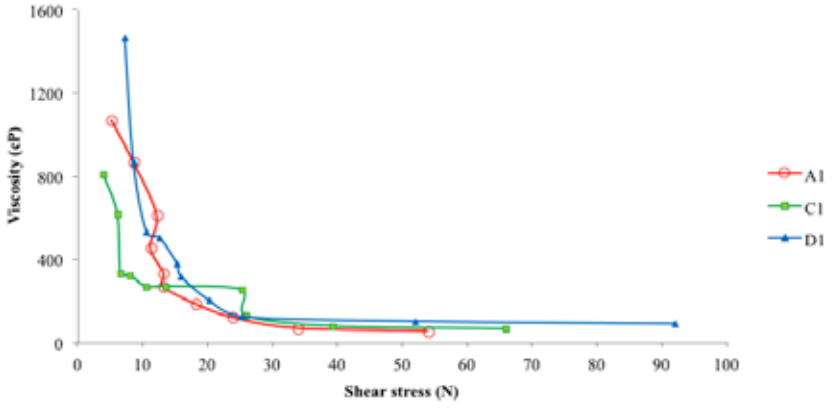
A1, B1, C1 and D1 are 0.0, 2.5, 5.0, and 7.5 % $^{w}/_{w}$ of *Hyptis suaveolens* aerial extract cream respectively

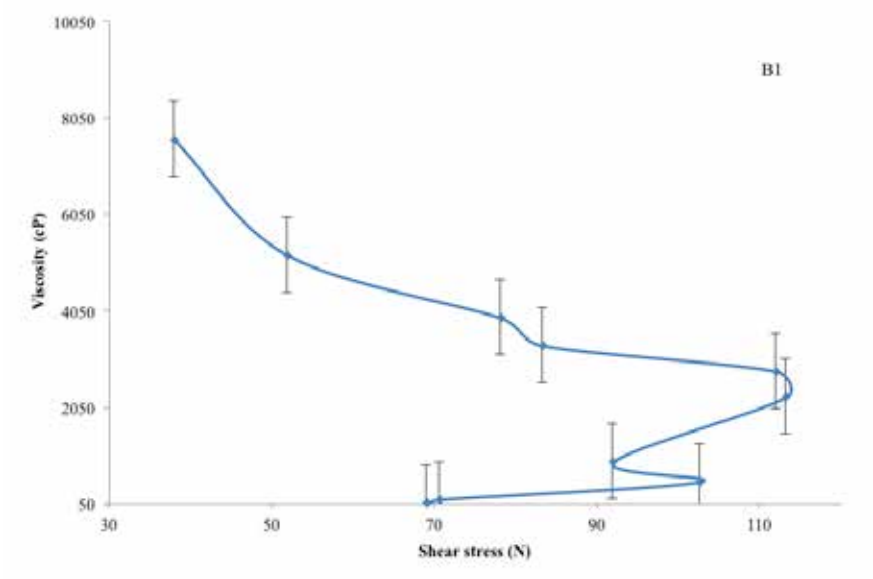
Figure 2. pH of the creams at Baseline (BL), after 1, 2 and 3 months at 25 ^{0}C

Viscosity

The cream formulations generally had high viscosity values thus reflecting the adherence property of semi-solid pharmaceutical dosage forms and the high yield stress involved such that it will not run off the skin surface after application. Yield stress is the minimum stress that must be applied before a material starts to flow[16]. The yield stress of cream formulations should be high enough so that they do not flow out of their container due to their own weight if the container is placed in an upside-down position. It should however not be too high to offer significant resistance during application onto the skin. In terms of viscosity values, the cream formulations are acceptable.

As shown in Figure 4, formulations A1, C1 & D1 exhibited reduced viscosity with increased shear stress, while the viscosity of B1, seem not to depend on shear stress as it increased irrespective of the force applied. Generally, semi-solid formulations undergo a wide variety of stress during removal from the container and application to the affected area[17]. Evaluating the effect of stress on the cream is thus important to determine its behaviour during transportation and use.

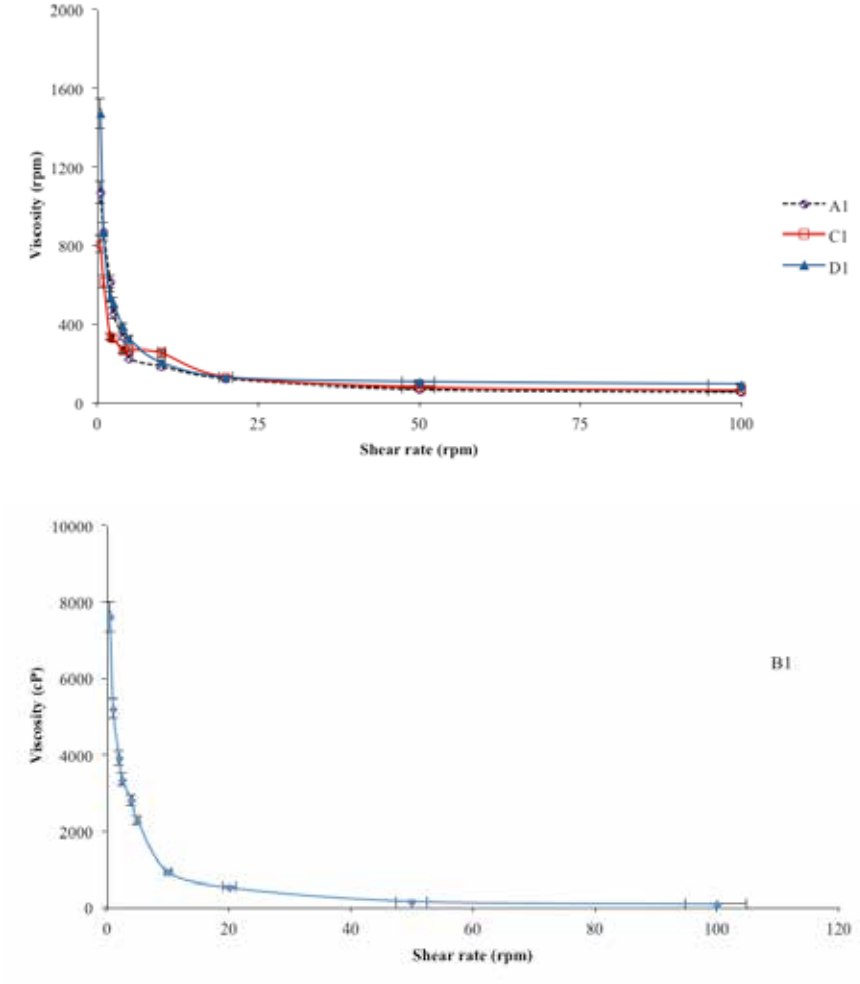




A1, B1, C1 and D1 are 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ respectively of *Hyptis suaveolens* dermatological formulations

Figure 4. The effect of shear stress on the viscosity of *Hyptis suaveolens* dermatological formulations

In addition, the viscosity of all the cream formulations generally reduced as the rate of shear increased as shown in Figure 5. The cream formulations therefore demonstrated pseudoplastic rheological pattern. Due to the peculiar rheological behaviour of semisolids, they can adhere to the skin or mucous membrane for sufficiently long periods before they are washed off. The viscosity of a semi solid formulation is the adherent property that enables it to cling as films until an external force act on it. This property enables semi-solid formulations to exhibit prolonged activity on the skin[17].



A1, B1, C1 and D1 are 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ respectively of *Hyptis suaveolens* dermatological formulations

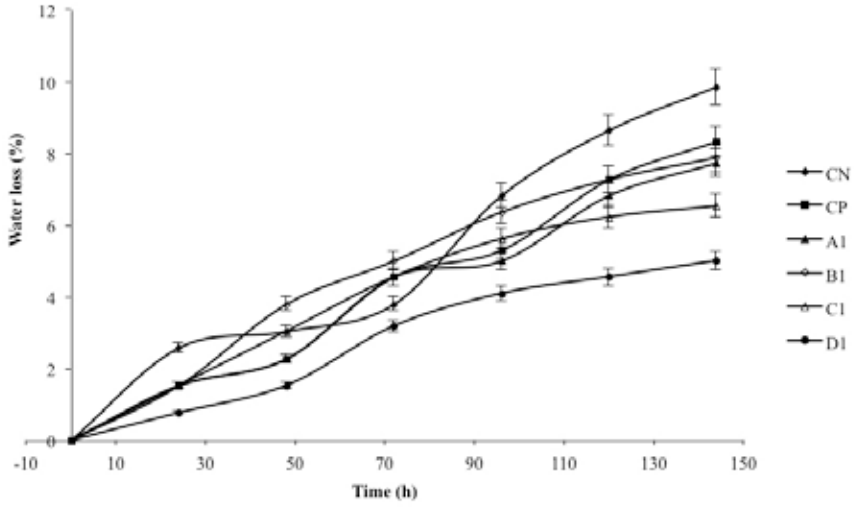
Figure 5. The effect of shear rate on the viscosity of *Hyptis suaveolens* dermatological formulations

Occlusion factor

The occlusion factor as presented in Table 3 showed that the cream formulations had occlusion properties ranging from fair to very high. When a topical pharmaceutical formulation is occlusive, such products form barrier on the skin and thus affects skin hydration. Occlusive products are usually oil-based semi-solid formulations and such provide skin barrier repair through restoration of lipids in the skin. In addition to the therapeutic activity of these cream formulations, they will also help in preventing skin dryness, maintenance of skin smoothness and elasticity[18]. This assertion will be further strengthened if *ex-vivo* and *in vivo* occlusion studies can be done.

