ACTA PHARMACEUTICA SCIENCIA

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Founded in 1953 by Kasım Cemal GÜVEN

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Contents

Aims and Scope of Acta Pharmaceutica Sciencia

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

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

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

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK

Editor

INSTRUCTIONS FOR AUTHORS

1. Scope and Editorial Policy

1.1. Scope of the Journal

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

The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part.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

All manuscripts has to be written in clear and concise English. Starting from 2016, the journal will be issued quarterly both in paper and online formates also publish special issues for national or international scientific meetings and activities in the coverage field.

1.2 Manuscript Categories

Manuscripts can be submitted as Research Articles and Reviews.

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

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

1.3 Prior Publication

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

1.4 Patents and Intellectual Property

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

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

1.5 Professional Ethics

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

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

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

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

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1 General Considerations

Manuscripts should be kept to a minimum length. Authors should write in clear,

concise English, employing an editing service if necessary. For professional assistance with improving the English, figures, or formatting in the manuscript before submission please contact to editorial office by e-mail for suggestions.

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

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

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

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

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

Authors may find the following sources useful for recommended nomenclature:

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

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

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

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

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

· Once in the abstract.

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

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

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

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

2.2 Manuscript Organization

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

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

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

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

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

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

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

The preferred forms for some of the more commonly used abbrevations are mp, bp, ^{o}C, K, min, h, mL, µL, g, mg, µg, cm, mm, nm, mol, mmol, µmol, ppm,

TLC, GC, NMR, UV, and IR. Units are abbreviated in table column heads and when used with numbers, not otherwise. (See section 4 for more abbreviations)

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

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

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

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

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

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

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

Acknowledgment: Authors may acknowledge people, organizations, and financial supporters in this section.

Abbreviations Used: Provide a list of nonstandard abbreviations and acronyms used in the paper, e.g., YFP, yellow fluorescent protein. Do not include compound code numbers in this list. It is not necessary to include abbreviations and acronyms from the Standard Abbreviations and Acronyms listed in section 4.

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

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

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

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

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

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

List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used.

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

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

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

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

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

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

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

- Black & White line art: 1200 dpi

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

- Color art (RGB color mode): 300 dpi

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

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

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

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

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

2.3 Specialized Data

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds.

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

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

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

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

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

3. Submitting the Manuscript

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

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

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

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

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

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

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

3.2.2 Institutions Authors should give their institutional information during submission.

3.2.3 Authors The authors' surnames, names, institutional information appear as entered order in the previous page. Filling all e-mail adresses are re-

quired. Institutional information is available in **Manuscript Details** table at the top of the screen. After filling all required fields, you may click the **Continue** button.

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

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

3.2.5 Abstract The summary of the article should be entered to **Abstract** window at this level. There must be an English summary for all articles and the quantity of words must be not more than 150. If special characters such as alpha, beta, pi or gamma are used in summary, they can be added by **Abstract** window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text. The abstract of the articles are accessible for arbitrators; so you should not add any information related to the institutions and authors in this summary part. Otherwise the article will returned without evaluation. Authors will be required to comply with the rules.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3.4 Page to Follow The Article The Main Page of Author ensures possibility to follow the article. This page consists three different parts; some infor-

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

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

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

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

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

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

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

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

ORIGINAL ARTICLES

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DOI: 10.23893/1307-2080.APS.05801

Cytotoxic Activity of the Root and Fruit Extracts of *Heptaptera Anisoptera* (DC.) Tutin

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2 Istanbul University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Turkey

ABSTRACT

Cytotoxic activities of the root and fruit extracts of *Heptaptera anisoptera* (DC.) Tutin were investigated on the colon cancer COLO205 and KM12 cell lines. The dichloromethane extract of the roots of *H. anisoptera* showed cytotoxic activity with IC_{50} values of 3.1 and 3.9 ug/mL on the COLO205 and KM12 cell lines, respectively. Cytotoxic activity of the dichloromethane extract of the fruits were similar to those of root extract with IC_{50} values of 5.5 and 4.8 ug/mL on the COLO205 and KM12 cell lines, respectively.

Keywords: Cytotoxic activity, *Heptaptera anisoptera*, Apiaceae

INTRODUCTION

The genus *Heptaptera* Marg. & Reuter (Apiaceae) is represented by 10 species worldwide, four of them; *H. cilicica* (Boiss. & Bal.) Tutin, *H. anisoptera* (DC.) Tutin, *H. anatolica* (Boiss.) Tutin and *H. triquetra* (Vent.) Tutin are growing in Turkey[1,2]. *Heptaptera* species are known to contain sesquiterpene coumarin derivatives[3,4], these compounds have various biological activities such as; cytotoxicity, P-glycoprotein inhibitory, cancer chemoprevention, anti-inflammatory, antibacterial, antileishmanial, antiviral, antidiabetic, etc.[5-9].

METHODOLOGY

Plant Material

The roots and fruits of *Heptaptera anisoptera* were collected in the vicinity of Kahramanmaraş in June 2013 and identified by Prof. A. Duran. A voucher specimen (A. Duran 9621) was deposited in the Herbarium of Selçuk University, Faculty of Sciences, Department of Biology (Konya).

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Extraction

Coarsely powdered roots (150 g) and fruits (50 g) of the plant were separately and sequentially extracted at room temperature with dichloromethane (CH_2Cl_2) and methanol. The extracts were individually concentrated in a rotary evaporator under reduced pressure to dryness. Dichloromethane and methanol extracts of the roots were 3.89 g, 2.59% and 9.59 g, 6.39%, respectively. Dichloromethane and methanol extracts of the fruits were 5.73 g, 11.46% and 1.97 g, 3.94 %, respectively. Methanol extract was redissolved in a mixture of methanol/water (10:90) and then partitioned with ethyl acetate (EtOAc), the resulting extracts were separately concentrated in vacuo to dryness. Ethyl acetate and aqueous-methanol extracts of the roots were 1.18 g, 0.79% and 8.41 g, 5.6%, respectively. Ethyl acetate and aqueous-methanol extracts of the fruits were 0.25 g, 0.5% and 1.72 g, 3.44%, respectively.

Cytotoxicity Assay on Colon Cancer Cells

The assay used for this study was a two-day, two cell line XTT bioassay[10], an in vitro antitumor colorimetric assay developed by the MTL Assay Development and Screening Section. Colon cancer cell lines used were COLO205 and KM12. Cells were maintained and passed weekly in RPMI-1640 medium with phenol red (Gibco, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine (Quality Biologicals, Inc., Gaithersburg, MD, USA) and 10% fetal bovine serum (Hyclone, Logan, UT, USA). Cells were placed in a humidified incubator with an atmosphere of 5% CO2 and 95% air and a temperature of 37° C. Cells were placed in a humidified incubator with an atmosphere of 5% CO2 and 95% air and a temperature of 37 °C. Cells used in the assay were harvested with RPMI-1640 medium, without phenol red (Gibco, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine (Quality Biologicals, Inc., Gaithersburg, MD, USA) and 10% fetal bovine serum without antibiotics. Harvested cells were counted using a Cellometer Auto T4 cell counter (Nexcelom Bioscience LLC, Lawrence, MA, USA) and plated in 384-well flat-bottom polystyrene microtiter plates (Nunc, Nunc A/S, Denmark), at a density of 5000 cells/well for COLO205 and 5000 cells/well for KM12. The cells were incubated in a 5% CO2/95% air and 37 °C incubator for 24 h. After incubation, test samples were added to plates using a Biomek FX robotic liquid handling workstation (Beckman/Coulter, Fullerton, CA, USA). The robot performed eight 2-fold serial dilutions of the sample and then transferred the sample from the source plate to the assay plate. The plates used were Costar 384-well round-bottom plates (Corning Inc., Corning, NY, USA). Cells were further incubated with samples for 48 h, at which time the XTT reagent was added. Viable cells reduced the

XTT to a colored formazan product, and after an additional 4 h incubation period the amount of formazan produced was quantified by absorption at 450 nm, using a 650 nm reference. Sanguinarine was used on each plate as a positive control.

RESULTS AND DISCUSSION

This is the first report on the cytotoxic activity of the roots and fruits of *H. anisoptera*. The dichloromethane extracts of the roots and fruits exhibited strong inhibitory activity on the colon cancer COLO205 and KM12 cell lines. The ethyl acetate extract of the fruits exhibited strong inhibitory activity on the COLO205 cell lines with IC50 value of 4.5 ug/mL but only a moderate inhibitor activity on KM12 cell lines. The cytotoxic activities observed with these extracts are shown in Table 1.

Table 1. Cytotoxic activities of extracts

Extracts	Cytotoxic activity (IC_{50} values in ug/mL)	
	COLO205	**KM12**
1	3.1	3.9
2	> 50	20.5
3	> 50	> 50
4	5.5	4.8
5	4.5	15.2
6	> 50	> 50

1: CH_2Cl_2 extract of the roots; 2: EtOAc extract of the roots; 3: aqueous-methanol extract of the roots

4: CH_2Cl_2 extract of the fruits; 5: EtOAc extract of the fruits; 6: aqueous-methanol extract of the fruits

The dichloromethane extract of the roots of *H. anisoptera* showed cytotoxic activity with IC_{50} values of 3.1 and 3.9 ug/mL on the COLO205 and KM12 cell lines, respectively. The cytotoxic activities of dichloromethane extract of the fruits were similar to those of root extract with IC_{50} values of 5.5 and 4.8 ug/mL on the COLO205 and KM12 cell lines, respectively. The ethyl acetate extract of the fruits showed cytotoxic activity with IC_{50} values of 4.5 and 15.2 ug/mL on the COLO205 and KM12 cell lines, respectively. Whereas, the ethyl acetate extract of the roots showed a moderate cytotoxic activ-

ity with IC_{50} values of 20.5 ug/mL on the KM12 cell lines but no inhibitor activity up to 50 ug/mL on the COLO205 cell lines. Previously, Appendino *et al.* reported umbelliprenin, badrakemin, badrakemone, colladonin (major compound), colladin, 14-acetoxybadrakemin, 14-acetoxybadrakemone, 14-hydroxycolladonin from the chloroform extract of the roots and more polar compounds samarcandin, samarcandone, conferol, conferone, feselol, 9,10,11-trihydroxyumbelliprenin, 9,10,11-5'-tetrahydroxyumbelliprenin, 9, 10, 5'-triacetoxy-11-hydroxyumbellipreni, 10, 11, 5'-trihydroxyumbelliprenin from the chloroform extract of the fruits of *H. anisoptera* collected from Diyarbakır in June 1991[3,11,12]. Cytotoxic activity of certain sesquiterpene coumarins were described earlier[4,5], thus, the cytotoxic compound(s) of the roots and fruits of *H. anisoptera* may be this type of compound(s). Bioactivity guided fractionation of the dichloromethane extracts of the roots and fruits of *H. anisoptera* is planned to isolate and identify their cytotoxic principles.

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Obturator Neuropathy Combined with Guillain-Barre Syndrome

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ABSTRACT

Obturator neuropathy caused by entrapment of obturator nerve is a disease with numbness, pain and weakness in legs. Guillain-Barre syndrome is an immune-mediated peripheral polyneuropathy characterized by nearly same symptoms.

51-year-old man presented with severe shortness of breath. He was admitted to ICU and treated with mechanical ventilation followed complete blood count (CBC). We observed inflammation, started required medications and, we provided improvement in a short time. When patient was visited, he explained numbness, pain in hip area, muscle weakness in all extremities. Cervical MRI and nerve conduction tests were performed. MRI showed oedema in sciatic nerve and entrapment of obturator canal, resulted to obturator neuropathy and nerve conduction tests revealed GBS. He was treated with plasmapheresis and IVIG, he was discharged without neurological recovery. The patient consent was taken orally.

In conclusion, treatments decreased complaints and provided to walk short distances. Reinnervation signals were observed in nerve conduction test.

Keywords: Guillain-Barre Syndrome, Obturator Neuropathy, IVIG, Nerve Conduction Test

INTRODUCTION

Obturator neuropathy (ON) is an illness presenting with sensory loss, pain or weakness with leg adduction. Guillain-Barre Syndrome (GBS) has similar symptoms. The most determinative diagnostic parameters are nerve conduction in both diseases.[1,2] Therefore, GBS may also leads to ON whether it was diagnosed late. There have been lots of studies in literatures about these illnesses,

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separately. However, this is the first report where GBS and ON are seen in combination. Our objective is to show IVIG therapy is effective for the treatment of both GBS and ON diseases. This report mentions about on GBS combined with Obturator neuropathy. This combination is the first in the globe according to the literature. Additionally, there is an important data that IVIG therapy which is used to treat GBS disease, also improved Obturator neuropathy.

METHODOLOGY

A 51-year-old man was admitted with loss of consciousness and shortness of breath (SOB). On hospitalization, his family also explained about muscle weakness and pain while sitting and walking. After admission to intensive care unit (ICU) of hospital, intubation, fluid replacement therapy and mechanical ventilation were provided. His first complete CBC showed increased CRP (39,5 mg/L) with leukocytosis ($15,2*10^9$/L) and thrombocytosis ($663*10^3$). All these complaints made us think about an inflammatory disease. First, IV isotonic NaCl 50 mL for hydration, teicoplanin 400 mg IV 3x1 for possible sepsis, salbutamol nebulizer for SOB, paracetamol IV for pain, pregabalin NG for neuropathic pain and NG nutrition because of his unconsciousness were administered.

A few hours later patient was recovered. Next day, he explained loss of feel, SOB, weakness in his extremities and severe pain in his hip. In that condition, we decided to perform full motor and sensorial studies and cervical MRI. Clinical and electrodiagnostic findings showed GBS with axonal damage at ligaments that seen in Table 1.