Percent loss of water from formulations

As shown in Figure 6, the negative control lost more water than all the formulations. Generally, the percentage water loss from all the formulations were not up to 10 % even after 144 hours. It shows that the creams probably had significant occlusive properties. This cannot however be ascertained until the occlusion factor (F) is calculated. The cream formulations also had water losses lower than the controls. The filter paper pores were occluded by the cream samples at varying degrees hence the loss of water differs. The formulation ingredients usually affect the properties exhibited by the product. The ranking of percentage water loss was CN>CP>B1>A1>C1>D1. This implies that formulation D1 will retain water more than the others and therefore maintain its physical outlook more than the other with faster tendency of drying up.

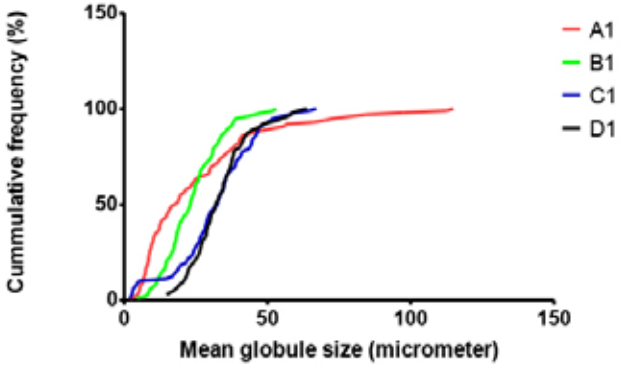


CN and CP are negative and positive controls while A1, B1, C1 and D1 are 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ respectively of Hyptis suaveolens dermatological formulations

Figure 6. The effect of time on water loss from *Hyptis suaveolens* dermatological formulations

Globule size distribution

The mean globule size of *Hyptis suaveolens* dermatological formulations are presented in Table 3, while the globule size distributions are in Figure 7. The results showed that the mean globule sizes were between 23.79 ± 9.93 to 33.24 ± 10.52 μm. The globule size distribution for all the formulations showed a sigmoidal pattern and least sizes ranged from 2.00-14.78 μm ranking in the order of C1<A1<B1<D1. The highest sizes also ranged from 52.93-114.90 μm in the order of B1<D1<C1<A1. Frequency statistics revealed that 75 % of the globules had sizes generally below 41 μm while 25 % were lower than 27 μm. Conventional creams have a mean droplet size ranging from 10 to 100 μm and such formulations generally demonstrate poor penetration of drug-loaded oil droplets into deep skin layers[19]. It has also been reported that microparticles with diameters ranging from 3 to 10 μm selectively penetrate follicular ducts, whereas particles >10 μm remain on the skin surface, and those < 3 μm are distributed randomly into hair follicles and stratum corneum[19]. This implies that the activity of the dermatological formulations prepared in this study will cut across the hair follicles, stratum corneum, follicular ducts and skin surface because of lower and higher sizes of the globules[20].



A1, B1, C1 and D1 contains 0.0, 2.5, 5.0 and 7.5 % $^{w}/_{w}$ of *Hyptis suaveolens* aerial extract

Figure 7. Globule size distribution for *Hyptis suaveolens* dermatological formulations.

Photomicrographs of the formulations

The Photomicrographs of the dermatological formulations are shown in Figure 8. The globules were seen to be spherical and the outer part took up the aqueous dye. The sphericity of the globules is generally a contributory factor to the stability of dispersed systems like creams. This could have assisted the cream formulation in maintaining stability shown when tested for 3 months. The uptake of the aqueous dye by the outer part of the globules leaving colourless internal part shows that the formulations had aqueous dispersion medium and the internal phase which could not be stained with the dye was the oil. This implies that the formulations were oil-in-water emulsions. Oil-in-water semi solid formulations are usually miscible with water, easily water-washable and readily rubs into the skin hence sometimes called vanishing cream[21]. Water washable creams are generally aesthetic and cosmetically acceptable compared to oily types[22]. The dermatological creams prepared in this study can therefore find application as vanishing cream in addition to therapeutic importance.

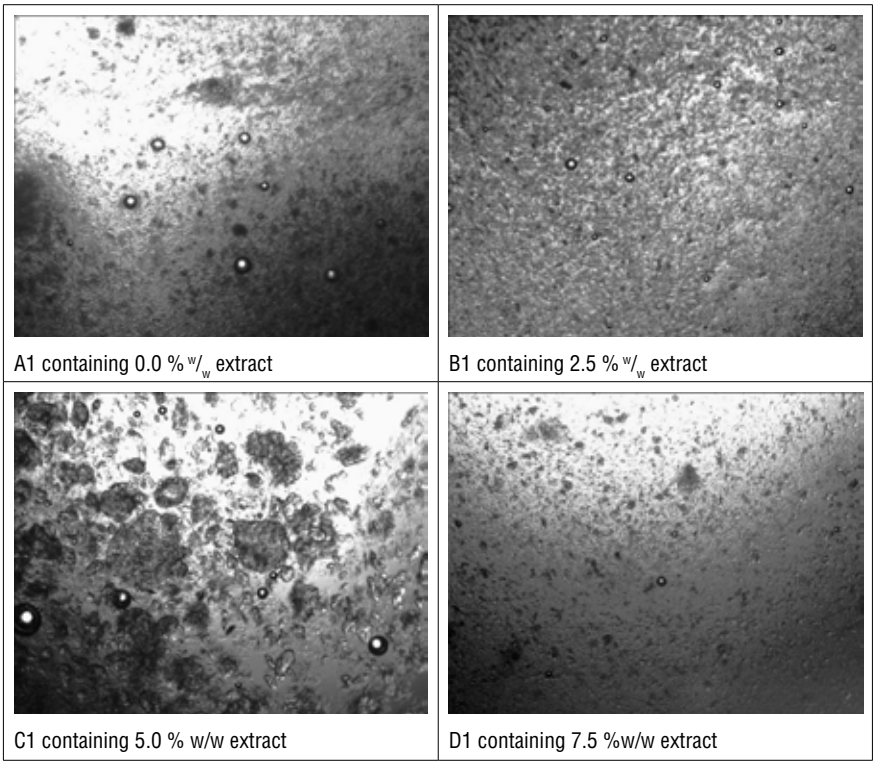


Figure 8. Photomicrographs of *Hyptis suaveolens* dermatological formulations showing dispersed globules

Antimicrobial studies

The results of the antibacterial activity of the dermatological formulations are presented in Table 4. The dermatological formulations of *Hyptis suaveolens* aerial extract showed antibacterial properties on both the clinical and standard isolates of *Staphylococcus aureus* (S. a). The activity was observed to be concentration dependent. While B1 containing 2.5 %w/w of the extract showed no activity on the microorganisms, C1 and D1 containing 5 and 7.5 %w/w of the extract had appreciable effect. All concentrations of C1 showed activity on the clinical strain of *S. aureus* while only 100 mg/mL and the undiluted form of C1 showed activity on the standard strain of the microorganism. This implies that the standard strain of *S. aureus* used in this study requires higher concentration to overcome its defences. Interestingly however, there were no significant differences ($p>0.05$) in the zone of inhibition of 100 mg/mL on the clinical and standard strains. In all cases, the undiluted cream gave higher zones of inhibition still showing a concentration-dependent response.

Table 4. The effect of *Hyptis suaveolens* dermatological formulation on bacteria strains (mean ± SD, n=3)

Formulation code	Cream concentration (mg/mL)	*Staphylococcus aureus* (clinical isolate)	*Staphylococcus aureus* (Standard microorganism)	*Pseudomonas aeruginosa* (standard microorganism)
		Zone of inhibition (mm)		
A1	25	0.0	0.0	0.0
	50	0.0	0.0	0.0
	100	0.0	0.0	0.0
	Undiluted (100 mg)	0.0	0.0	0.0
B1	25	0.0	0.0	0.0
	50	0.0	0.0	0.0
	100	0.0	0.0	12.3 ± 1.2
	Undiluted (100 mg)	0.0	0.0	15.0 ± 1.5
C1	25	15.00 ± 1.54	0.0	10.0 ± 2.4
	50	17.30 ± 1. 33	0.0	13.0 ± 1.1
	100	21.20 ± 1.04	20.50 ± 2.72	18.0 ± 1.5
	Undiluted (100 mg)	25.50 ± 2.34	27.00 ± 1.23	20.5 ± 2.1
D1	25	6.0 ± 5.29	0.0	0.00
	50	17.83 ± 0.76	15.0 ± 0.0	11.0 ± 2.5
	100	22.17 ± 1.26	23.5 ± 1.04	28.0 ± 3.1
	Undiluted (100 mg)	27.32 ± 0.98	33.0 ± 2.3	39.5 ± 2.5

There was no activity on Pseudomonas aeruginosa clinical isolate hence not shown on the Table.
Reference cream used is Betamethasone-neomycin cream (Table 6).

Generally, dermatological formulations are used on application sites without dilution, except otherwise prescribed by the physician; it is understandable therefore that the undiluted showed higher inhibitory responses. Dilution was however done to observe what would happen when lower quantities are used by the patient. Lower amount of the cream formulation will definitely contain reduced quantity of the bioactive extract. None of the formulations showed activity on the clinical isolate of *Pseudomonas aeruginosa* while diluted B1 (100 mg/mL) and undiluted showed activity on the standard strain in addition to formulations C1 and D1.

Comparing the activity of the cream formulations to the negative control (distilled water), it was observed that the formulations showed superior properties because the negative control showed no zone of inhibition. Furthermore, comparing the activity of the undiluted formulations to that of the positive control (Betamethasone-neomycin cream), which was also not diluted, the for-

mulations demonstrated significantly improved ($p<0.05$) properties both for the clinical and standard isolates. However, the positive control showed activity against both clinical and standard microorganisms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Formulation A1 is also a control (base) since it has 0.0 %w/w of the extract and it showed no activity on all the microorganisms used. This showed that whatever activity is observed in the dermatological formulations may be attributed to the extract incorporated.

The antifungal activity of the dermatological formulations is presented in Table 5. The results showed that all concentrations used were active although the undiluted was significantly higher ($p<0.05$). The activity of all the formulations on the fungal strains was also concentration-dependent within each formulation and among B1, C1 and D1. Comparing the activity of the formulations with that of the positive control (ketoconazole), the undiluted formulations demonstrated significantly ($p<0.05$) higher activity on both *Candida albicans* and *Trychophyton rubrum* (Table 6). This shows that the dermatological formulations of *Hyptis suaveolens* aerial extract can be further developed for antimicrobial application in dermatophytes infection.

Table 5. The effect of *Hyptis suaveolens* dermatological formulation on fungal strains (mean ± SD, n=3)

Formulation code	Cream concentration (mg/mL)	*Candida albicans*	*Trychophyton rubrum*
		Zone of inhibition (mm)	
A1	25	0.00	0.00
	50	0.00	0.00
	100	0.00	0.00
	Undiluted	0.00	0.00
B1	25	10.00 ± 1.25	9.12 ± 2.34
	50	14.37 ± 1.24	13.25 ± 0.76
	100	16.55 ± 2.19	16.50 ± 0.57
	Undiluted	22. 58 ± 2.34	19.17 ± 1.09
C1	25	12.07 ± 1.23	10.35 ± 0.87
	50	15. 53 ± 0.99	13.00 ± 1.25
	100	20.75 ± 2.13	18.05 ± 0.77
	Undiluted	24.50 ± 0.78	24.17± 1.22
D1	25	15.59 ± 1.05	12.25 ± 2.08
	50	21.44 ± 0.69	22.50 ± 1.23
	100	23.15 ± 0.89	27.09 ± 0.56
	Undiluted	29.75 ± 0.97	33.50 ±.2.43

Reference cream used was ketoconazole cream (Table 6)

Table 6. Activity of controls (positive and negative) on the microorganisms

Description of control	*S. aureus* (clinical isolate)	*S. aureus* (standard microorganism)	*Pseudomonas aeruginosa* (Clinical isolate)	*Pseudomonas aeruginosa* (standard microorganism)	*Candida albicans*	*Trychophyton rubrum*
	Zone of inhibition (mm)					
Bethamethasone-neomycin cream	24.0 ± 1.5	14.50 ± 1.25	20.00 ± 2.20	12.50 ± 1.50	NA	NA
Ketoconazole cream	NA	NA	NA	NA	18.35 ± 2.50	15.24 ± 2.15
Distilled water	0.0	0.0	0.0	0.0	0.0	0.0

100 mg of control was used undiluted and placed in the well.

The dermatological formulations of Hyptis suaveolens aerial extract were successfully prepared, they were oil-in-water emulsions, washable and cosmetically acceptable hence can find application as vanishing cream in addition to therapeutic importance. In addition, the pH of the formulated creams after exposure to different storage conditions had no significant change showing stability of the active ingredient in the base. The creams had high viscosity values indicating the adherent property to the skin surface after application. The fair to very high occlusion values showed that in addition to the therapeutic activity of these cream formulations, they will also help in preventing skin dryness, maintenance of skin smoothness and elasticity.

Formulation D1 will retain water more than the others and therefore maintain its physical outlook more and drying up will be prevented. The globule size distribution for all the formulations implied that the activity of the dermatological formulations will cut across the hair follicles, stratum corneum, follicular ducts and skin surface.

The activity of the formulations against bacterial clinical isolates was not as encouraging as that of the standard strains. The cream formulations especially at higher concentrations of 5.0 and 7.5 %w/w exhibited significant antifungal activity against the tested microorganisms. Overall assessment indicates that the formulations have potential for development as a standardized dosage form for the applicable for the treatment of skin infections where the interrogated organisms are implicated. It could therefore be developed for commercial use.

CONFLICT OF INTEREST

The authors all agreed that there was no conflict of interest of whatsoever.

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Design, Synthesis, In Vivo and In Silico Anticonvulsant Activity Studies of Derivatives of 6-Amino-4-Hydroxy-2-Thio-Pyrimidine

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ABSTRACT

The aim of the given research was the targeted synthesis of anticonvulsants - (4-amino-6-hydroxy-pyrimidin-2-yl)thio-N-acetamides derivatives. Perspective of the search of anticonvulsants was evaluated by the ADMET method and docking study. The starting 6-amino-2-thiopyrimidine was obtained in result of reaction between thiourea and ethyl cyanoacetate in sodium ethoxide environment. The targeted thioacetamide derivatives were synthesized by alkylation of 6-amino-2-thiopyrimidine with the corresponding 2-chloroacetamides in DMFA environment in potassium carbonate presence. The structure of the synthesized compounds was determined using ^{1}H and ^{13}C NMR spectroscopy, LS/MS, and elemental analysis. A screening study of anticonvulsant activity on the model of pentylenetetrazole-induced seizures in rats was carried out. A lead compound – 2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methylphenyl)acetamide – was found. The mentioned compound has shown its ability to prevented lethality, reduce the number and severity of seizures, as well as to increase latency period. Some correlation features between structure and anticonvulsant activity were determined. The obtained results of molecular docking study have shown the affinity of the lead compound to $GABA_A$, $GABA_{AT}$, Carbonic Anhydrase II, and NMDA receptors, and a possible mechanism for anticonvulsant action.

Keywords: 6-amino-2-thiopyrimidine, Acetamides, Anticonvulsant activity, ADMET, Docking.

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INTRODUCTION

In modern neurological practice, several generations of anticonvulsants have been used for a long time.[1] Insufficient efficiency of existing antiepileptic drugs, severe side effects[2], as well as development of refractory epilepsy[3,4] point to the relevance of the search for new potential anticonvulsants.

Scientific achievements of the last decades concerning the establishment of anticonvulsant action mechanisms, crystalline structure of target proteins, and amino acid composition of active receptor sites, diversity of the developed *in silico* methods for analysis and evaluation of the ligand and receptor affinity, ADMET parameters, as well as formulated pharmacophore models of "structure-activity" correlation[5], allows rationalizing the search for new AEDs.

The analysis of the structure of second- and third-generation anticonvulsants in accordance with the basic positions of the pharmacophore models of AEDs[5,6] search has shown that most of them, namely lamotrigine, vigabatrine, gabapentin, pregabaline, retigabine[7,8], have in their structure free amino group, which has amide nature in the case of felbamate and levetiracetam (Fig. 1).

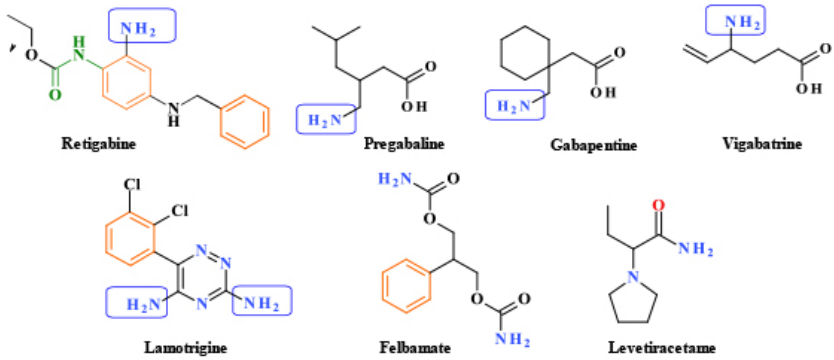


Figure 1. Chemical structure of the known anticonvulsants

The amino group acts as an electron donor by a lone electron pair, and forms conventional hydrogen bonds, which increases the affinity between the ligand and the active receptor site. The role of the amino group in interaction between the receptor is well illustrated both by *in silico* methods, and is proven *in vitro* method, for example, by interrelation between vigabatrin and GABA aminotransferase[9] active site and gabapentin with branched-chain aminotransferase.[10]

Structural modification of the known drugs is one of the most priority areas of the rational drug design of new anticonvulsants[6], it is necessary for creation of the optimal conformation between the receptor and the ligand to improve

its pharmacological profile. Our attention was focused on modifying the structure of the first-generation anticonvulsant – phenobarbital, which is still widely used for convulsive status epilepticus treatment in infants and children[11] and is considered as the drug of choice for refractory epilepsy treatment.[12] Pyrimidin-4(3*H*)-one derivatives, as well as its annelated derivative – quinazoline-4(3*H*)-one, having a pronounced anticonvulsant properties, have been already obtained by us using modification of its structure. Some correlation parameters between structure and anticonvulsant activity were determined has already yielded pyrimidine-4(3*H*)-one and with pronounced anticonvulsant properties and some patterns of structure-anticonvulsant activity have been established (Fig. 2).[13,14]

In the present study, our decision was to synthesize the structural analogues of the previously synthesized thiopyrimidine-4(3*H*)-one acetamides by changing methyl radical from the 6th position of pyrimidine cycle to an unsubstituted amino group (Fig. 2). The donor in a hydrogen bond, which is an amino group, can significantly improve anticonvulsant activity markers, due to formation of stronger and more beneficial receptor-ligand conformation.