Table 1. Clinical and electrodiagnostic findings

Motor nerve studies

Nerves and Record sites	Latency (ms)		Amplitude (mV)		NCV (m/sec)	
Median Nerve	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**
Wrist	4,1	3,8	6,1	3,2	-	-
Elbow	8,5	8,5	3,8	2,0	52,1	49,3
Ulnar Nerve						
Wrist		4,3		2,4		-
Elbow		9,3		1,9		66,3
Peroneal Nerve						
Ankle		4,0		3,2		-
Fibula head		9,6		3,1		50,1
Pop.fos.		11,9		2,8		42,9
Tibial Nerve						
Ankle		4,3		7,4		-
Pop.fos.		12,2		6,5		46,7

Sensory nerve studies

Nerve	Recod site	Stimulation Site	Latency (ms)		Amplitude (μV)		NCV (m/sec)	
Sural Nerve	**Wrist**	**Mid-calf**	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**
				2,9		10,7		48,3
Median/ Ulnar/ Radius	Wrist	Index finger		2,7		12,0		44,4
		5th digit		2,3		8,3		45,7

F-wave Studies

Nerve	Record site	Stimulation site	Latency (ms)		
			M wave	**F wave**	**F-M wave**
Right Median	APB	Wrist	4,08	30,83	26,75
Right Ulnar	ADM	Wrist	3,25	35,17	31,92
Right Tibial	AH	Ankle	3,83	51,00	47,17

ADM: Abductor digiti minimi; AH: Abductor hallucis; APB: Abductor pollicis brevis; NCV: Nerve conduction velocity; Pop.fos.: Popliteal fossa

Cervical MRI expressed common T2 signal increase in all muscles and fascial areas, but mainly at the obturator externa and interna. Furthermore, oedema was also observed in sciatic nerve (Figure 1).

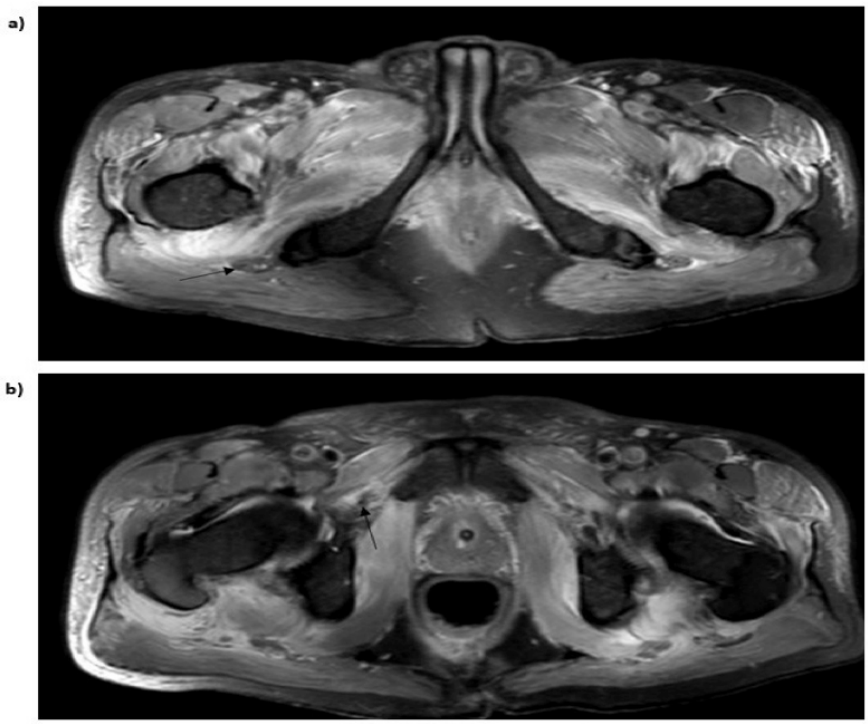

Figure 1. a) Entrapment of obturator channel; b) Oedema in sciatic nerve

We thought that these situations occurred as a secondary change to polyneuropathy associated with GBS. Immediately, plasmapheresis treatment was administered for 7 days. Plasmapheresis therapy resulted in clinical improvement and patient was discharged from hospital. However, due to insufficient neurological healing, he admitted rehabilitation programs. 3 weeks later, he came back with same complaints and then given 5 g x 1x5 vials/daily dose of IV immunoglobulin (IVIG) therapy. Motor and sensorial studies were repeated, and it was observed that motor abilities and other symptoms of patient became better. However, little progression was determined in axonal damage. After from 5-day-follow up process, he was discharged to home with his medications He used pregabalin for peripheral neuropathy, salbutamol inhaler for SOB and prednisolone for inflammation of obturator externa and interna. 5 months later he came back to neurology ward and he was controlled to see the progression. He was observed polyneuropathy with subacute axonal damage.

However, it was significantly better than before. Moreover, reinnervation potentials were also observed in the examination mainly in obturator nerves. Finally, oedema in sciatic nerve and inflammation of obturator joint was treated completely and he was able to walk short distances using 2 crutches.

RESULTS AND DISCUSSION

Obturator neuropathy (ON) is a severe and common illness. There are plenty of conditions that cause also lead to suppression of nerve and results in obstetrical ON.[3,4] Entrapment of nerve should be considered as cause of pain, loss of sense and weakness in muscle. Guillain-Barre syndrome (GBS) is a progressive and immune-mediated peripheral polyneuropathy characterized by motor and sensory weakness. Documentations reports estimated incidence of GBS for all ages combined vary from 0.16 to 3.0 per 100,000 person-years.[5] As we all know basic complaints of GBS is managed by use of IV immunoglobin therapy and plasmapheresis. Detailed anamnesis, physical examination and clinical findings are very important to recognise the illnesses correctly as soon as possible, because, the prognosis of both GBS and ON is dependent on early diagnosis.[6] Motor and sensory tests and MRI scanning should be done. Additionally, if it is possible, diagnosis should be supported by magnetic resonance neuroimaging (MRN).[7] In studies performed by Riva et al. and Van Asseldonk et al., IV immunoglobulin treatment healed motor axonal neuropathy transiently and marginally.[8, 9] We also observed the similar clinical ameliorations in our patient. Obturator neuropathy has similar symptoms; flaccid weakness of lower extremities, loss of sense and fulminant pain while sitting. To improve these, analgesics, pregabalin (for peripheral neuropathy) and physiotherapy are suggested.[10] Addition to these, it should be studied in future whether IV immunoglobulin may be beneficial in both GBS and ON treatment.

In conclusion, not only symptoms and diagnosis process, but also treatment of both diseases is very similar. In our report, patient has two separate polyneuropathic diseases. However, we diagnosed them early and began to treat. Although obturator joint inflammation and sciatic nerve oedema were treated successfully, obturator neuropathy was not recovered completely. Although this condition, patient could walk short distances.

On the other hand, unfortunately, GBS was not treated completely. However, reinnervation signals were observed at electrophysiological test.

This report is very interesting and important. Since according to our knowledge, obturator neuropathy combined GBS case was encountered for the first time and we showed that IVIG also heals obturator neuropathy beside GBS.

CONFLICT OF INTEREST

The authors have no potential conflict of interest relevant to this article.

Informed Consent: Verbal informed consent was obtained from patient and his parents who participated in this study.

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ETHICAL PUBLICATION STATEMENT

We confirm that we have read and understood the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. This study has ethical approval with the protocol number of 594/01.08.2019.

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Effect of Avocado *(Persea gratissima)* Leaf Extract on Calcium Oxalate Crystallization

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ABSTRACT

The aim of this study was to investigate the effect of avocado (*Persea gratissima*) leaf extract on calcium oxalate crystallization. For this purpose, calcium oxalate was synthesized in the absence and presence of avocado leaf extract media. The precipitated crystals were characterized by XRD, FTIR, SEM, TEM, zeta potential, and thermogravimetric analysis. The XRD results indicated that the calcium oxalate precipitated in pure media consisted of crystals in monohydrate form with monoclinic structure. The SEM and TEM images proved that the crystals underwent a morphological change in the extract media. The calcium oxalate crystals had a zeta potential of -3.2 mV and the surface of the crystals prepared in the extract media was more negative (-31.8 mV) when compared with crystals prepared in pure media. Thus, it can be concluded that the formation of calcium oxalate monohydrate crystal, a major component of urinary stones, is reduced, and the morphology and size of the crystals are changed by avocado leaf extract media.

Keywords: Calcium oxalate, crystallization, avocado leaves, morphology

INTRODUCTION

Kidney stones or nephrolithiasis is one of the most common urologic diseases worldwide and places a heavy burden on human health. The annual prevalence of kidney stones is estimated at 3–5% and the approximate lifetime prevalence is 15–25% worldwide[1]. Kidney stone formation is a biological process involving the crystallization of several urinary minerals. These urinary stones develop when the minerals in urine aggregate and grow instead of being dissolved and passed out of the body[2]. Kidney stones are predominantly (approximately 80%) composed of calcium oxalates and calcium phosphates, of which 70% are calcium oxalate stones[3]. Calcium oxalate exists in three forms—monohydrate (COM, $CaC_2H_4 \cdot H_2O$), dihydrate (COD, $CaC_2H_4 \cdot 2H_2O$), and trihydrate (COT,

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$CaC_2H_4 \cdot 3H_2O$). The most stable form under normal atmospheric conditions is calcium oxalate monohydrate. Specific solution conditions (e.g., supersaturation) and temperatures are required for the other forms[4-6]. Calcium oxalate monohydrate crystals are known to be the main component of stone formation in the urinary tract and kidneys. Kidney stones are a significant clinical problem so a large body of research is focused on their biomineralization, especially on preventing kidney stone formation by the use of various crystal growth modifiers[7-12]. As well as using chemical additives to reduce kidney stone formation, long-term treatment of kidney stones using herbs and plants is becoming more popular[6]. In this study, avocado leaf extract, a traditional, natural, harmless treatment was investigated as a crystal growth modifier. Avocado leaves are a natural ingredient used in traditional medicine as a diuretic, anti-inflammatory, antihypertensive, and antioxidant[13]. They contain a range of active compounds, including alkaloids, tannins, saponins, triterpenoids, and flavonoids. Avocado leaves have been demonstrated to effectively prevent calcium oxalate crystallization[14,15]. Thus, in this work, the researcher investigated the effect of avocado leaf extract on calcium oxalate crystallization. The crystalline structure and morphology of crystals prepared in solutions with and without avocado leaf extract were determined and compared via in vitro experiments.

METHODOLOGY

Analytical-grade calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium oxalate ($Na_2C_2O_4$) were obtained from Merck Company. Distilled water was used throughout the experiments.

The crystallization experiments were done in a cylindrical jacketed crystallizer with a working capacity of 0.5 L. The experimental set-up included a thermostat, pH control system, stirrer, peristaltic pump, and syringe pump. The temperature was controlled using a thermostat. During the crystallization experiments, the temperature and pH of the suspension were kept at 37 °C and 7.4. A three-blade stirrer was installed in the center of the crystallizer to ensure the desired stirring rate (400 rpm). In each experiment, 200 mL of $CaCl_2 \cdot 2H_2O$ was placed in the crystallizer and then left to reach thermal equilibrium. Afterwards, 200 ml of $Na_2C_2O_4$ solution was fed into the crystallizer using a peristaltic pump to produce calcium oxalate monohydrate crystals. During the crystallization process, the crystallizer was kept closed.

To evaluate the effect of avocado leaf extract on calcium oxalate crystals in vitro, a 5 wt% aqueous extract was used. Firstly, dried avocado leaves were ground to a fine powder, 2.5 g of the powder was added to 50 mL of boiling

distilled water, and the mixture was boiled for 5 min. The solution was allowed to cool to room temperature and then filtered. The prepared extracts were then utilized in the experiments and the extracts were added to the crystallizer via a syringe pump. At the end of the experiments, the suspension was removed from the crystallizer and a portion of the sample was dried. The remaining portion of the sample was subjected to aging at 37 °C at pH 7.4 for 2 months.

To characterize the calcium oxalate end-products, various techniques were used. X-ray diffraction measurements (XRD) were performed using a Bruker D2 Phaser tabletop diffractometer in the diffraction angle range of 2θ = 10–60°. The surface morphology of the calcium oxalate was explored using scanning electron microscopy (SEM; Zeiss EVO LS 10) and transmission electron microscopy (TEM, JEOL JEM 1220). To determine the functional groups in the crystals, a Fourier-transform infrared spectrometer (FTIR; Shimadzu IR Affinity-1) was used. The zeta potential of the sample was measured using a Malvern Zeta Sizer Nano Series Nano-ZS. Thermal analysis was performed under a nitrogen atmosphere using a Setaram LABSYS Evo thermogravimetric (TG) analyzer. The sample (15 ± 0.1 mg) was placed in a crucible and heated from 30 °C to 800 °C at a constant heating rate of 10 °C/min.

RESULTS AND DISCUSSION

Characterization of the Avocado Leaves

Prior to the crystallization experiments, the general properties of the avocado leaves were investigated by FTIR, XRD, SEM, and TG analysis. The FTIR spectrum shown in Figure 1 was collected in the range of 600–4000 cm^{-1} to identify the chemical structure of the avocado leaves.

The wide transmittance band at 3313.71 cm^{-1} represents the O–H stretching vibrations. The peaks at 2927.94 cm^{-1} and 2850.79 cm^{-1} were assigned to the asymmetric and symmetric vibrations of CH_2, respectively.

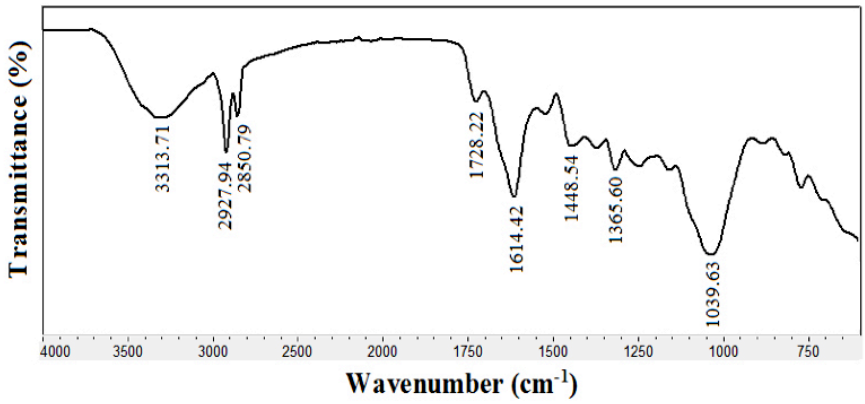


Figure 1. FTIR spectrum of the avocado leaves

The band at 1728.22 cm^{-1} was associated with the C=O stretching vibration in carboxylic acids, aldehydes, and ketones due to polyphenols. The strong band at 1614.42 cm^{-1} can be attributed to the C=C vibrations in the aromatics. The peaks shouldered at 1448.54 cm^{-1} and 1365.60 cm^{-1} are related to the CH_2 and CH_3 bending vibrations, respectively. A band at 1039.63 cm^{-1} indicated the presence of esters in the sample. The functional groups detected on the leaves indicated the presence of polyphenols and flavonoids[16].