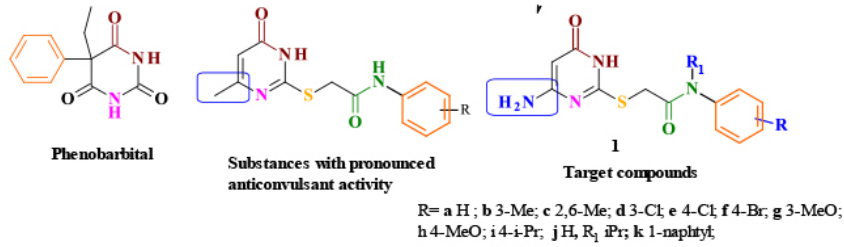


Figure 2. Modification direction and pharmacophore fragments enhancing anticonvulsant activity.

The available tools and models can predict the following properties: absorption, distribution, metabolism, excretion, and toxicity (ADMET). Structure-based *in silico* tools, as molecular docking, can predict possible interactions with the target of study.[15] Therefore, for the further substantiation of expediency and prospects of the search of anticonvulsants among the substances planned for the synthesis, we carried out docking study into the $GABA_A$ receptor active site and evaluated ADMET properties – physicochemical, pharmacokinetic, drug-like and related parameters.

METHODOLOGY

ADMET

Physicochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness and other medicinal chemistry parameters were calculated using SwissADME[16] online free tool (swissadme.ch).

Molecular docking study

Flexible molecular docking tool for the search of molecules with affinity to a specific biological target was used in the present study. The following Protein Data Bank (PDB) macromolecules were used as biological targets: $GABA_AR$ (PDB ID - 4COF), $GABA_{AT}$ (PDB ID -1oHW), CA II (PDB ID - 1EOU and 3IEO), NMDAR (PDB ID - 5TP9).[17] The biological targets were selected according to the literature data concerning the mechanism of action of anticonvulsants.[18]

IsisDraw 2.4 software was used to prepare ligands chemical structure, which were saved in mol format. Chem3D software, by means of molecular mechanical MM2 algorithm, was used to optimize the obtained structures, and saved as pdb-files. The obtained pdb-files were converted into PDBQT by AutoDockTools-1.5.6, and the number of active torsions was set as default.[19]

PDB files of proteins were downloaded from the protein data bank. To delete water molecules and ligand from the crystal we used discovery Studio V17.2.0.16349. Structures of proteins were saved as pdb-files. Polar hydrogens were added and saved as PDBQT in AutoDockTools-1.5.6 software. Docking study was carried out by Vina.[19] For visualization, Discovery Studio V17.2.0.16349 was used.

Chemistry

Sigma-Aldrich (USA) reagents were used in the present research. Standard techniques were used for purification of the reagents. Thin layer chromatography (TLC) method using aluminium silica gel plates was used to monitor the reactions progress. Electrothermal IA9100X1 (Bibby Scientific Limited, Staffordshire, UK) digital melting point apparatus was used for determination of the melting points (°C) in a capillary. Varian Mercury-400 (Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz) was used to record ^{1}H NMR spectra in hexadeuterodimethyl sulfoxide (DMSO-*d6*) using tetramethylsilane (TMS) as an internal standard (chemical shifts are in ppm). Bruker Avance 400 (100.6 MHz) was used to record ^{13}C NMR spectra. Chemical shifts were reported in ppm downfield from TMS as internal standards. Euro Vector EA-3000 (Eurovector SPA, Redavalle, Italy) microanalyzer was used for elemental analysis. The ob-

tained results of the elemental analyses were within ± 0.4% of the theoretical values. PE SCIEX API 150EX chromatograph was used to obtain LC/MS spectra.

6-Amino-2-thio-4-hydroxy-pyrimidine was obtained according to the previously described method.[20]

General procedure of the synthesis of S-alkylated derivatives of 6-amino-2-thio-4-hydroxy-pyrimidine 1a-k

6-amino-2-thiouracil (10 mmol) and potassium carbonate (20 mmol) mixture in 10 ml of DMF was stirred at 70-80°C for 1 hour, then the obtained mixture was cooled to room temperature, and an appropriate chloroacetamides **(**10 mmol) solution in 10 ml of DMF was added and stirred for 5 hours. The reaction mixture was filtered, and the obtained filtrate was evaporated in a vacuum. After that, the obtained residue was treated with 100 ml of cold water. The formed precipitate was filtered, air dried, and recrystallized from an acetone-DMF mixture.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-phenylacetamide 1a. Yield: 89.0% white crystals; mp = 245-247 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 11.65 (1H, br. s, OH), 10.01 (1H, s, NHCO), 7.59-7.27 (4H, m, H-2',3',5',6'), 7.05 (1H, t, *J*=7.5, H-4'), 6.56 (2H, s, NH_2), 5.04 (1H, s, CH-5), 4.07 (2H, s. SCH_2). ^{13}C NMR (100 MHz, DMSO-d_6,): 169.4, 162.8, 161.8, 158.0, 156.4, 138.5, 128.9, 128,5, 121,6, 121.4, 94.9, 56.4, 38.8. LC-MS: m/z =277.07 [M+1]. Anal. Calcd. for $C_{12}H_{12}N_4O_2S$: C, 52.16; H, 4.38; N, 20.28; O, 11.58; S, 11.60. Found: C, 52.01; H, 4.36; N, 20.323; S, 11.58.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methylphenyl) acetamide 1b. Yield: 73%; white crystals; mp = 227-229 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 11.56 (1H, br. s, NH-3), 10.00 (1H, s, NHCO), 7.28 (1H, s, H-2'), 7.12 (1H, t, *J*=8.2, H-5'), 7.02 (1H, d, *J*=7.5, H-6'), 6.52 (1H, d, *J*=7.5, H-4'), 5.05 (1H, s, CH-5), 4.00 (2H, s. SCH_2), 2.13 (3H, s, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6,): 169.2, 168.2, 162.7, 161.7, 138.4, 128.5, 124.6, 120.1, 138.6, 118.6, 94.9, 38.8, 21.3. LC-MS: m/z = 291.09 [M+1]. Anal. Calcd. for $C_{13}H_{14}N_4O_2S$: C, 53.78; H, 4.86; N, 19.30; S, 11.04. Found: C, 53.58; H, 4.84; N, 19.33; S, 11.00.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(2,6-dimethylphenyl)acetamide 1c. Yield: (89.0%); white crystals; mp = 256-258 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 11.58 (1H, br. s, OH), 10.02 (1H, s, NHCO), 7.55-7.38 (3H, m, H-3', 4', 5'), 6.56 (2H, s, NH_2), 5.05 (1H, s, CH-5), 4.01 (2H, s, SCH_2), 2.23 (6H, s, $2CH_3$). LC-MS: m/z = 305.10 [M+1]. Anal. Calcd. for $C_{14}H_{16}N_4O_2S$: C, 55.25; H, 5.30; N, 18.41; S, 10.53. Found: C, 55.06; H, 5.28; N, 18.47; S, 10.49.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-chlorophenyl) acetamide 1d. Yield: 77.0%; white crystals; mp = 234-236 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 11.60 (1H, br. s, OH), 10.02 (1H, s, NHCO), 7.30 (1H, s, H-2'), 7.12 (1H, t, *J*=8.2, H-5'), 7.02 (1H, d, *J*=7.5, H-6'), 6.56 (2H, s, NH_2), 6.52 (1H, d, *J*=7.5, H-4'), 5.07 (1H, s, CH-5), 4.02 (2H, s. SCH_2). LC-MS: m/z = 311.23 [M+1]. Anal. Calcd. for $C_{12}H_{11}ClN_4O_2S$: C, 46.38; H, 3.57; N, 18.03; S, 10.32. Found: C, 46.22; H, 3.56; N, 18.09; S, 10.28.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(4-chlorophenyl) acetamide 1e. Yield: 85.0%; white crystals; mp = 245-247 °C; ^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 11.65 (1H, br. s, OH), 10.27 (1H, s, NHCO), 7.60 (2H, d *J*=9, H-3',5'), 7.37 (2H, d, *J*=9, H-2',5'), 6.56 (2H, s, NH_2), 5.12 (1H, s, CH-5), 4.02 (2H, s. SCH_2). ^{13}C NMR (100 MHz, DMSO-d_6,): 169.4, 168.2, 162.8, 161.8, 136.6, 133.3, 129.0 (2C), 129.0, 120.4 (2C), 38.8. LC-MS: m/z = 311.13 [M+1]. Anal. Calcd. for $C_{12}H_{11}ClN_4O_2S$: C, 46.38; H, 3.57; N, 18.03; S, 10.32. Found: C, 46.24; H, 3.56; N, 18.06; S, 10.29.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(4-bromophenyl) acetamide 1f. Yield: 80.0%; white crystals; mp = 258-260 °C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.68 (1H, br. s, OH), 10.22 (1H, s, NHCO), 7.61 (2H, d, *J*=8, H-3',5'), 7.42 (2H, d, *J*=8, H-2',6'), 6.85 (2H, s, NH_2), 5.05 (1H, s, CH-5), 4.04 (2H, s. SCH_2). LC-MS: m/z = 356.98 [M+1]. Anal. Calcd. for $C_{12}H_{11}BrN_4O_2S$: C, 40.58; H, 3.12; N, 15.77; S, 9.03. Found: C, 40.44; H, 3.10; N, 15.82; S, 9.02.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methoxyphenyl) acetamide 1g. Yield: 73.0%; white crystals; mp = 208-210 °C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.52 (1H, br. s, OH), 10.01 (1H, s, NHCO), 7.28 (1H, s, H-2'), 7.12 (1H, t, *J*=8.2, H-5'), 7.02 (1H, d, *J*=7.5, H-6'), 6.89 (1H, d, *J*=7.5, H-4'), 6.51 (2H, s, NH_2), 5.04 (1H, s, CH-5), 4.00 (2H, s. SCH_2), 3.75 (3H, s, OCH_3). LC-MS: m/z = 307.08 [M+1]. Anal. Calcd. for $C_{13}H_{14}N_4O_3S$: C, 50.97; H, 4.61; N, 18.29; S, 10.47. Found: C, 50.81; H, 4.60; N, 18.36; S, 10.44.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(4-methoxyphenyl) acetamide 1h. Yield:76.0%; white crystals; mp = 255-257°C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.52 (1H, br. s, OH), 10.20 (1H, s, NHCO), 7.54 (2H, d *J*=8, H-3',5'), 7.30 (2H, d, *J*=8, H-2',6'), 6.50 (2H, s, NH_2), 5.10 (1H, s, CH-5), 4.09 (2H, s. SCH_2), 3.75 (3H, s, OCH_3). LC-MS: m/z = 307.06 [M+1]. Anal. Calcd. for $C_{13}H_{14}N_4O_3S$: C, 50.97; H, 4.61; N, 18.29; S, 10.47. Found: C, 50.89; H, 4.60; N, 18.36; S, 10.44.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(4-isopropylphenyl)acetamide 1i. Yield: 66.0%; white crystals; mp = 255-257°C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.60 (1H, br. s, OH), 10.01 (1H, s, NHCO), 7.48 (2H, d, *J*=8.4, H-3',5'), 7.17 (2H, d, *J*=8.4, H-2',6'), 6.51 (2H, s, NH_2), 5.04 (1H, s, CH-5), 3.99 (2H, s. SCH_2), 2.85-2.79 (1H, m, CH), 1.18 (6H, s. $2CH_3$). LC-MS: m/z = 319.12 [M+1]. Anal. Calcd. for $C_{15}H_{18}N_4O_2S$: C, 56.59; H, 5.70; N, 17.60; S, 10.07. Found: C, 56.49; H, 5.68; N, 17.58; S, 10.02.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-isopropyl-N-phenylacetamide 1j. Yield: 72.0%; white crystals; mp = 235-237°C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.60 (1H, br. s, OH), 7.62-7.30 (4H, m, H-2',3',5',6'), 7.05 (1H, t, *J*=7.5, H-4'), 6.56 (2H, s, NH_2), 5.02 (1H, s, CH-5), 4.02 (2H, s. SCH_2), 2.85-2.79 (1H, m, CH), 1.18 (6H, s. $2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6,): 169.2, 165.7, 162.8, 161.8, 158.0, 137.9, 128.9, 128.5, 128,0, 127.5 , 94.6, 59,8, 36.9, 20.6 (2C) LC-MS: m/z = 319.12 [M+1]. Anal. Calcd. for $C_{15}H_{18}N_4O_2S$: C, 56.59; H, 5.70; N, 17.60; S, 10.07. Found: C, 56.45; H, 5.69; N, 17.64; S, 10.00.

2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(naphthalen-1-yl) acetamide 1k. Yield: 70.0%; white crystals; mp = 297-299°C; ^{1}H NMR (300 MHz, DMSO-d 6, δ (ppm)): 11.62 (1H, br. s, OH), 10.02 (1H, s, NHCO), 7.98-7.79 (4H, m, Ar), 7.68-7.56 (3H, m, Ar), 6.56 (2H, s, NH_2), 5.06 (1H, s, CH-5), 4.08 (2H, s. SCH_2). LC-MS: m/z = 327.09 [M+1]. Anal. Calcd. for $C_{16}H_{14}N_4O_2S$: C, 58.88; H, 4.32; N, 17.17; S, 9.82. Found: C, 58.78; H, 4.30; N, 17.19; S, 9.80.

Anticonvulsant activity

Animals

75 adult male rats (130-150 g) and adult random-bred albino mice of either sex weighing 130-150g were used for research. The animals were kept in the National Pirogov Memorial Medical University (Vinnytsya, Ukraine) vivarium, under the standard conditions with a temperature of (22±1°C), relative humidity of (55±15%), free access to food and water, and a 12-hour light/darkness cycle (8.00-20.00), respectively. The animals were treated in accordance with «Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes», with the procedures and requirements of the State Expert Centre of the Ministry of Health of Ukraine and with the rules of European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), resolution of the First National Congress on Bioethics (Kyiv, 2001), with the Law of Ukraine National Congress on Bioethics

(Kyiv, 2001) and with the Law of Ukraine №3447-IV «On Protection of Animals from Cruel Treatment» dated 02.21.2006.

Animals were divided to experimental and control groups at random. The test substances were and injected with an oral cannula through a probe (volume 0.5 ml / 100 g body weight of rats) after dissolution in 1% starch gel. 20 mg / kg dose was chosen for the screening. Phenobarbital and lamotrigine were used as the reference compounds and were administered in the same manner at their mean anticonvulsant doses of 20 mg/kg body weight. The experiment duration was calculated according to the data about the maximum anticonvulsant activity level of the drug described in the literature data.[21] In the control group of animals, an equivalent amount of solvent was administered. Pentylentetrazole-induced seizures were caused in period 9:00 – 11:00, in order to minimize circadian rhythms influence.[22]

Pentylenetetrazole-Induced Seizures

Pentylentetrazole («Sigma», USA) at a dose of 80 mg/kg was used as a single subcutaneous injection to the laboratory animals to start a convulsive attack. A freshly prepared suspension of experimental compounds and reference drugs phenobarbital and lamotrigine (20 mg/kg) were given to the animals. The anticonvulsant activity level was estimated according to the latent period duration, the severity and duration of seizures in minutes and the lethality rate of mice.

The severity of seizures was evaluated by a 5-point scale, considering the following criteria (including the number of died animals):[23] 0 - no seizure activity; 1 - hyperkinesia; 2 - trembling, twitching; 3 - clonic seizures of upper limbs with the rise on their lower limbs; 4 - pronounced tonic-clonic seizures, the animal's fall to the side, available phase of tonic extension; 5 - repeated tonic-clonic seizures, loss of posture, death. Anticonvulsant effect was considered an animal protection based on the clonic and tonic seizures and the lethality.

Statistical analysis

The obtained data was reported as the mean ± standard error of the mean. Comparison among the studied groups was analyzed by one-way ANOVA with the post hoc Dunnett's multiple comparison test to judge significance of the observed effect (Statistical package for social sciences, SPSS 16.0, USA). P values ≤ 0,05 was considered as significant.

RESULTS AND DISCUSSION

Construction of possible candidates' virtual database for the synthesis was carried out using logical and structural analysis of the literature[24] and our own

experience[13,14] regarding the effect of amide fragment substituents on anticonvulsant activity. As it is shown on Figure 2, modification of the 6-amino-2-thioxo-pyrimidin-4-one basic structure occurred by implementation of arylacetamide fragment with 11 different bioactive substituents.

Scheduled for synthesis compounds are barbiturates structural analogues, whose mechanism of action is realized by allosteric site binding to the proteins type-A γ-aminobutyric acid receptors ($GABA_A$Rs). AutoDockTools1.5 virtual screening was used to predict the affinity of the studied compounds **1a-k** with $GABA_A$R (PDB 4COF)[25]. Phenobarbital was used as the reference ligand to compare binding energy, due to its binding energy to $GABA_A$R active site of was -7.6 kcal/mol. All the tested **1a-k** ligands showed a significant $GABA_A$R affinity with low scoring functions at the same or slightly worse level (1g, h, j) with the reference drug (Table 1). The obtained data confirm the prospect of the search for anticonvulsants among the mentioned derivatives, and allow us to choose pentylenetetrazole (PTZ) model of seizures for primary screening, since PTZ is a classic proconvulsant which action is caused by $GABA_A$ site of the benzodiazepine receptor complex suppression and the intensity of GABA-ergic inhibitory processes in the CNS reducing.

Another confirmation of the scheduled studies expediency was the obtained results of ADMET parameters calculation (Table 1), which predict **1a-k** acetamides to be orally bioavailable (i.e. the drug-likeness). Table 1 shows just several calculated parameters, namely the numerical features of the physicochemical properties, lipophilicity and drug likeness according to 6 different filters.