XRD analysis was used to identify the structure of the avocado leaves and XRD result is given in Figure 2. As seen from the patterns, the intense peaks of sample were identified at 2θ of 29.4, 38.4, 44.5, 64.9 and 77.9°.

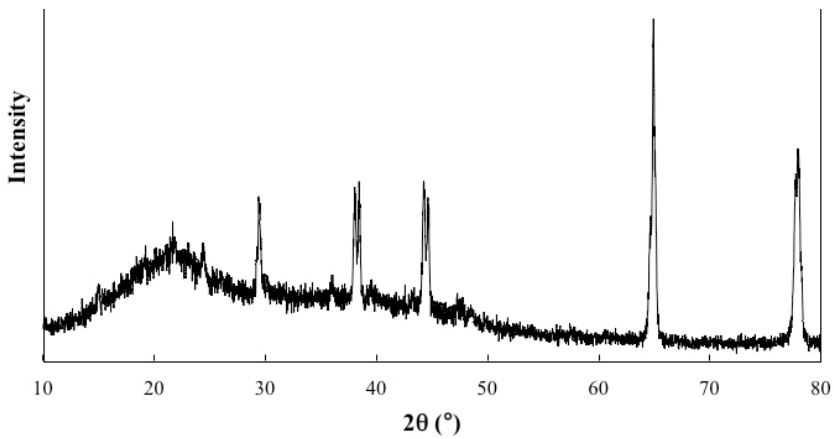


Figure 2. XRD spectrum of the avocado leaves

SEM analysis was used to determine the morphology of the avocado leaves. Figure 3 demonstrates that the surface of the leaves was heterogeneous with non-uniform sized and long cylindrical shaped particles.

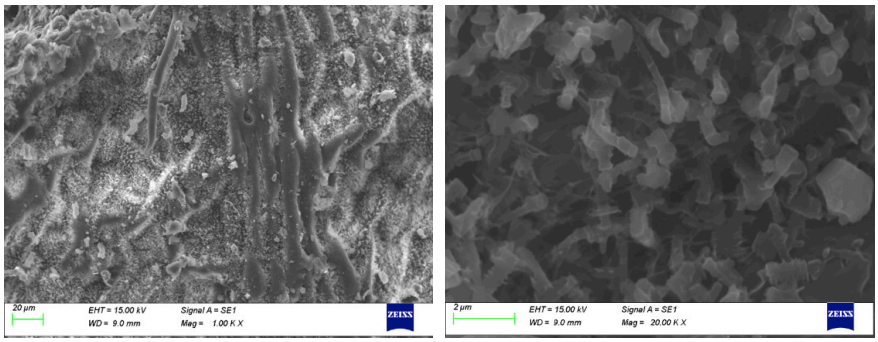


Figure 3. SEM image of the avocado leaves (1000X and 20000X)

The thermal structural evolution of the leaves was investigated by using a thermogravimetric analyzer. The TG and differential thermogravimetric (DTG) curves of the sample are shown in Figure 4.

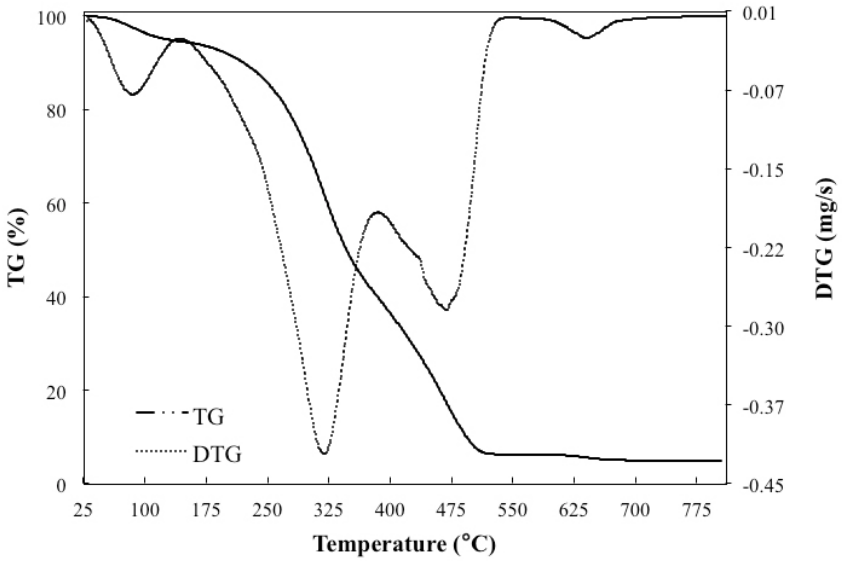


Figure 4. TG and DTG curves of the avocado leaves

The thermal degradation of the sample showed four weight-loss regions. The first stage in the temperature range from approximately 35 °C to 150 °C represents the evaporation of moisture content, whilst the second stage (165-395 °C) is related to the decomposition of hemicellulose, which is the main component of the leaves. The third (395-550 °C) and fourth (590-710 °C) steps were attributed to the decomposition of cellulose and lignin, respectively. Hemicellulose is thermally less stable than cellulose and lignin owing to its amorphous structure and so it starts decomposing readily in the lower temperature range. The cellulose decomposition requires higher temperature owing to the presence of strong hydrogen bonding. Lignin, being the most complex structure, is assumed to decompose slowly over a broad temperature range from ambient to 800 °C.

Structure Analysis

Figure 5 depicts the XRD patterns of the calcium oxalate crystals precipitated with and without the avocado leaf extract media. The sample crystallized in the absence of the extract was monohydrate form with no other crystalline phases nor phase transformations, and the major diffraction peaks of the sample were in agreement with JCPDS card no: 00–020-0231.

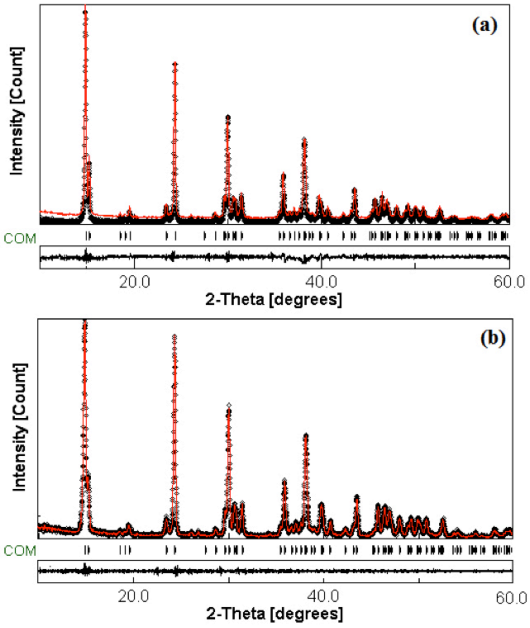


Figure 5. XRD patterns of calcium oxalate crystals precipitated in the absence (a) and the presence (b) of extract media

As can be seen from the patterns of the crystals, four dominant peaks at 2θ = 14.9, 24.5, 30.1, and 38.3° attributed to the (101), (020), (220), and (130) planes of the monoclinic structure were detected in each pattern, in agreement with the results of previous studies[17]. Materials Analysis Using Diffraction (MAUD) software was utilized to determine the unit cell parameters and detect the crystal system. The computed lattice parameters of the crystals precipitated in pure media were determined as a = 9.989 Å, b = 7.298 Å, c = 6.310 Å, and β = 107.0°, which were in agreement with the results of a previous study[18]. The crystal system was monoclinic with space group P21/n.

With avocado leaf extract added to crystallization media, the crystals were all in the monohydrate form as in the pure media. There was no change in the crystal structure of the calcium oxalate monohydrate crystals formed in the presence of the extract. However, differences in the intensities of the dominant peaks were observed in the extract media compared to those in pure media. The lattice parameters for the extract media were a = 10.004 Å, b = 7.309 Å, c = 6.307 Å, and β = 107.0°.

FTIR and Zeta Analysis

The FTIR spectra of the crystals precipitated with and without avocado leaf extract were collected for the determination of their functional groups. Figure 6 displays the FTIR spectra of the samples in the range of 600–4000 cm^{-1}.

The wide transmittance bands in the range of 3000–3500 cm^{-1} represent the asymmetric and symmetric O–H stretching of the water molecules. Two bands at approximately 1600 cm^{-1} and 1310 cm^{-1} denoted the presence of symmetrical and anti-symmetrical C=O stretching vibrations. The peaks at ~950 cm^{-1} and ~880 cm^{-1} were assigned to C–C stretching vibrations. The band at 659 cm^{-1} was associated with the bending and wagging modes of water[19,20]. Figure 6, it can be clearly seen that the extract introduced new peaks in the range of 1000–1150 cm^{-1}. Thus, the avocado leaf extract adsorbed on the calcium oxalate surface and affected the surface properties of the crystals. Therefore, in addition to FTIR analysis, zeta potential measurements were carried out to identify the surface charge of the samples.

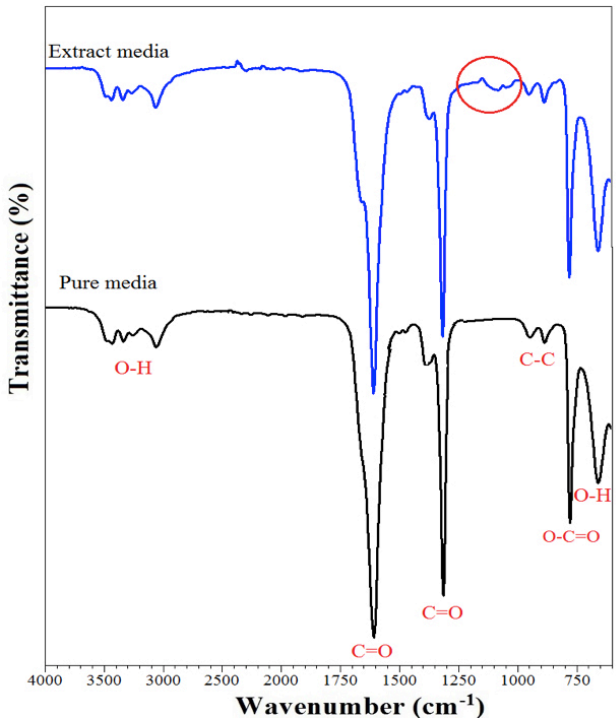


Figure 6. FTIR spectra of calcium oxalate crystals precipitated in the absence and the presence of extract media

The zeta potential profiles of the crystals versus time are shown in Figure 7. Zeta potential measurements showed that the calcium oxalate crystals precipitated in pure media had a surface charge of –3.2 mV.

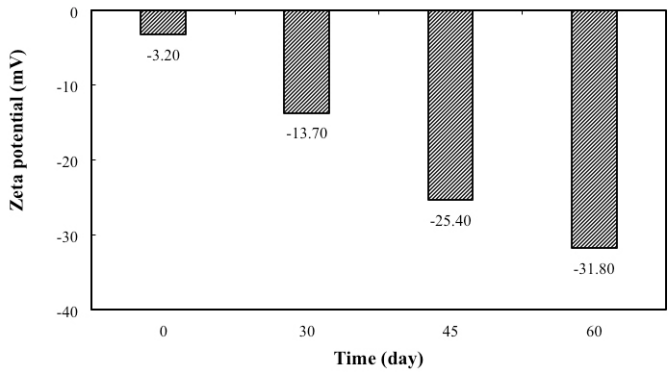


Figure 7. The zeta potential changes of calcium oxalate in the absence and the presence of extract media

The negativity of the zeta potential value increased significantly in the presence of the avocado leaf extract media with time, reaching –31.8 mV at the end of day 60.

The growth and agglomeration of urinary crystals are inhibited by a more negative crystal surface, in agreement with the literature[21]. Thus, with the increment of the surface charge negativity, the crystals aggregate less readily because of their high electrostatic repulsion and stability, which indicates the inhibitory effect of the avocado leaf extract on calcium oxalate and kidney stone formation. In other words, this extract shows potential for preventing kidney stone formation by inhibiting crystal formation. The results will make a useful contribution to advancing research efforts on kidney stone formation, a significant clinical issue and an important area of biomineralization research. This study provides guidance for future studies in this area as well as experimental evidence for the inhibition of pathological crystal formation in the urinary tract.

Morphology Analysis

Figure 8 shows SEM images of the calcium oxalate monohydrate crystals precipitated in pure media with the corresponding energy dispersive X-ray (EDX) spectra.

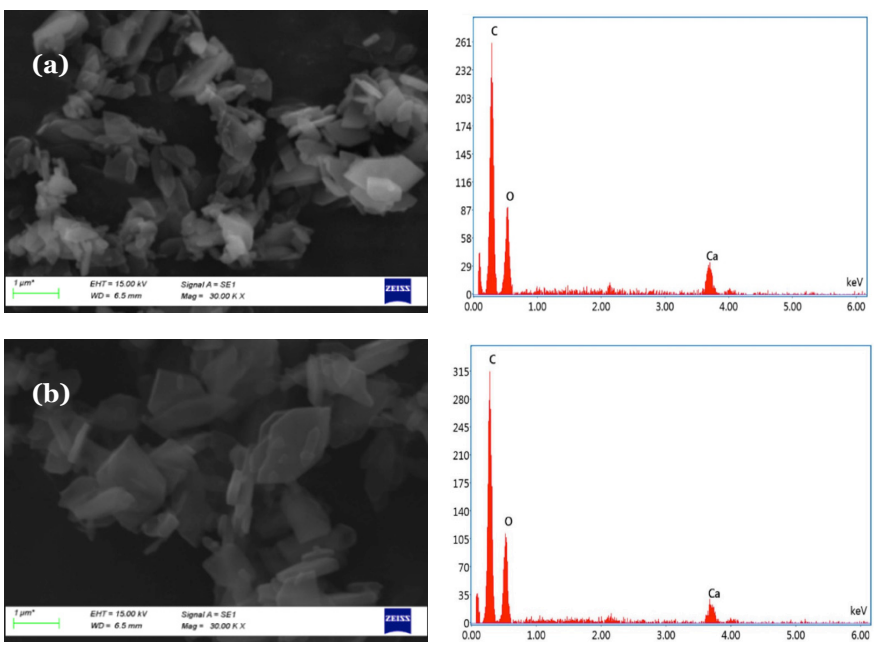
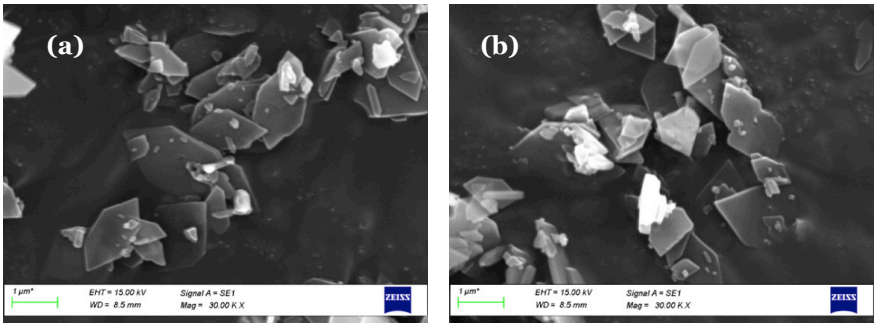


Figure 8. SEM images and EDX results of calcium oxalate crystals precipitated in pure media at t = 1 day (a) and 60 days (b)

The images were taken at different times. In Figure 8a, the crystals precipitated in pure media at the end of 1 day were in a hexagonal form and form robust aggregates on one another. The mean particle size of the crystals was 1.3 μm. In order to evaluate the morphological changes over time, the SEM image of the calcium oxalate monohydrate precipitated at the end of 1 day was compared to those of the crystals kept in solution for 2 months. The crystals preserved their aggregated form but grew bigger to a mean particle size of 2.1 μm. In both measurements, the surface of the crystals was smooth, and the crystals were similarly regular shaped and uniform sized. In addition, the elemental contents of the crystals were determined as Ca, C, and O, in good agreement with the literature[6].

Figure 9 displays the morphological features of the calcium oxalate crystals precipitated in the avocado leaf extract media at different times.

As shown in Figure 9, the surface properties and morphology of the crystals were directly related to the duration of the sample exposure to the extract media. It was observed that the crystals were still in hexagonal form after being exposed to the extract for 30 days, but they started to lose their aggregation tendency. The mean particle size of the crystals was 1.6 μm. Moreover, the precipitated crystals lost their smooth surfaces, started to deform, and surface nucleation occurred in the extract media. After 45 days, the nuclei formation on the crystal surface increased and these nuclei grew to form new crystal shapes. Additionally, the longer precipitation time further enhanced the breaking and deformation tendencies of the crystal surface. The mean particle size of the crystals was approximately 850 nm. After being exposed to the extract for 60 days, the surface became rougher, fractured, and more layered. The mean particle size continued to fall with time, being measured as 720 nm.



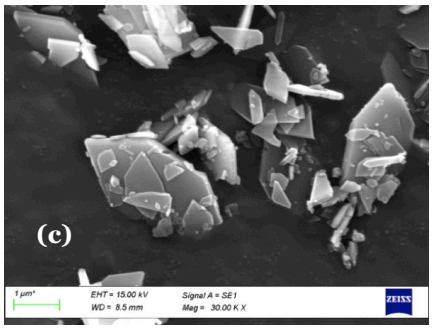


Figure 9. SEM images of calcium oxalate crystals precipitated in the presence of avocado leaf extract at t = 30 days (a), 45 days (b), and 60 days (c).

In order to detect these surface changes more precisely and to gain more insight into the effect of the extract on the crystal morphology, TEM images were obtained at the end of 60 days in pure media and in the presence of avocado leaf extract. The results are presented in Figure 10.

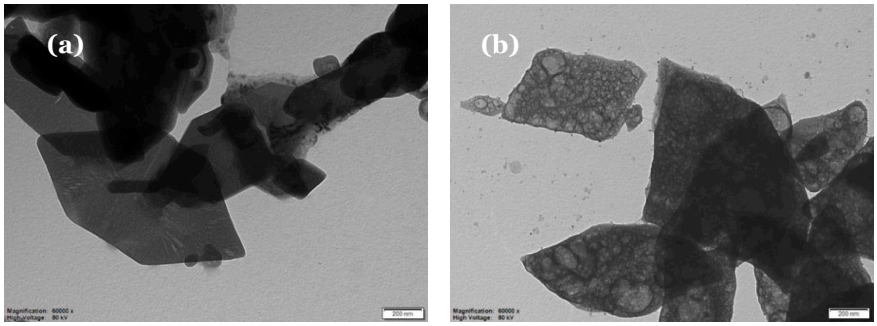


Figure 10. TEM images of calcium oxalate crystals precipitated in the absence (a) and presence (b) of avocado extract.

It can be clearly seen in Figure 10a that the surfaces of the crystals precipitated in pure media were smooth, in parallel with the SEM results, and there was no surface nucleation or defects. The calcium oxalate crystals were in a regular hexagonal shape, in agreement with the literature[22]. On the other hand, the crystals precipitated in avocado leaf extract media underwent both morphological changes and surface nucleation (Figure 10b). Furthermore, they completely lost their evenly distributed appearance.

From the evaluation of the SEM and TEM images together, it can be concluded

that the extract had a significant modifying effect on the calcium oxalate morphology. The regular hexagonal-shaped crystals with smooth surface precipitated in pure media transformed into weaker crystals with a deformed surface. It was also noted that the duration of exposure to the extract also had an effect on the morphology of the calcium oxalate crystals.

Thermal Analysis

The thermal decomposition behavior of the calcium oxalate crystals was investigated in this study. The TG and DTG curves of the crystals precipitated with and without the extract with a heating rate of 10 °C/min are shown in Figure 11. Thermal decomposition of the samples indicated three weight loss steps—dehydration, decarbonylation, and decarbonation—respectively.

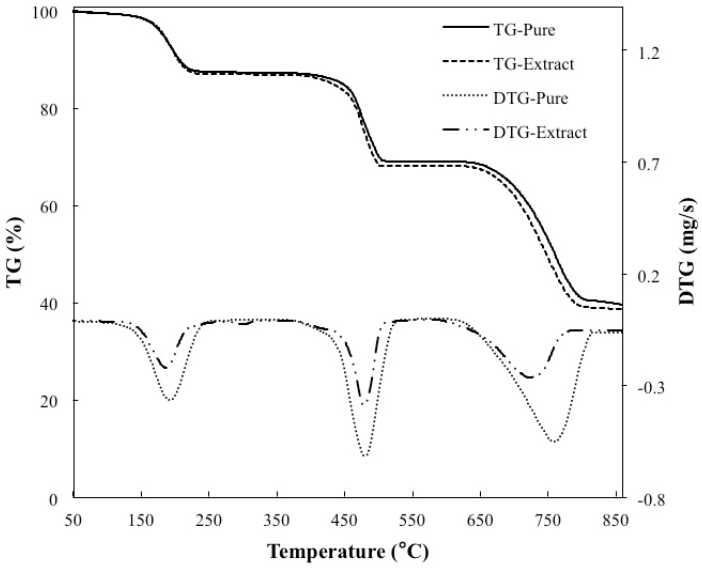


Figure 11. TG and DTG curves of calcium oxalate crystals precipitated in the absence (a) and presence (b) of avocado extract

A similar decomposition profile was also detected for the extract media. The weight loss of the crystals precipitated in pure media was 61.5%, which was in agreement with the literature[23]. Increment of 0.3%, were observed for struvite crystals prepared in avocado extract media, respectively. The increase in the weight loss was attributed to the decomposition of the traces of the extract remaining the sample.

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Synthesis and Investigations of Antimicrobial, Antioxidant Activities of Novel Di-[2-(3-alkyl/aryl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)-azomethinephenyl] Isophtalates and Mannich Base Derivatives

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ABSTRACT

In this study, the synthesis of di-[2-(3-alkyl/aryl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)-azomethinephenyl] isophtalates (**2a-g**) from the reactions of 3-alkyl/aryl-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**1a-g**) with di-(2-formylphenyl) isophtalate is described. Then, the compounds **2** were treated with morpholine in the presence of formaldehyde to synthesize di-{2-[1-(morpholine-4-yl-methyl)-3-alkyl(aryl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]-azomethinephenyl} isophtalates (**3a-g**). The newly synthesized compounds were characterized using IR, ^{1}H-NMR and ^{13}C-NMR spectral data. In addition, the compounds synthesized were screened for their antimicrobial activities. Furthermore, the antioxidant properties of the newly synthesized compounds were analysed for their in-vitro potential antioxidant activities in three different methods (reducing power, free radical scavenging and metal chelating activity). These antioxidant activities were compared to those from standard antioxidants, such as BHA, BHT, EDTA and α-tocopherol.

Keywords: Schiff base, Mannich base, Antimicrobial activity, Antioxidant activity.

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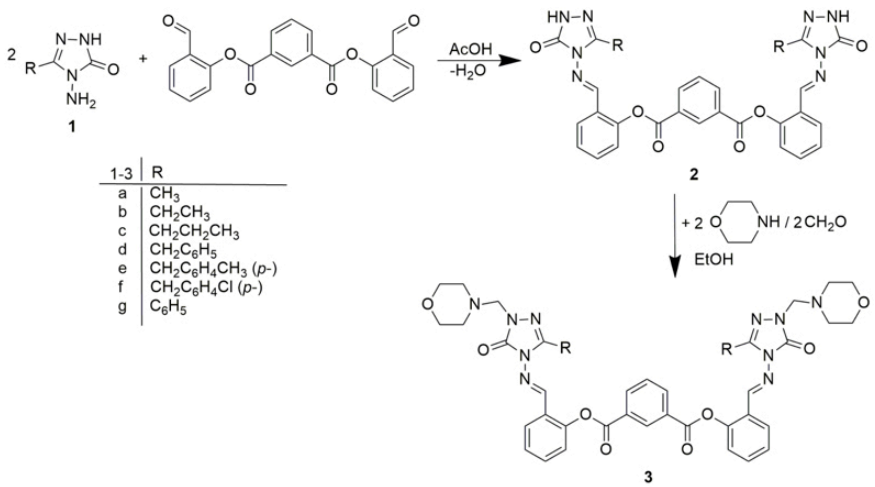
INTRODUCTION

A large number of heterocyclic compounds containing the 1,2,4-triazole ring, are associated with diverse biological properties such as antioxidant, anti-convulsant, anti inflammatory, antimicrobial and anti-viral activity. Mannich bases have applications the field medicinal chemistry, the product synthetic polymers, the petroleum industry, as products used in water treatment, cosmetics, the dyes industry, etc [1]. In addition, Mannich bases have biological activity such as anticancer [2,3], antibacterial [4–6], antimycobacterial [7–9], anti inflammatory [10–12], analgesic [13,14], antifungal [15,16], antitumor [17,18]namely the 1-aryl-2-dimethylaminomethyl-2-propen-1-one hydrochlorides 1a-e and 1-aryl-3-dimethylamino-2-hydroxymethyl-1-propanone hydrochlorides 2a-e. A number of these compounds possess marked cytotoxic potencies (IC(50, antiviral [19–21], antidepressant [22,23], antiulcer [24], anticonvulsant [25], antimalarial [26,27] and antioxidant activities [28].

Antioxidants were defended organisms and cells from damage induced by oxidative stress. Thus, significant research has been ruled to investigate this feature. Scientists have dealt with the new compounds in recent years. Natural sources provide the effective components forestall or decrease the influence of oxidative stress on cells have been used [29]. Exogenous chemicals and endogenous metabolic steps in human body or in food system might produce highly reactive free radicals, particularly oxygen provided radicals, which are capable of oxidizing biomolecules, resulting in cell death and issue damage. Oxidative damages play a considerable pathological role in human diseases. It has been an important pathological effect of oxidative damage in human disease. For example, cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative injury. In addition to, excessive generation of ROS (reactive oxygen species) induced by various stimuli and which exceeds the antioxidant capacity of the organism leads to a diversity of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer [30]. In this paper, in order to define antioxidant activity of the synthesized Mannich Bases were researched different antioxidant method; iron binding effect, reducing power and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity [31]. Furthermore, the antimicrobial activities of novel Mannich bases were investigated with agar well diffusion method [32]. In the present paper, the starting materials (**1a-g**) were synthesized from the reactions of the corresponding ester ethoxycarbonylhydrazones with an aqueous solution of hydrazine hydrate [33,34] and di-[2-(3-alkyl/aryl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)-azomethinephenyl] isophtalates (**2a-g**) were obtained by the reactions of compounds (**1a-g**) with di-(2-formylphenyl) isophtalate. Then, the

compounds **2** reacted with formaldehyde and morpholine to afford di-{2-[1-(morpholine-4-yl-methyl)-3-alkyl(aryl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]-azomethinephenyl} isophtalates (**3a-g**) (**Scheme 1**).



Scheme 1. The synthetic pathway of the compounds **2** and **3**.