Table 1. ADMET properties for target compounds **1a-k**

Properties	Compounds										
	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**	**1j**	**1k**
Docking in active site of $GABA_A$ (PDB 4COF)											
Binding energy (kcal/mol)	-7.4	-7.5	-7.3	-7.2	-7.3	-7.4	-6.2	-6.7	-7.4	-6.2	-7.8
Physicochemical Properties											
Molecular weight	276	290	304	310	310	355	306	306	318	318	326
Rotatable bonds	5	5	5	5	5	5	6	6	6	6	5
H-bond acceptors	4	4	4	4	4	4	5	5	4	4	4
H-bond donors	3	3	3	3	3	3	3	3	3	2	3
Molar Refractivity	74.0	78.9	83.9	79.0	79.0	81.7	80.5	80.5	88.5	88.5	91.5
TPSA[1] (Å)	126.43	126.4	126.43	126.43	126.43	126.43	135.66	135.66	126.43	117.64	126.43
Lipophilicity											
iLOGP	1.29	1.87	1.97	1.79	1.80	1.94	1.80	1.76	2.37	2.50	1.97
XLOGP3	1.58	1.95	2.31	2.21	2.21	2.27	1.55	1.55	2.71	2.56	2.83
WLOGP	1.31	1.62	1.93	1.97	1.97	2.07	1.32	1.32	2.44	2.31	2.47
MLOGP	0.73	0.59	0.86	1.27	1.27	1.40	0.06	0.06	1.11	1.11	1.58
SILICOS-IT Log P	0.76	1.26	1.76	1.41	1.41	1.45	0.81	0.81	1.84	1.26	1.79
Consensus Log P	1.14	1.46	1.77	1.73	1.73	1.83	1.11	1.10	2.09	1.95	2.13
Druglikeness											
Lipinski violations	0	0	0	0	0	0	0	0	0	0	0
Ghose violations	0	0	0	0	0	0	0	0	0	0	0
Veber violations	0	0	0	0	0	0	0	0	0	0	0
Egan violations	0	0	0	0	0	0	1	1	0	0	0
Muegge violations	0	0	0	0	0	0	0	0	0	0	0
Bioavailability Score violations	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
[1]Total polar surface area;											

In result of water solubility parameter test, all the studied compounds were moderately soluble. According to pharmacokinetics parameters – high gastrointestinal absorption, partial blood-brain barrier permeability (except **1g, h** - do not penetrate the BBB), the absence of inhibitory effect on CYP450 enzymes and are not P-glycoprotein substrates. **1f** compound having 4-bromophenyl substituent showed one violation in lead likeness (medicinal chemistry) parameter: its molecular weight was 355.21 instead of the desired 250 ≤ MW ≤ 350. Since molecular weight deviation was not significant, **1f** compound was left for the further research. Compounds **1g, h** having methoxy groups in phenyl radical also had one violation in the drug likeness parameter, namely the Egan filter: topological polar surface area was TPSA> 131.6, while the optimal range was 20

and 130 Å^2. The given descriptor is quite important, due to its correlation with passive molecular transport through membranes including blood-brain barrier (BBB). Given the fact that the deviation was only 1.6 Å^2 and, according to Kelder[26] data, the allowable PSA value was <150Å^2, **1g, h** compounds were left for the further studies. Negative prognosis for BBB penetration of compounds may be associated only with relatively high TPSA values. It's worth mentioning that other criteria are rather important for effective AEDs, and their calculation for the test substances **1a-k** was positive:

- lipophilicity, an indicator that is considered to be the key property for drug development, especially CNS affecting ones; [27,28]

- H-bond donors and acceptors quantity and molecular weigh, directly affecting on ligand binding sites formation in proteins-targets;[29]

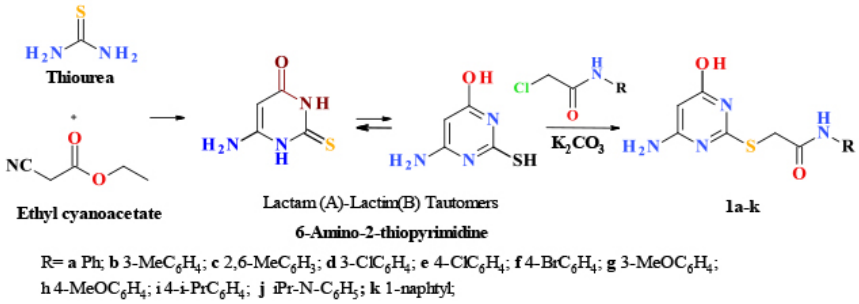
- whether the substance is a glycoprotein P-gp substrate, because one of the key P-gp roles is the CNS protection from xenobiotics[30], including AED.[31] Most of the modern AED (gabapentin, felbamate, topiramate, etc.) are P-gp substrates, and this is considered to be a reason of pharmacoresistant epilepsy;

- whether the substance is a cytochromes P450 inhibitor. All AEDs methabolism, excluding levetiracetam and lamotrigine, is realized by cytochromes P450 subfamily CYP1-3, moreover, the most important enzymes are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A3, CYP3A4.[32] Therefore, inhibition of the mentioned enzymes may lead to a large number of side effects.

To form key intermediate rings – 6-amino-2-thioxo-pyrimidine-4-one – we used already developed approach[13], but ethyl acetoacetate was substituted to cyanoacetate (Scheme 1). The reagents condensation was carried out in refluxing absolute ethanol with a 2.6-2.8-fold molar excess of sodium ethoxide.

The existence of 6-amino-2-thio-pyrimidin-4-one in several tautomeric forms (lactam **A**/lactim **B**) (scheme 1) substantiates the possibility of several alkylation directions depending on the reaction conditions.[33,34] Since we decided to modify the structure of previously obtained methyl derivatives, for the target products **1a-k** synthesis, we used the same conditions leading to S-alkylated thiopyrimidines.[13,35] We carried out the interaction between 6-amino-2-thiopyrimidine and the corresponding 2-chloroacetamides in dimethylformamide environment and the presence of potassium carbonate excess at a temperature of 70-80˚C. According to the TLC, mass-spectroscopy and ^{1}H NMR-spectroscopy data, the alkylation is selective with the following S-derivatives formation. The synthesized **1a-k** substances are white crystalline substances, easily soluble in dioxane, dimethylformamide, and insoluble in water. The structure and purity

of the obtained compounds were confirmed by elemental analysis, ^{1}H NMR, ^{13}C NMR spectroscopy, and LC/MS. ^{1}H NMR spectra of the synthesized **1a-k** compounds have shown the singlet signal of the acetamide residue proton group at δ 10.16-10.01 ppm (excluding **lj** substance), the singlet signal of methylene SCH_2-group in area of 4.12-4.00 ppm, and a two-proton singlet signal of an amino group in the 6 position at 6.51-6.32 ppm.



Scheme 1. Alkylation of 6-amino-2-thiopyrimidine of 2-chloroacetamides

The aryl radical protons resonate in the region of δ 7.61 -7.22 ppm according to the substituent's location. Other signals were interpreted in comparison with the spectra of the previously described 6-methyl-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one acetamides: extended singlet signal at 11.50-11.52 ppm. was attributed to OH group proton, since proton NHC=O in the third position of the pyrimidine cycle resonates in a weaker field δ 12.52-12.43 ppm at its existence in the lactam form; CH signal shift at the 5 position of the pyrimidine cycle into the strong field region by almost 1 ppm - 5.02-4.99 ppm in 4-hydroxypyrimidines **1a-k** against 6.05-5.98 ppm in pyrimidin-4-ones[13], indicating that there is no effect of a strong electron-withdrawing carbonyl group on compounds **1a-k**, which also allows pointing to the fact that the synthesized compounds are 2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-acetamides.

The synthesized **1a-k** compounds were examined for anticonvulsant activity using pentylenetetrazole-induced model of seizures in rats. Pentylenetetrazole administration led to convulsions development in all animals of the control group: the latent period duration averaged 4.7 minutes; convulsion attack duration was 9.7 minutes (Table 2). Convulsions were accompanied by tonic-clinical seizures, a clear phase of tonic extension was also observed, and lethality in the group was 100%. Phenobarbital significantly prevented convulsions development in all animals. After lamotrigine administration in rats, pentylenetetrazole caused some manifestations of seizures (convulsive twitching, jumps and tonic contractions of the forelimbs), but statistically significantly prolonged the

latency period in 5.8 times; the severity and duration of seizures decreased significantly, compared to the control group, and lethality in the group was 20%.

Pharmacological screening resulted that all the studied compounds **1a-k** in one or another way demonstrated their anticonvulsant effect on the model of pentylenetetrazole-induced seizures in rats. After their administration, a tendency to prolong the latency period at least in 2.3 times was found, lethality has changed from 60% to 100%, and severity and duration of seizures decreased (Table 2).

Compound **1b** with a 3-methyl substituent in phenyl radical considered being the absolute lead. It significantly improved all experimental convulsion indicators without exception: the latency period increased in 5.6 times ($p < 0.05$), the severity of seizures decreased in 12.4 times (0.4 points instead of 4.96 in the control group), and duration of convulsions decreased more than in 12 times (up to 0.80 ± 0.8*min instead of 9.70 ± 0.90 min, $p < 0.05$). Comparing of the obtained screening results of 3-methyl-(**1b**) and 2,6-dimethylphenylacetamides (**1c**) points to a worsening of anticonvulsant activity markers as a result of increase of methyl groups number: the latency period increased only in 3.5 times, lethality protection was 80%, and any influence on duration of seizures was not observed.

Table 2. Influence of the investigated substances on the pentylenetetrazole-induced seizures in rats

Groups of animals	Number of rats in the group	Dose, mg/kg	Duration of the latent period, min	Duration of seizures, min	Lethality abs. units (%)	Severity of seizures, (points)
Control	n=10	-	4.7 ± 0.30	9.70±0.90	10 (100%)	4,96
1a	n=5	20	11.2 ± 1.6*	10.8 ± 2.2	0*	2.1
1b	n=5	20	26.6 ± 0.7*	0.80 ± 0.8*	0*	0.4
1c	n=5	20	16,0 ± 5,8*	8,2 ± 3,4*	1 (20%)	2,0
1d	n=5	20	14.4 ± 4.0*	7.6 ± 2.0	2 (40%)	3.2
1e	n=5	20	12,4 ± 2.6*	13.8 ± 3.5*	2(40%)	3.2
1f	n=5	20	20.0 ± 3.0*	1.0 ± 1.0*/#	0*	0.4
1g	n=5	20	11,2 ± 1,3*	4,4 ± 0,5*	1 (20%)	2,2
1h	n=5	20	16.0 ± 5,8	8.2 ± 3,4	1 (20%)	2.4
1i	n=5	20	12,8 ± 2,8*	9,0 ± 0,8	1 (20%)	2,0
1j	n=5	20	16.0 ± 1.2*	6.6 ± 2.5	1 (20%)	2.30
1k	n=5	20	11.2 ± 1.3*	4.4 ± 0.5*	0*	2.2
Phenobarbital	n=5	20	30.0 ± 0.0*	0*	0*	0
Lamotrigine	n=5	20	27.6 ± 0.8*	2.40±0.40*	1 (20%)	2.20

Footnotes: n - a number of animals in the group

* - compared to control group indicators $p<0.05$

Compound **1f** with 4-bromophenyl substituent also showed significant anticonvulsant properties: it significantly extended the latency period in 4.25 times, duration of seizures was only 1 minute, instead of 9.7 in the control group, and their severity was 0.4 points, it's also worth mentioning that this compound completely prevented lethality in the appropriate group of animals.

Compound 1a with unsubstituted phenyl radical completely prevented lethality in the appropriate group of animals, prolonged the latency period by 2.4 times, but it did not affect the duration of seizures, although their severity was much lower - 2.1 points instead of 4.96 in the control group. Our expectations concerning the introduction of an additional hydrophobic ring and, as a result, increasing anticonvulsant effect of **1k** compound, did not materialized, because a significant difference in anticonvulsant protection of animals between phenyl-**1a** and the naphthyl-substituted **1k** derivatives was not found (Table 2).

The obtained results of 3- and 4-methoxyphenyl substituted derivatives, **1g** and **1h**, respectively, were similar and showed a tendency of the mentioned compounds for anticonvulsant activity. It's worth mentioning that for compounds containing chlorine atoms (**1d** and **1e**), the highest lethality in animals was observed (40% of deaths) and the highest among the studied compounds severity of seizures was determined - 3.2 points. In the present study, we were not surprised by the given results, due to results were compared to the obtained before markers concerning the influence of chlorine atoms on lethality increasing in the groups of animals.[13]

To sum it up, it is possible to make conclusions about advisability of implementation of free amino group into the structure of potential anticonvulsants, because 6-amino derivatives demonstrated more significant and stable results consisted of protection of experimental animals in the chemo convulsive model, compared to 6-methyl-substituted derivatives. The obtained results of pharmacological screening indicate GABA-positive properties of the synthesized acetamides.

Since the nature and mechanisms of seizures are different, "perfect" anticonvulsant should act on different models of seizures and show a multifactorial mechanism of action. The advantage of phenobarbital is its wide range of anticonvulsant effect, which is demonstrated on various models of seizures[18]: PTZ, MES and kindling models, for example, amygdala kindled rat, hippocampal kindled rat and corneal kindled mouse. Structural similarity of the lead compound 2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methylphenyl)acetamide **1b** with phenobarbital gives reason to predict its multifactor anticonvulsant effect. To confirm this assumption, previously we carried out *in silico* research

using molecular docking. The choice of targets was made in accordance with the known mechanisms of action of modern anticonvulsants.

It is known, that $GABA_A$Rs are the principal mediators of rapid inhibitory synaptic transmission in the human brain.[25] A decline in $GABA_A$R signaling triggers hyperactive neurological disorders such as epilepsy. Therefore, we carried out docking study for **1b** compound into the active $GABA_A$ site, comparing the obtained scoring function with native ligand binding results – benzamidine, which is new $GABA_A$R modulator (Table 3, Fig. 3).

Table 3. Docking results of compound 1b and native ligands in anticonvulsant biotargets active sites

Receptor	Binding energy (kcal/mol)	Hydrophobic interaction	Hydrogen interaction	Binding energy (Native ligand)
	Ligand **1b**			
$GABA_AR$ (4COF)	-7.5	Tyr205, Tyr157, Ala201	Gln64, Ser156, Tyr157, Glu155, Thr176	-8.5 (benzamidine)
$GABA_{AT}$ (1OHW)	-8.2	Cys135, Val300, Trp354 Phe189, Lys329	His190, Ser137, Phe189, Gly191, Gly136, Thr353	-6.7 (vigabatrine)
CA II (1EOU)	-6.7	His64, Leu197, Pro201	Thr199/198, Pro200, His64	-7.4 (sulfamate)
CA II (3IEO)	-5.7	His64 His94 Phe131 Leu198 Ile91	Gln92, Thr200, Thr199, Gln92	-5.8 (lacosamide)
NMDAR (5TP9).	-8.8	Tyr144, His273, Ile128, Pro141, Pro129, Val266	Thr242	-7.7 (felbamate)

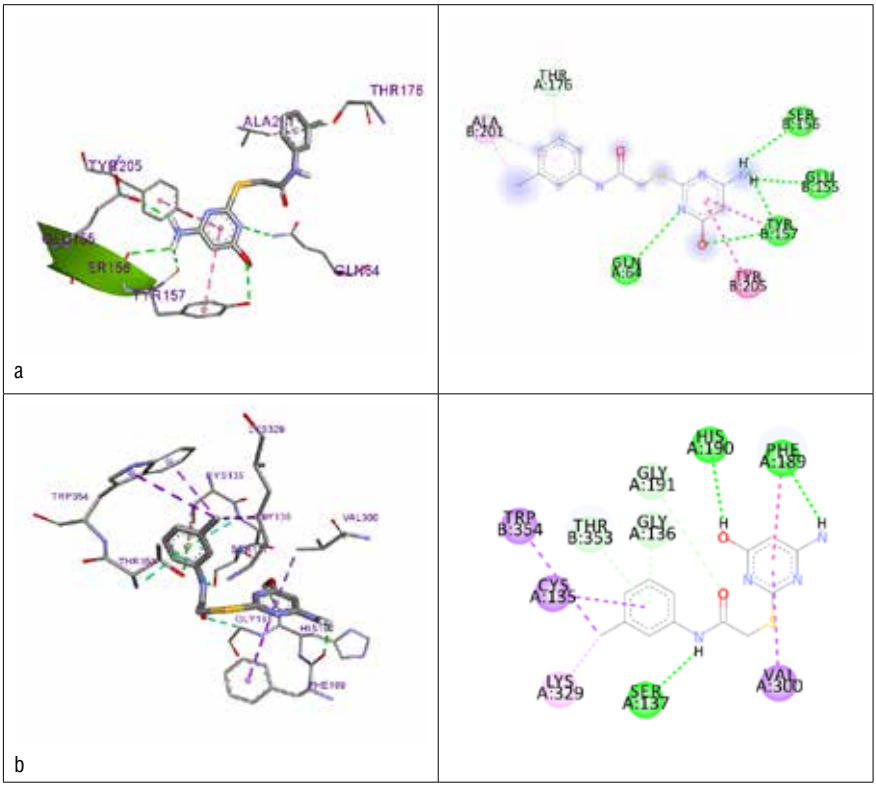


Figure 3. 2D and 3D interaction between GABAAR ($_4$COF) (a), GABAAT (10HW) (b) and ligand 1b: hydrogen bonds are indicated by green dotted lines, hydrophobic interactions – purple dotted lines.

Table 3 shows scoring functions of docking scores of the investigated studied **1b** ligand and the native ligands with the corresponding target proteins, also it shows which amino acids of the receptor active site are involved in the interaction between ligand **1b,** and the bonds nature. As can be seen from the docking data of ligand **1b** into $GABA_AR$ protein active site, 4 hydrophobic bonds are typical for it: 2 of them are formed with alanine (Ala201) and 2 – between the pyrimidine cycle and aromatic rings of tyrosine residues (Tyr205, 157). A significant number of hydrogen bonds with glutamine (Gln64), serine (Ser156), tyrosine (Tyr157), glutamic acid (Glu 155), and tryptophan (Thr176) are characterized by low binding energy - 7.5 kcal/mol, but higher than in native ligand - 8.5 kcal/mol.