METHODOLOGY

Synthesis

*Preparation of Compounds **2a-g**:* 3-Alkyl/Aryl-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**1**) (0.01 mol) was dissolved in acetic acid (20 mL) and treated with di-(2-formylphenyl) isophtalate (0.01 mol). The mixture was refluxed for 1.5 hours and then evaporated at 50-55°C *in vacuo*. Several recrystallizations of the residue from ethanol gave pure compound di-[2-(3-alkyl/aryl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)-azomethinephenyl] isophtalate (**2**) as white colour crystals.

*Preparation of Compounds **3a-g**:* Compound **2** (5 mmol) was dissolved absolute ethanol and to this solution were added to formaldehyde (% 37, 10 mmol) and morpholine (6 mmol). The reaction mixture was refluxed for 4 hours and filtered. The crude product was recrystallized from ethanol.

Physical data of the new compounds (**2a-g** and **3a-g**) are presented in Table **1**. IR, ^{1}H-NMR and ^{13}C-NMR spectral data are given in Tables **2-6**.

Table 1. Physical data of the compounds **2** and **3**

Compounds	2a	2b	2c	2d	2e	2f	2g	3a	3b	3c	3d	3e	3f	3g
% Yield	99	98	90	96	97	96	90	92	91	91	94	90	92	90
Melting Point (°C)	271	233	226	273	260	243	251	224	183	165	176	158	155	206

Table 2. IR data of the compounds **2** and **3** (cm^{-1}).

Compounds	n_{NH}	$n_{C=O}$	$n_{C=N}$	n_{COO}	$n_{1,4\text{-disubstituted benzenoid ring}}$	$n_{1,3\text{-disubstituted benzenoid ring}}$	$n_{1,2\text{-disubstituted benzenoid ring}}$	$n_{monosubstituted benzenoid ring}$
2a	3191	1744, 1713	1604	1208	-	871 and 789	754	-
2b	3188	1739, 1705	1598	1203	-	902 and 800	761	-
2c	3180	1710	1598	1207	-	902 and 824	752	-
2d	3183	1709	1596	1202	-	904 and 819	752	752 and 694
2e	3173	1745, 1705	1598	1200	829	903 and 796	755	-
2f	3185	1740, 1705	1598	1201	821	903 and 792	749	-
2g	3180	1705	1603	1203	-	905 and 802	756	-
3a	-	1742, 1704	1597	1215	-	856 and 768	768	-
3b	-	1742, 1700	1593	1204	-	897 and 765	765	-
3c	-	1744, 1700	1591	1205	-	897 and 765	757	-
3d	-	1742, 1700	1590	1207	-	904 and 762	762	762 and 709
3e	-	1746, 1701	1596	1206	841	904 and 790	757	-

3f	-	1744, 1701	1598	1211	820	907 and 801	745	-
3g	-	1740, 1697	1604	1207	-	896 and 800	766	766 and 687

Table 3. ^{1}H-NMR data of the compounds **2** (DMSO-d_6, δ/ppm)

Compounds	$2CH_3$	$2CH_2$	$2PhCH_3$	$2CH_2$	Aromatic H	2N=CH	2NH
2a	2.10(s)	-	-	-	7.48-7.53(m,4H), 7.66(td,2H,J=8.00,1.60 Hz), 7.90(t,1H,J=8.80 Hz), 8.04(dd,2H,J=8.00 Hz), 8.54(dd,2H,J=8.00,1.60 Hz),8.84(t,1H,J=1.60 Hz)	9.93(s)	11.75(s)
2b	1.09 (t,J= 7.60Hz)	-	-	2.47(q, J=7.60 Hz)	7.48-7.52(m,4H), 7.66(td,2H,J=8.40,1.60 Hz), 7.91(t,1H,J=8.00 Hz), 8.02(d,2H,J=8.00 Hz), 8.55(dd,2H,J=8.00,2.00 Hz), 8.85(s,1H)	9.93(s)	11.75(s)
2c	0.85 (t,J= 7.20Hz)	1.57(sext, J=7.20 Hz)	-	2.43(t,J= 7.20 Hz)	7.49-7.53(m,4H), 7.67(t,2H,J=8.00 Hz), 7.91 (t,1H,J=8.00 Hz), 8.02(dd,2H,J=8.00,1.20 Hz), 8.55(dd,2H,J=8.00,1.60 Hz), 8.85(s,1H)	9.92(s)	11.79(s)
2d	-	-	-	3.92(s)	7.19-7.31(m,10H), 7.47-7.50(m,4H), 7.65(td,2H,J=8.00,1.60 Hz), 7.86(t,1H,J=8.00 Hz), 7.99(d,2H,J=8.00 Hz), 8.51(dd,2H,J=8.00,1.60 Hz), 8.82(s,1H)	9.91(s)	11.91(s)
2e	-	-	2.23(s)	3.86(s)	7.08(d,4H,J=8.00 Hz), 7.14(d,4H,J=8.00 Hz), 7.46-7.51 (m,4H), 7.65(td,2H,J=8.00,1.60 Hz), 7.86(t,1H,J=8.00 Hz), 8.00(dd,2H,J=7.60,1.20 Hz), 8.51(dd,2H,J=8.00,1.60 Hz), 8.83(s,1H)	9.90(s)	12.00(s)
2f	-	-	-	3.93(s)	7.27(d,4H,J=8.40 Hz), 7.35(d,4H,J=8.40 Hz), 7.47-7.51(m,4H), 7.66(t,2H,J=8.00 Hz), 7.86(t,1H,J=8.00 Hz), 7.99(d,2H,J=7.60 Hz), 8.51(dd,2H,J=8.00,1.60Hz), 8.82(s,1H)	9.92(s)	11.93(s)
2g	-	-	-	-	7.48-7.55(m,10H), 7.67-7.82(m,7H), 7.99(dd,2H,J=8.00,1.20 Hz), 8.40(dd,2H,J=7.60,1.60 Hz), 8.68(s,1H)	9.88(s)	12.32(s)

Table 4. ^{13}C-NMR data of the compounds **2** (DMSO-d_6, δ/ppm)

Comp.	2COO	2Triazole C_5	2N=CH	2Triazole C_3	Aromatic C	C3-Aromatik C	Aliphatic C
2a	163.54	149.51	148.91	144.09	123.64(2CH), 126.01(2C), 126.95(2CH), 127.70(2CH), 129.42(2C), 130.16(CH), 131.00(CH), 132.54(2CH), 135.22(2CH), 151.16(2C)	-	10.85($2CH_3$)
2b	163.54	149.46	149.09	147.86	123.66(2CH), 126.03(2C), 126.98(2CH), 127.86(2CH), 129.42(2C), 130.14(CH), 131.01(CH), 132.53(2CH), 135.21(2CH), 151.30(2C)	-	9.87($2CH_2\underline{CH_3}$), 18.31($2\underline{CH_2}CH_3$)
2c	163.53	149.45	149.23	146.71	123.64(2CH), 126.03(2C), 127.00(2CH), 127.87(2CH), 129.44(2C), 130.19(CH), 131.02(CH), 132.54(2CH), 135.21(2CH), 151.23(2C)	-	13.31($2CH_2CH_2\underline{CH_3}$), 18.65 ($2CH_2\underline{CH_2}CH_3$), 26.46($2\underline{CH_2}CH_2CH_3$)
2d	163.51	149.68	148.46	146.09	123.54(2CH), 125.97(2C), 126.67(2CH), 127.12(2CH), 129.32(2C), 130.13(CH), 131.03(CH), 132.59(2CH), 135.20(2CH), 151.10(2C)	126.94(2CH), 128.37(4CH), 128.72(4CH), 135.58(2C)	30.82($2CH_2Ph$)
2e	163.50	149.66	148.43	146.23	123.52(2CH), 125.98(2C), 126.93(2CH), 127.14(2CH), 129.32(2C), 130.11(CH), 131.03(CH), 132.56(2CH), 135.18(2CH), 151.17(2C)	128.58(4CH), 128.94(4CH), 132.46(2C), 135.74(2C)	20.55($2PhCH_3$), 30.43($2CH_2Ph$)

2f	163.49	149.67	148.59	145.75	123.35(2CH), 125.92(2C),126.96 (2CH), 127.21(2CH), 129.32(2C), 130.13(CH), 131.00(CH), 132.62(2CH), 135.19(2CH), 151.14(2C)	128.31(4CH), 130.65(4CH), 131.43(2C), 134.53(2C)	30.14($2CH_2$Ph)
2g	163.51	151.47	149.80	144.64	123.69(2CH), 125.88(2C),127.01 (2CH), 127.44(2CH), 129.24(2C), 129.82(CH), 131.02(CH), 132.78(2CH), 134.95(2CH), 151.29(2C)	126.42(2C), 127.89(4CH), 128.42(4CH), 130.04(2CH)	-

Table 5. ^{1}H-NMR data of the compounds **3** (DMSO-d_6, δ/ppm)

Comp.	$2CH_3$	$2CH_2$	$2CH_2$	$2CH_2NCH_2$	$2CH_2OCH_2$	$2NCH_2N$	Aromatic H	2N=CH
3a	2.11(s)	-	-	2.54(m)	3.48(m)	4.38(s)	7.49-7.53(m,4H), 7.67(td,2H, J=8.40,1.60 Hz), 7.91(t,1H, J=8.00 Hz), 8.05(d,2H, J=7.60 Hz), 8.54(dd,2H,J=8.00,1.60 Hz), 8.87(s,1H)	9.93 (s)
3b	1.09(t, J=7.60 Hz)	2.50(q,J= 7.60 Hz)	-	2.55(m)	3.49(m)	4.40(s)	7.49-7.53(m,4H), 7.67(t,2H,J =8.00 Hz), 7.92(t,1H,J=8.00 Hz), 8.03(d, 2H,J = 7.60 Hz), 8.55 (d,2H, J =7.60 Hz), 8.87(s,1H)	9.92 (s)
3c	0.85(t, J=7.20 Hz)	1.57 (sext,J = 7.20 Hz)	2.48(t,J = 7.20 Hz)	2.55(m)	3.49(t, J = 4.40 Hz)	4.41(s)	7.50-7.53(m,4H), 7.65-7.68(m,2H), 7.92(t,1H,J=8.00 Hz), 8.00-8.05(m,2H), 8.54(dd,2H,J=8.00,1.60 Hz), 8.87(d,1H,J=1.60 Hz)	9.92 (s)
3d	-	-	3.95(s)	2.48(t,J = 4.40 Hz)	3.47 (m)	4.42(s)	7.18-7.31(m,10H), 7.47-7.51(m,4H), 7.66(td,2H,J=7.60,1.20 Hz), 7.85(t,1H, J=8.00 Hz), 7.99(d,2H, J=7.60 Hz), 8.49(d,2H,J=7.60,1.60 Hz), 8.83(s,1H)	9.89 (s)

3e	2.22 (s)	-	3.90(s)	2.46(t,J = 4.40 Hz)	3.47(t,J = 4.40 Hz)	4.41(s)	7.07-7.14(m,8H), 7.46-7.51(m,4H), 7.66(td,2H,J =7.60,1.60 Hz), 7.85(t,1H, J =7.60 Hz), 8.00(dd,2H,J=8.00,1.60 Hz), 8.49(dd,2H,J=8.00,1.60 Hz), 8.83(m,1H)	9.88 (s)
3f	-	-	3.96(s)	2.46(t,J = 4.40 Hz)	3.47(t,J = 4.40 Hz)	4.41(s)	7.27(d,4H,J=8.40Hz), 7.35(d,4H, J=8.40 Hz), 7.47-7.49(m,4H), 7.64-7.69(m,2H), 7.85(t,1H,J=8.00 Hz), 7.97-8.01(m,2H), 8.49(dd,2H,J=7.60, 1.60 Hz), 8.83(m,1H)	9.91 (s)
3g	-	-	-	2.54(m)	3.49(t,J = 4.40 Hz)	4.52(s)	7.47-7.55(m,12H), 7.70 (td,2H,J =8.40, 1.60 Hz), 7.76-7.82(m,3H), 7.99(dd,2H,J=8.00,1.60 Hz), 8.38(dd,2H,J=8.00,1.60 Hz), 8.71(m,1H)	9.88(s)

Table 6. ^{13}C-NMR data of the compounds **3** (DMSO-d_6, δ/ppm)

Comp.	2COO	a	2N=CH	b	Aromatic C	C3-Aromatik C	c	d	e	Aliphatic C
3a	163.53	149.53	149.18	142.97	123.66(2CH), 125.84(2C), 126.97(2CH), 127.90(2CH), 129.48(2C), 130.23(CH), 130.97(CH), 132.67(2CH), 135.29(2CH), 150.15(2C)	-	65.96	65.79	49.86	10.81 ($2CH_3$)
3b	163.53	149.50	149.35	146.68	123.67(2CH), 125.86(2C), 127.00(2CH), 127.99(2CH), 129.48(2C), 130.21(CH), 130.97(CH), 132.67(2CH), 135.28(2CH), 150.28(2C)	-	65.96	65.82	49.88	9.88 ($2CH_2CH_3$), 18.20 ($2CH_2CH_3$)

3c	163.52	149.48	149.40	145.47	123.66(2CH), 125.85(2C), 127.02(2CH), 127.97(2CH), 129.49(2C), 130.25(CH), 130.98(CH), 132.68(2CH), 135.28(2CH), 150.22(2C)	-	65.97	65.77	49.88	13.30 ($2CH_2CH_2CH_3$), 18.67($2CH_2CH_2CH_3$), 26.25 ($2CH_2CH_2CH_3$)
3d	163.50	149.71	148.75	144.81	123.56(2CH), 125.81(2C), 126.75(2CH), 127.25(2CH), 129.36(2C), 130.19(CH), 130.96(CH), 132.72(2CH), 135.25(2CH), 150.16(2C)	126.96(2CH), 128.44(4CH), 128.68(4CH), 135.44(2C)	65.96	65.96	49.88	30.61 ($2CH_2Ph$)
3e	163.50	149.70	148.75	144.96	123.55(2CH), 125.97(2C), 126.96(2CH), 127.16(2CH), 129.36(2C), 130.18(CH), 130.96(CH), 132.56(2CH), 135.14(2CH), 150.15(2C)	128.55(4CH), 129.01(4CH), 132.30(2C), 135.83(2C)	65.95	65.89	49.88	20.55 ($2PhCH_3$), 30.37 ($2CH_2Ph$)
3f	163.53	149.70	148.88	144.50	123.57(2CH), 125.76(2C), 126.97(2CH), 127.34(2CH), 129.36 (2C), 130.18(CH), 130.98(CH), 132.76(2CH), 135.24(2CH), 150.15(2C)	128.38(4CH), 130.62(4CH), 131.49(2C), 134.39(2C)	65.95	65.95	49.85	30.09 ($2CH_2Ph$)
3g	163.54	151.72	149.84	143.31	123.69(2CH), 125.69(2C), 127.01(2CH), 127.51(2CH), 129.30(2C), 129.86(CH), 130.99(CH), 132.88(2CH), 134.98(2CH), 150.36(2C)	125.90(2C), 127.87(4CH), 128.46(4CH), 130.03(2CH)	66.29	65.97	49.86	-

a) *2Triazole C_5,* ***b)*** *2Triazole C_3,* ***c)*** *$2CH_2OCH_2$,* ***d)*** *$2NCH_2N$,* ***e)*** *$2CH_2NCH_2$*

Biological Methods

Antioxidant Activity

Trichloroacetic acid (TCA), α-tocopherol, butylated hydroxyanisole (BHA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-pyridyl)-5,6-bis(phenylsulfonic acid)-1,2,4-triazine (ferrozine) and ferrous chloride, were acquired from E. Merck. and Sigma–Aldrich.