It was found, that γ-Aminobutyrate aminotransferase ($GABA_{AT}$) is responsible for inhibitory neurotransmitter GABA degradation, and is a target for antiepileptic drugs because its selective inhibition raises GABA concentrations in brain.[9] GABA-AT exactly is irreversibly inhibited by the modern AED vigaba-

trin,[18] which was used as a native ligand, and the active site coordinates were determined according to its location. Molecular docking between $GABA_{AT}$ (1OHW) target and ligand **1b** resulted that the studied compound is characterized by significantly less binding energy (high docking scores) with $GABA_{AT}$ (-8.2 kcal/mol) target, comparing to the reference drug vigabatrin binding energy (-6.7 kcal/mol). Figure 3 points to the fact, that ligand **1b** completely occupies the active site in the target protein and engages in hydrophobic interaction with cysteine (Cys135), valine (Val300), tryptophan (Trp354), and phenylalanine (Phe189), and is additionally stabilized by hydrophilic bonds with residues of the following amino acids: histidine (His190), phenylalanine (Phe189) and serine (Ser137), with carboxyl group of which the NH group of the acetamide fragment interacts. The obtained docking results show high probability of anticonvulsant activity realization of the studied 2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methylphenyl)acetamide **1b**, namely due to GABA-ergic system, especially by $GABA_{AT}$ inhibition.

Carbonic anhydrase II is a zinc-containing enzyme catalysing the reversible hydration of carbon dioxide.[36] This reaction is essential for important physiological anion-exchange processes. Carbonic anhydrase inhibition in brain leads to CO_2 accumulation and inhibition of excessive paroxysmal discharges of the neurons, which causes the drugs' antiepileptic activity.[37,38] Evaluation of ligand **1b** binding to the active site CA II (PDB code 1EOU) was carried out in comparison to sugar sulphamate, which is analogue of the modern and widely used AED named topiramate[36], showing inhibitory effect on carbonic anhydrase. Although immersion into the target protein active site is quite deep, the formed bonds are rather strong, and there is also an additional interaction between the sulphur atom of the studied ligand **1b** and histidine amino acid imidazole cycle, but the binding energy of compound **1b** is higher than the one of native sulfamate ligand (- 6.7 and - 7.4 kcal/mol, respectively)(Fig. 4.).

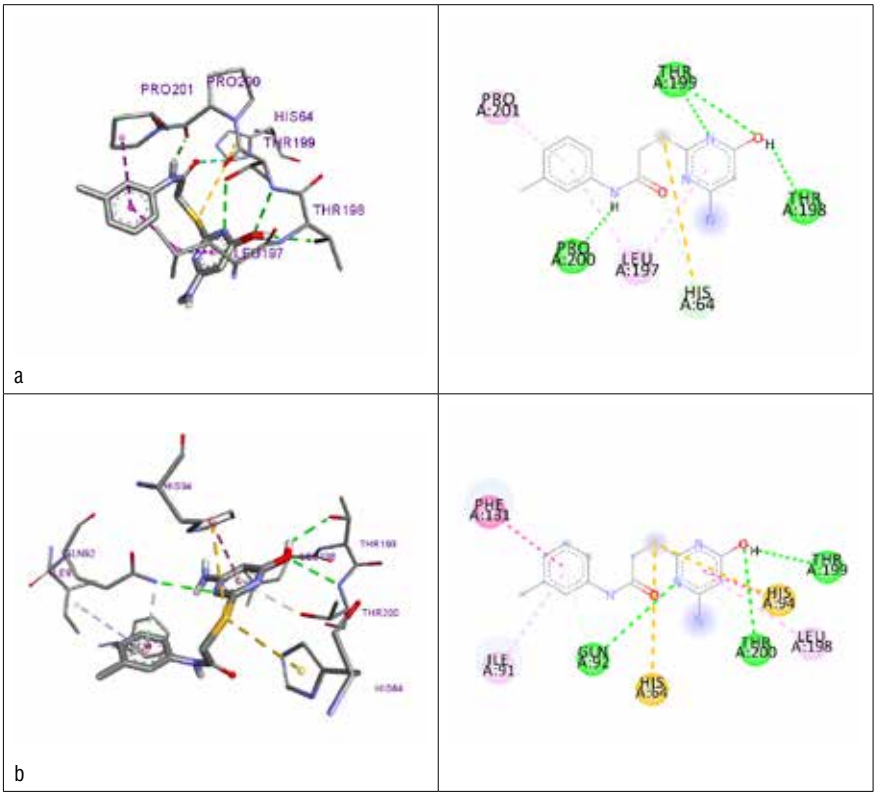


Figure 4. 3D and 2D interaction between Carbonic Anhydrase II 1EOU (a), 3IEO (b) and ligand 1b: hydrogen bonds are indicated by green dotted lines, hydrophobic interactions – purple dotted lines, Pi-S – orange.

It also has been found that modern AED lacosamide interacts with the coumarin site of carbonic anhydrase binding,[39] which may be one of its anticonvulsant activity mechanisms. Docking study of 2-((4-amino-6-hydroxypyrimidin-2-yl) thio)-N-(3-methylphenyl)acetamide **1b** into coumarinic site of carboxylic anhydrase (PDB code 3IEO) demonstrated affinity to the mentioned protein at the reference drug level: -5.7 and -5.8 kcal/mol, respectively. Hydrophobic bonds between ligand **1b** and phenylalanine (Phe131), leucine (Leu198) and isoleucine (Ile91), and hydrophilic ones with glycine (Gln92), tryptophan (Thr200 and Thr199) form stable conformation, which is also being stabilized by two Pi-S bonds between the thio group of ligand **1b** and the imidazole cycle of histidine 64 and 94. It should be noted that, as in lacosamide case, there was no interaction with zinc ions Zn (II) in adduct CA II-ligand **1b**. Obtained calculations show that compound **1b** anticonvulsant activity by its inhibition mechanism of carbonic anhydrase II is less reliable than GABA aminotransferase inhibition.

N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor, gated by the endogenous co-agonists glutamate and glycine, permeable to Ca2+ and Na+. NMDAR dysfunction is associated with numerous neurological and psychiatric disorders, including schizophrenia, depression, Alzheimer's disease and epilepsia.[40] To predict the possibility of glutamatergic mechanism of anticonvulsant activity of the studied pyrimidine derivative **1b**, its affinity for the active site NMDAR (PDB code 5TP9) was determined (Fig. 5).

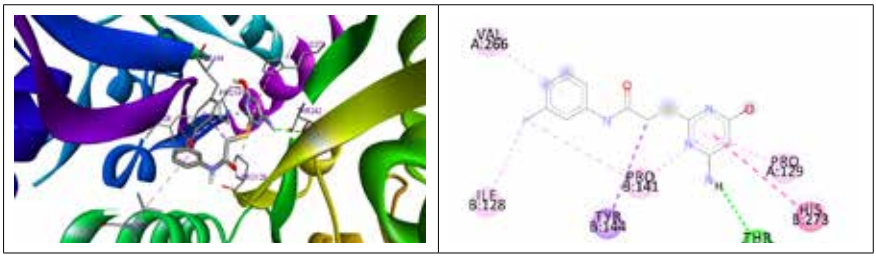


Figure 5. 3D and 2D interaction between NMDAR (5TP9) and ligand 1b: hydrogen bonds are indicated by green dotted lines, hydrophobic interactions – purple dotted lines.

Deep immersion into NMDA receptor hydrophobic pocket with formation of strong hydrophobic bonds (distance Å≤ 5) was found: between CH_2 group and tyrosine aryl ring (Tyr144), between the pyrimidine cycle of compound **5.2** and histidine imidazole fragment (His273), methyl radical of the acetamide residue and isoleucine (Ile128), and proline pyrrolidine fragment (Pro141), compound **1b** pyrimidine cycle, and proline pyrrolidine residues 141 and 129, the ligand phenyl radical, and valine (Val266). Ligand **1b** forms only one hydrophobic bond between tryptophan oxygen atom (Thr242) and ligand 5.2 amino group (distance 2,78 Å). Scoring function points to high affinity of ligand **1b** to NDMA receptor active site: -8.8 kcal/mol (Tabl. 3.), while a known inhibitor of NDMA receptor felbamate is characterized by -7.7 kcal/mol value. Considering that, anticonvulsant activity of compound **1b** by its inhibitory effect on glutamate NDMA receptor is quite probable.

According to *in silico* ADMET analysis and docking into the active $GABA_A$ receptor site, synthesis of 2-(4-amino-6-hydroxy-pyrimidin-2-yl)thio-N-acetamides as a potential anticonvulsants was scheduled. The synthesis was carried out by 6-amino-2-thiopyrimidine alkylation with appropriate 2- chloroacetamides in DMF environment in potassium carbonate presence. According to the TLC, mass-spectroscopy and ^{1}H NMR-spectroscopy data, the corresponding thioacetamide derivatives were alkylation products. All the synthesized compounds have shown a tendency to anticonvulsant activity on the model of pentylenetetrazole seizures in rats. Positive effect on anticonvulsant activity of pyrimidine

cycle amino group in the 6 position was found. The estimated affinity of the lead compound to anticonvulsant biotargets allows providing a multifactor mechanism for anticonvulsant activity implementation: due to the inhibitory effect on GABA aminotransferase and the ionotropic glutamate receptor NMDA. The obtained *in vivo* data and *in silico* calculations substantiate the prospect of in-depth study of the anticonvulsant and concomitant pharmacological and toxicological properties of the lead compound 2-((4-amino-6-hydroxypyrimidin-2-yl)thio)-N-(3-methylphenyl)acetamide.

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