Reducing Power

The reducing power of the compounds **2a-g** and **3a-g** were determined using the method of Oyaizu[35] as explained in the literature [31] and were shown in Table 8.

Table 7. The reducing power method

Reagents	S_1	S_2	S_3	N_1	N_2	N_3	Blank
Compound	-	-	-	100μL	250μL	500μL	-
Standard	100μL	250μL	500μL	-	-	-	-
Phosphate Buffer	2,4 mL	2,25 mL	2,0 mL	2,4 mL	2,25 mL	2,0 mL	-
$K_3Fe(CN)_6$	2,5 mL	2,5 mL	2,5 mL	2,5 mL	2,5 mL	2,5 mL	-

Free Radical Scavenging Activity

Free radical scavenging effect of the compounds **2a-g** and **3a-g** were estimated by DPPH·, by the method of Blois [36] as explained in the literature [31] and were summarized in Table 8.

Table 8. The free radical scavenging effect method

Reagents	S_1	S_2	S_3	N_1	N_2	N_3	Blank	Control
Compound	-	-	-	50μL	100μL	150μL	-	-
Standard	50μL	100μL	150μL	-	-	-	-	-
Ethyl Alcohol	2,95 mL	2,90 mL	2,85 mL	2,95 mL	2,90 mL	2,85 mL	-	3 mL
DPPH	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	4 mL	1 mL

Metal Chelating Activity

The chelating of ferrous ions by the compounds **2a-g** and **3a-g** and references were measured according to the method of Dinis et al. [37] as explained in the literature [31] (Table 9).

Table 9. The metal chelating activity method

Reagents	S_1	S_2	S_3	N_1	N_2	N_3	Blank	Control
Compound	-	-	-	30μL	60μL	90μL	-	-
Standard	30μL	60μL	90μL	-	-	-	-	-
Ethyl Alcohol	3,75 mL	3,75 mL	3,75 mL	3,75 mL	3,75 mL	3,75 mL	3,75 mL	3,75 mL
$FeCl_2 4H_2O$	0,05 mL	0,05 mL	0,05 mL	0,05 mL	0,05 mL	0,05 mL	0,05 mL	0,05 mL
Ferrozine	0,2 mL	0,2 mL	0,2 mL	0,2 mL	0,2 mL	0,2 mL	0,2 mL	0,2 mL

Antimicrobial Activity

Antimicrobial activities of **2a-g** and **3a-g** compounds were investigated simple susceptibility screening test using agar-well diffusion method [32] as adapted earlier [38]. All microorganisms present in the test were provided from the Microbiologic Environmental Protection Laboratories Company in France. These microorganisms: *Klebsiella pneumoniae* ATCC4352, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC259222, *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC11774, *Bacillus cereus* ATCC11778.

RESULTS AND DISCUSSION

The synthesized seven new Schiff bases and seven new Mannich bases were identified using IR, ^{1}H-NMR, ^{13}C-NMR spectral data.

Antioxidant Acivity

In vitro antioxidant activities of fourteen new compounds **2a-g** and **3a-g** were investigated. Antioxidant activities were determined by the methods showed below.

Total reductive capability using the potassium ferricyanide reduction method

The reducing power of the compounds **2a-g** and **3a-g** was determined as described in [39, 40]. All compounds in different amounts showed lower absorption rates in this study than the standard compounds. Consequently, no activity was observed with respect to the reduction of metal ion complexes to their lower oxidation states or their involvement in an electron transfer reaction. In summary, synthesized compounds were not involved in reductive activities as seen in Figures **1** and **2**.

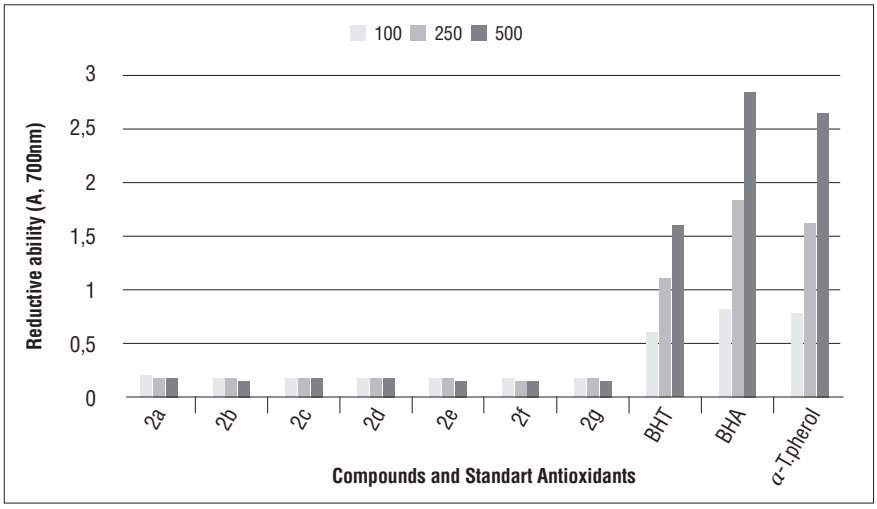


Figure 1. Total reductive potential of different concentrations of the compounds **2**, BHT, BHA and α-tocopherol.

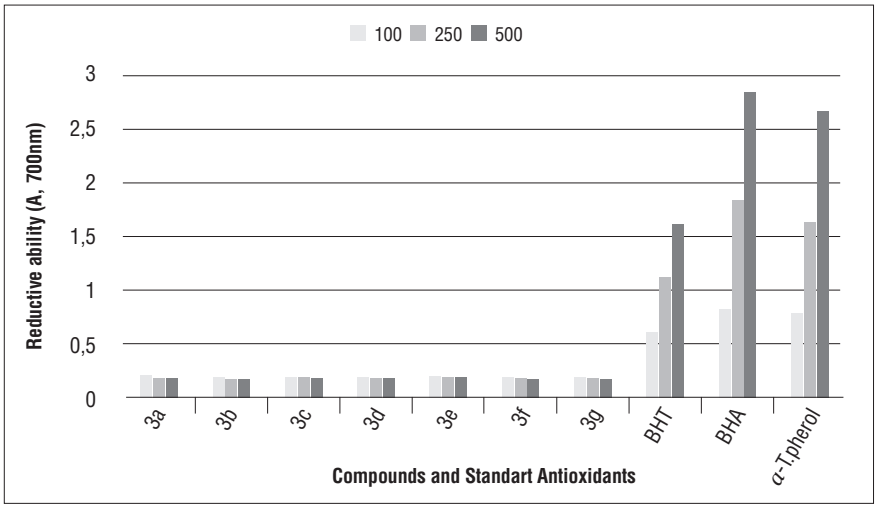


Figure 2. Total reductive potential of different concentrations of the compounds **3**, BHT, BHA and α-tocopherol.

DPPH· radical scavenging activity

The scavenging effect of compounds **2a-g** and **3a-g** was estimated by DPPH as explained in [41-43]. The DPPH method was used to determine the antiradical activity of compounds and standard antioxidants such as BHA, BHT and α-tocopherol in the study. It has been found that recently synthesized compounds have no activity as radical scavengers as shown in Figures **3** and **4**.

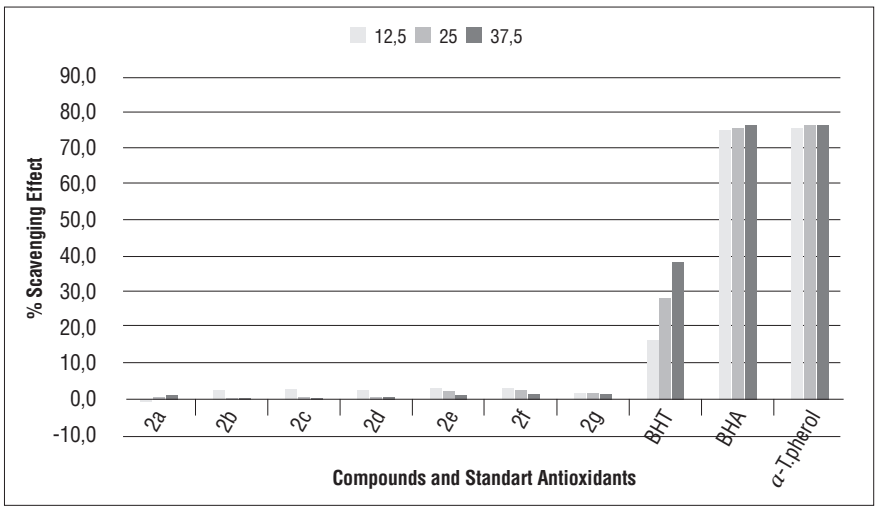


Figure 3. Scavenging effect of the compounds **2**, BHT, BHA and α-tocopherol at different concentrations (12.5–25–37.5 µg/mL).

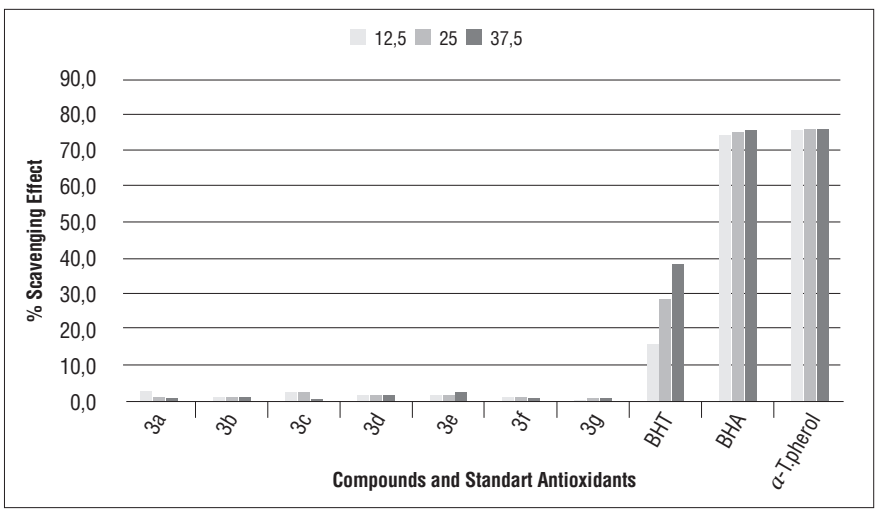


Figure 4. Scavenging effect of the compounds **3**, BHT, BHA and α-tocopherol at different concentrations (12.5–25–37.5 µg/mL).

Ferrous ion chelating activity

The chelation effect against iron ions by the compounds and standards was determined. Ferrozin can form complexes quantitatively with Fe^{2+}. In the presence of chelating agents, the complex formation is disturbed, so that the red colour of the complex decreases. The measurement of colour reduction thus allows the estimation of the chelating activity of the coexisting chelator [44]. Iron ion chelating activities of the compounds **2**, **3**, EDTA and α-tocopherol are shown in Figures **5** and **6**, respectively.

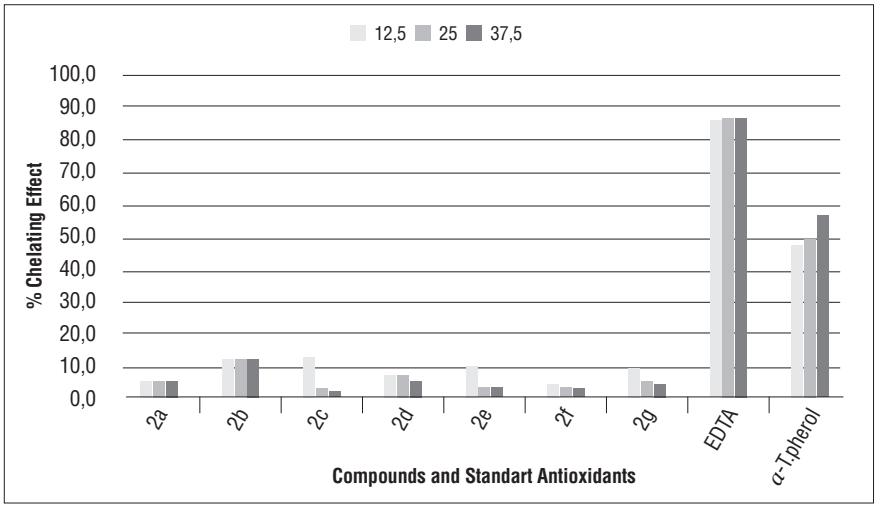


Figure 5. Metal chelating effect of different amount of the compounds **2**, EDTA and α-tocopherol on ferrous ions.

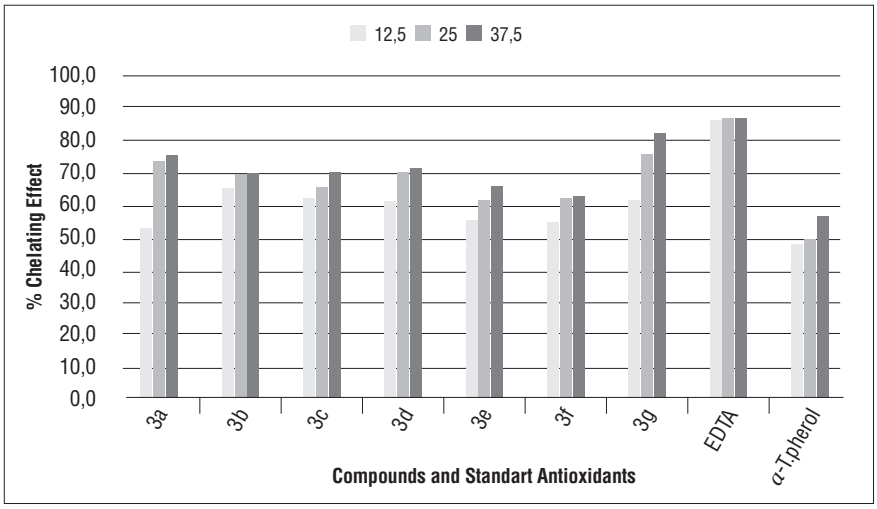


Figure 6. Metal chelating effect of different amount of the compounds **3**, EDTA and α-tocopherol on ferrous ions.

The high metal-chelating activity refers to a low absorption level at 562 nm. From the data in Figure 3, it can be deduced that the metal-chelating effects of compounds are concentration-dependent. As a result, compounds with significant iron-binding capacities can prove that their action as peroxidation inhibitors stems from their iron-binding capacities. The order of metal chelation of compounds and standards decreases when EDTA > **3g** > **3a** > **3d** > **3b** ≈ **3c** > **3e** > **3f** > α-tocopherol. Despite the low solubility rate for free iron, chelated iron complexes are known to have higher solubility rates in solutions that can be readily attributed to the ligand. Due to their potential involvement in iron-catalysed reactions, compound iron complexes could also be active.

Antimicrobial Activity

The antimicrobial activity of the compounds **2** and **3** were investigated. The results are shown in Table **10** and **11**.

Table 10. Zone diameters for antimicrobial activity of the **2,3** and Standart compounds

Compound	Microorganisms and Inhibition Zone (mm)					
	K.p.	P.a.	E.c.	S.a.	B.s.	B.c.
2a	-	-	-	-	-	-
2b	-	-	-	-	-	-
2c	-	-	-	-	-	-
2d	-	-	-	-	-	-
2e	-	-	-	-	-	-
2f	-	-	-	-	-	-
2g	-	-	-	-	-	-
3a	12	16	19	10	-	-
3b	13	17	13	12	-	-
3c	17	14	17	17	-	-
3d	15	13	17	15	-	-
3e	15	12	16	14	-	-
3f	12	11	15	11	-	-
3g	23	21	22	24	-	-
Ampicillin (X3261)	35	36	34	37	33	36
Neomycin (X3385)	16	17	16	13	17	17
Step-tomycin (X3385)	11	12	10	21	12	12

Table 11. Screening for antimicrobial activity of the compounds **2** and **3**.

Compound	Microorganisms and Inhibition Zone (mm)					
	K.p.	P.a.	E.c.	S.a.	B.s.	B.c.
2a	-	-	-	-	-	-
2b	-	-	-	-	-	-
2c	-	-	-	-	-	-
2d	-	-	-	-	-	-
2e	-	-	-	-	-	-
2f	-	-	-	-	-	-
2g	-	-	-	-	-	-
3a	++	++	+++	+	-	-
3b	++	+++	++	++	-	-
3c	+++	++	+++	+++	-	-
3d	++	++	+++	++	-	-
3e	++	++	++	++	-	-
3f	++	++	++	++	-	-
3g	+++	+++	+++	+++	-	-

The inhibition zone: (–): <5.5 mm; (+): 5.5–10 mm; (++): 11–16 mm; (+++): ≥17 mm.

K.p.: Klepsiella pneumoniae (ATCC4352), P.a.: Pseudomonas aeruginosa (ATCC27853), E.c.: Escherichia coli (ATCC25922). S.a.: Staphylococcus aureus (ATCC6538), B.s.: Bacillus subtilis (ATCC11774), B.c.: Bacillus cereus (ATCC11778).

All of the Schiff Bases (**2a-g**) showed no effect against six bacteria. All of the Mannich Bases (**3a-g**) showed no effect against *B. Subtilis* ATCC11774 and B. *cereus* ATCC11778 bacteria strains. The antimicrobial activity of **3a-g** compounds against *K. pneumoniae*, *P. Aeruginos, E. coli*, *S. aureus* is lower than Ampicillin and higher than Neomycin and Streptomycin standards and listed in Table **10**. While compounds **3b**, **3g** showed high activity against *P. Aeruginosa* ATCC27853, compounds **3a, 3c, 3d, 3e** and **3f** showed moderate activity to this strain. On the other hand, different results were obtained from *K. pneumoniae* ATCC4352 strain. While compounds **3c**, **3g** showed high activity against *K. pneumoniae* ATCC4352, *Staphylococcus aureus* (ATCC6538) and *Escherichia coli* (ATCC25922) compounds **3a, 3b, 3d, 3e** and **3f** showed moderate activity to this strain. The compound **3a, 3c, 3d, 3g** showed high activity against *Escherichia coli* (ATCC25922) but, compound **3b, 3e, 3f** showed moderate activity against same bacteria. Compound **3g** showed high activity against first four bacteria and summarized in Table **10**, **11**.

The synthesis and in-vitro antioxidant valuation of fourteen new compounds

are explained. Antioxidant activity (the metal chelate activity) and anti-microbial activity of Mannich bases (**3a-g**) were higher than Schiff base compounds (**2a-g**). Synthesis of these new Mannich Bases can play especially a safety role in modern medicinal chemistry. These results may also ensure some lead for the improving of Mannich Bases curative aim.

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Formulation and Characterization of Fast Dissolving Tablets Using *Salvia Hispanica* (Chia Seed) Mucilage as Superdisintegrant

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ABSTRACT

Gums and mucilage are mainly explored for its various applications in dosage form development. *Salvia hispanica* (Lamiaceae) i.e. Chia seed mucilage mainly composed of xylose, glucose and methyl glucuronic acid that forms a branched polysaccharide of high molecular weight. The present study strives to investigate the disintegration ability of *Salvia hispanica* mucilage in the formulation of fast dissolving tablets of selected model drug indomethacin. Indomethacin belonging to the category of NSAIDs and is used as a therapeutic agent for relieving pain, inflammation and joint stiffness. *Salvia hispanica* mucilage was isolated and characterized by physicochemical parameters. Indomethacin tablets (F1-F5) were formulated by direct compression method using *S. hispanica* mucilage powder in varying concentration range of 2%, 4%, 6%, 8% and 10% respectively. Crospovidone (6%) was used as synthetic disintegrating agent for comparison. Micro crystalline cellulose was used as tablet diluent, mannitol, talc and magnesium stearate were used to enhance mouth feel and as lubricants respectively. All the formulations were evaluated for their pre and post compression parameters like tablet hardness, thickness, % friability, wetting time, drug content, disintegration time which was found to be in permissible limits. Drug excipient compatibility study was carried out using FTIR technique. The formulation F3 containing 6% of *Salvia hispanica* mucilage exhibit maximum drug release of 98.5% in 30 minutes, F4 and F5 formulations containing 8% and 10% of *Salvia hispanica* mucilage showed 82.4% and 80.5% drug release in 30 minutes. Results of *in vitro* drug released kinetic study indicated that the optimized formulation F3 followed Korsmeyer Peppas model with R^2 value of 0.9793. Stability studies performed on F3 formulation indicated that the prepared tablets remain stable for the period of 90 days and showed no change

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in *in vitro* drug release pattern. Various investigations have demonstrated that the *S. hispanica* seed mucilage can be successfully used as a natural superdisintegrant which is comparable to various synthetic disintegrants used in the formulation of fast dissolving drug delivery systems.

Keywords: Indomethacin, chia seed mucilage, *Salvia hispanica,* superdisintegrant, fast dissolving tablets

INTRODUCTION

Oral administration is the most familiar and preferential route for the delivery of therapeutic agents to the patients.[1] Mainly, tablets are medications of choice for patients because of dose precision, self-medication, avoidance of fear of needles, economical therapy, compactness, easy administration and patient compliance. No requirement for the maintenance of sterile conditions is there, therefore manufacturing cost is low.[2] Tablet formulations include the therapeutic active component i.e., active pharmaceutical ingredient (API) and the excipients which are the pharmaceutically inactive components. Regardless of their inactivity, excipients possess specific functionalities affecting the biopharmaceutical action of the formulation. The excipients incorporated into the tablet formulations are binders, diluents, disintegrants, lubricants, preservatives, colorants and flavoring agents.[3] Disintegration of the tablet is the important step involved in the release of the drug, therefore, the most essential excipient amidst all is the disintegrant which is responsible for the breaking up of the dosage form into smaller particles after it has been ingested and further allowing the dissolution and ultimately the absorption of the drug.[4]The disintegrant property can be modified for the development of fast dissolving/disintegrating tablets (FDT) which are beneficial for paediatric, geriatric, dysphagic, bedridden and uncooperative patients as conventional tablets might pose a difficulty in swallowing because of coughing, motion sickness, abrupt episodes of allergic attack or in case of unavailability of water. FDTs are novel dosage form which utilizes superdisintegrants for immediate and complete disintegration of the tablet, generally within the range of a few seconds to less than a minute, when placed on the tongue, thereby leading to drug release in the saliva. They are also known as 'quick-dissolving', 'orodispersible', 'melt-in-mouth' or 'mouth-dissolving tablets'.[5]Diverse factors influencing the disintegration process of the tablet include the particle size, moisture content, compression force, type and properties of the disintegrant, tablet hardness, nature of the drug, compatibility with other ingredients and the process of addition as well as mixing.[6,7]The crucial mechanisms involved in the tablet disintegration process include swelling, deformation, wicking, enzymatic reactions and repulsions between the particles.[8]

Different categories of superdisintegrants include natural (like Gums and mucilage) and synthetic superdisintegrants (croscarmellose sodium, crospovidone, carmellose, sodium starch glycollate etc.). Various Gums and mucilage such as *Mangifera indica* gum, Locust Bean gum, Fenugreek seed mucilage, *Ficus indica* fruit mucilage, *Lepidium sativum* mucilage, etc. have been utilized as superdisintegrant in the formulation of FDT. Additional advantages of natural polymers include easy accessibility, biocompatibility and lower cost as compared to synthetic ones.[9]

Chia seeds are obtained from *Salvia hispanica*, a herbaceous plant belonging to the family Lamiaceae. It is a consumable seed having remarkable therapeutic effects as well as nutritional value due to the presence of high quality carbohydrate (41%), fat (30-33%), protein (15-25%) and dietary fibre (18-30%) and mucilage. It is an excellent source of omega-3 fatty acid, antioxidants as well as various polyphenolic compounds such as caffeic acid, quercetin, chlorogenic acid, kaempferol and myricetin.[10]

The polysaccharides that make up the mucilage network of chia seeds are located in the outer seed coat layers and are composed of β-D-xylopyranosyl, α-D-glucopyranosyl and 4-O-methyl-α-D-glucopyranosyluronic acid. Upon addition of water the polysaccharides exudate, absorb water and unravel to full extension. Chia seeds i.e. *Salvia hispanica* mucilage gels can be used as pharmaceutical excipients in drug delivery.[11]

The present study strives to investigate the disintegration ability of *Salvia hispanica* mucilage in the formulation of FDT of the selected model drug Indomethacin. Indomethacin belongs to NSAID and is used as a therapeutic agent for relieving pain, inflammation and joint stiffness. The drug is included in BCS class II drug (poorly aqueous soluble and highly permeable), so it is worth to develop FDT of Indomethacin to enhance its solubility and bioavailability.

METHODOLOGY

Materials

Salvia hispanica seeds were procured from the local market of Patiala and authentication of the sample was confirmed from Department of Pharmacognosy, Chitkara University Punjab. Indomethacin was obtained as gift sample from Triko Pharmaceutical Rohtak. Crospovidone, Microcrystalline cellulose, Mannitol, magnesium stearate was purchased from Loba Chemie Ltd. Mumbai, India.

Isolation of mucilage

The seeds of *Salvia hispanica* were soaked in water for overnight (seed-solvent ratio was 1:20), boiled for half an hour and mixed on magnetic stirrer for 1 hour

so that the mucilage releases completely into water. The resulting mixture was centrifuged at 5000rpm for 50 min, after which three different layers were formed.[11] Only the gel layer was collected and dried in a hot air oven at 50°C. The product was grounded, passed through the sieve no. 80 and then stored at room temperature in desiccators for further use.

Physicochemical evaluation of mucilage

Isolated mucilage was evaluated for various physicochemical properties.[12]

Drug excipient compatibility studies

Drug excipient compatibility studies were performed with the help of Fourier transforms infrared (FTIR) spectroscopy. FTIR spectra (range of 3500-500 cm^{-1}) of the Indomethacin, *Salvia hispanica* mucilage and combined mixture of both (1:1) were taken (Bruker Alpha T).

Standard calibration curve of Indomethacin

For preparing the standard calibration curve of Indomethacin, 100 mg drug was weighed and transferred to 100 ml of volumetric flask. The drug was dissolved in 100 ml of phosphate buffer pH 6.8 (stock I). 10 ml solution was withdrawn from stock I and diluted upto 100 ml with phosphate buffer pH 6.8(stock II). Different dilutions were prepared by pipetting out 0.2, 0.4, 0.6, 0.8, 1 and 2 ml from stock solution II and then finally adjusting the volume upto 10 ml. The absorbance of all the resulting solutions was measured at 325 nm with the help of UV-VS spectrophotometer (Systronics).[13]

Formulation of Fast Dissolving Tablets of Indomethacin using *Salvia hispanica* mucilage

Fast dissolving tablets of Indomethacin (F1-F6) were prepared by direct compression method. The mucilage of *Salvia hispanica* was used in varying concentrations such as 2%, 4%, 6%, 8% and 10% as superdisintegrant. Microcrystalline cellulose was used as diluent, mannitol as filler, talc as glidant and magnesium stearate as lubricant. All the constituents were weighed according to the composition given in **Table 1**, sifted through mesh # 60 and mixed thoroughly in ascending order. The formulated blend was then examined for its pre-compression parameters. These formulations were compared with the standard formulation F6 containing crospovidone as superdisintegrant.

Table 1. Composition of fast dissolving tablets of Indomethacin.

Ingredients (mg)	Formulation batches					
	F1	F2	F3	F4	F5	F6
Indomethacin	150	150	150	150	150	150
Salvia hispanica mucilage	2%	4%	6%	8%	10%	-
Crospovidone	-	-	-	-	-	6%
MCC 102	112	106	100	94	88	82
Mannitol	25	25	25	25	25	25
Talc	2	2	2	2	2	2
Magnesium Stearate	5	5	5	5	5	5
Total	300	300	300	300	300	300

Pre compression parameters

The different powder blends were evaluated for pre compression parameters by finding the bulk density, tapped density, Carr's index, Angle of repose and Hausner ratio according to methods specified in pharmacopoeia.[14]

Evaluation of tablets

The prepared tablets were characterized for different post compression parameters such as weight variation, hardness, % friability, thickness, wetting time, drug content and disintegration time. The hardness of the tablets was assessed with the help of Monsanto hardness tester. It was done by taking the average value of any three tablets from every batch. Friability was tested by taking 20 tablets and placing the weighed tablets in the friability chamber (Roche friability tester) for 4 min at 25±1 rpm, and then percentage of the weight loss was determined. Disintegration test was performed in phosphate buffer of pH 6.8 at 37 ± 1 °C.[15]Thickness of tablets was determined using vernier caliper (Mitutoyo Dial Thickness Gauge, Mitutoyo, Japan). Wetting time was recorded by taking 10 ml of phosphate buffer pH 6.8 in petridish, over this fourfold tissue paper was placed and a tablet was carefully placed over this. Time required for a tablet to wet was recorded as wetting time. For determination of drug content ten tablets from each formulation batch was taken randomly and triturated to fine powder. Weight of powder equivalent to 150mg was taken in phosphate buffer pH 6.8 and assayed for drug content uniformity using double beam UV –visible spectrophotometer at λ_{max}325 nm.

In vitro drug dissolution study

In vitro release of the drug was evaluated by Lab India Dissolution Apparatus (LABINDIA DS 80 00, India), using phosphate buffer of pH 6.8 as the dissolution media (900 ml) which was maintained at a temperature of 37±1°C for half an hour, at 75 rpm. After specified time interval, 5ml of sample was withdrawn and concurrently replaced with the same volume of dissolution media. Spectrophotometrical analysis of the samples were measured at a wavelength of 250 nm, and % cumulative drug release was calculated.[16]

Drug release kinetics

Mechanism of drug release and kinetic followed was studied by fitting the data of drug release in different kinetic models i.e. zero order, first order, Higuchi, Hixson–Crowell and Korsmeyer- Peppas model.[17,18]

Zero order equation:

$Q_t = K_o t$

Q_t = percentage of drug release in time t

K_o = release rate constant.

First order equation:

$In (100- Q_t) = In100-K_1 t$

K_1= release rate constant

Higuchi's equation:

$Q_t = k_H t^{½}$

k_H= Higuchi constant

Hixson Crowell:

$(100-Qt)^{1/3} = 100^{1/3}- k_{HC} t$

k_{HC} = Hixson Crowell constant

Korsmeyer- Peppas:

$Q_t/ Q_n = k_{Hp} t^n$

Q_t/ Q_n= fraction of drug release in time t

n = release exponent

k_{Hp} = constant.

Stability studies

Stability testing of F3 formulation selected on the basis of release studies was carried out as per the ICH stability testing guidelines at 40°C with 75% relative humidity, tablet samples were loaded in stability test chamber (Remi Instruments, India) and samples were withdrawn at different time points 15, 30, 45, 60 and 90 days.[19] Samples were evaluated for disintegration time, *in vitro* drug release and uniformity in content.

RESULTS AND DISCUSSION

Physicochemical evaluation of mucilage

Mucilage from the seeds of *Salvia hispanica* was isolated with the help of standard procedure. The total yield of mucilage obtained was 12 %. The isolated mucilage was greyish white in colour and mucilaginous in taste. The physicochemical properties of isolated mucilage were analyzed and the results are listed in **Table 2**.

Table 2. Physicochemical evaluation of *Salvia hispanica* mucilage.

S.No	Properties evaluated	Observations
1	Colour	Greyish white
2	Taste	Mucilaginous
3	Odour	Odourless
4.	Appearance	Flaky appearance
4	Solubility	Forms viscous solution in water
5.	pH (By Digital pH meter)	6.4*
6.	Swelling ratio	9*
7.	Viscosity (1% solution)	416 cps*
8.	Test for Carbohydrates (Molisch's test)	+ve
9.	Test for Tannins (Ferric chloride test)	-ve
10.	Test for proteins (Ninhydrin test)	-ve
11.	Test for alkaloids (Wagner's test)	-ve
12.	Test for glycosides	+ve
13.	Test for mucilage (Ruthenium red test)	+ve
14.	Test for reducing sugar (Fehling's test)	+ve
15.	Mounting with Iodine Solution	+ve
16.	Test for uronic acid	+ve

*Results are expressed as mean (n=3), +ve=Positive, -ve = Negative

Standard calibration curve of Indomethacin

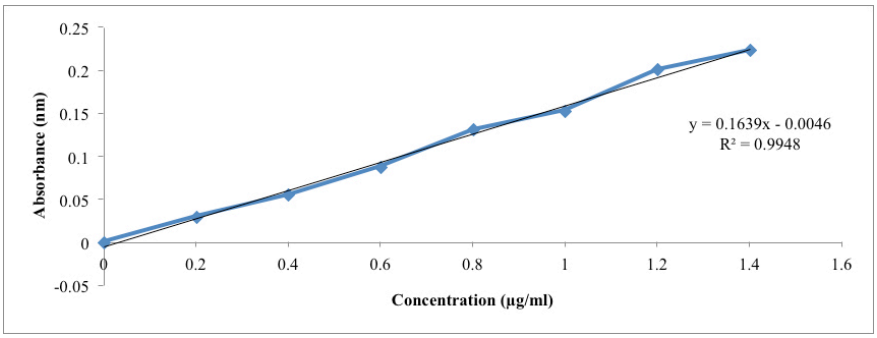

Figure 1. Standard calibration curve of Indomethacin.

Figure 1 shows the standard calibration curve of Indomethacin by plotting the absorbance on y-axis and concentration on x-axis of the graph. The correlation coefficient determined by the standard calibration curve was found to be 0.994.

Drug-excipients compatibility study

FTIR spectra of indomethacin, *Salvia hispanica* mucilage and mixture of drug and mucilage are given in Figure 2. Spectral analysis indicated all the important peaks of drug in the FTIR of drug and mucilage mixture which indicates that indomethacin is functionally compatible with the mucilage.

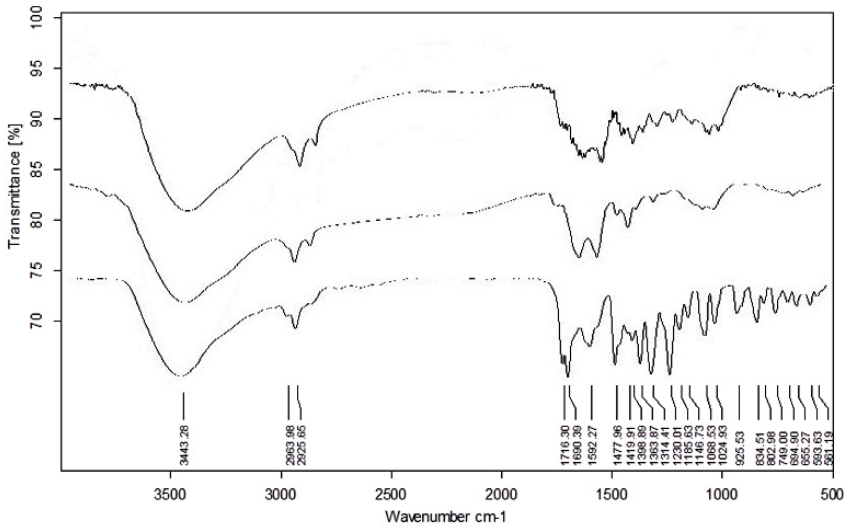

Figure 2. FTIR Spectra of a) Indomethacin b) *Salvia hispanica* mucilage c) Indomethacin+ *Salvia hispanica* mucilage.

Pre-compression parameter

Six formulation batches of indomethacin tablets (F1-F6) were formulated, each having different concentration of mucilage. The flow properties of different powder blends were analyzed by angle of repose, Carr's index and hausner ratio. The angle of repose values lie within the range 28.65 - 21.6, which indicates excellent flow ability. Carr's index between 19 – 11% and hausner ratio values ranging between 1.29-1.21 shows good flow characteristics. **Table 3** depicts the outcome of pre compression parameters of formulation blends (F1-F6).

Table 3. Evaluation of precompression parameters of formulation blends of Indomethacin tablets.

Parameters	F1	F2	F3	F4	F5	F6
Bulk Density (g/cm^3)*	0.036±0.06	0.034±0.01	0.032±0.02	0.031±0.01	0.031±0.02	0.034±0.01
Tapped Density (g/cm^3)*	0.048±0.5	0.052±0.07	0.054±0.05	0.05±0.05	0.05±0.02	0.054±0.03
Angle of Repose*	25.7±0.51	28.65±0.03	23.9±0.1	27.9±0.2	27.7±0.1	21.6±0.01
Carr's Index	19%	13%	11%	18%	18%	17%
Hausner's Ratio	1.21	1.24	1.25	1.26	1.27	1.29

*mean±SD, n=3

Post compression parameter

The hardness value of all the tablets was in the range of 1.5-2.5 kg/cm^2. The friability test was done to examine the durability and depict how much mechanical stress the tablets can withstand during their manufacturing, distribution and handling by the customer. According to IP, the percent friability value should not exceed above 1%. All the prepared tablet formulations show values between 0.3-0.75percent, which means the friability test, was passed by all of them as per the standard value. The disintegration time has a range of 19-72 sec and it decreased with an increase in concentration of mucilage. **Table 4** depicts the results for all the evaluation parameters.

Table 4. Evaluation of post compression parameters of Indomethacin fast dissolving tablet.

Formulation	F1	F2	F3	F4	F5	F6
Hardness (kg/cm^2)[a]	4.2±0.05	3.8±0.01	3.2±0.02	3.6±0.03	3.5±0.04	3.5±0.02
% Friability	0.74	0.23	0.5	0.4	0.4	0.32
Disintegration Time(sec)[a]	71.6 ±0.01	61.3 ±0.03	25.2±0.03	49.6 ±0.04	52.6 ±0.06	21.2 ±0.03
Thickness (mm)[a]	5.5±0.13	5.3±0.15	5.2±0.02	5.5±0.08	5.1±0.21	5.3±0.02
Wetting time[a] (sec)	52.3 ±0.03	42.3±0.04	14.05±0.02	32.23±0.03	30.05±0.11	18.92±0.12
Drug content[a]	98.2±0.23	99.3±0.24	99.5±0.01	98.5±0.08	98.2±0.25	99.3±0.06

[a] mean±SD, n=3

Drug release

In vitro drug release study is shown in Figure 3. The formulation F3 containing 6% of *Salvia hispanica* mucilage exhibit maximum drug release of 98.5% in 30 minutes, F4 and F5 formulations containing 8% and 10% of *Salvia hispanica* mucilage showed 82.4% and 80.5% drug release in 30 minutes, which indicated that the amount of drug released from the tablets decreased as the concentration of mucilage increased. This can be due to the fact that as the concentration of mucilage increases, it causes an increase in the apparent viscosity. Further, the intermolecular motion of the particles gets restricted and leads to the formation of an interfacial film which slows down the drug release.[20]

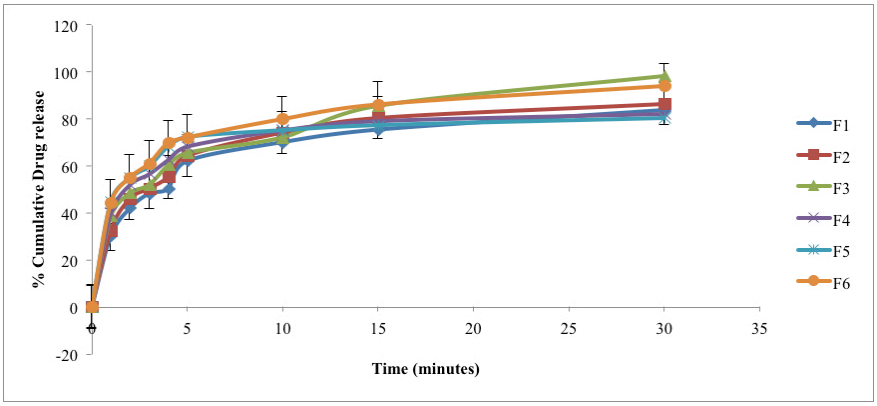

Figure 3. *In Vitro* drug release plot of F1- F6 formulations.

Release kinetic study

Results of *in vitro* drug released kinetic study as shown in Table 5 indicated that the optimized formulation F3 followed Korsmeyer Peppas model with R^2 value of 0.9793.

Table 5. Release kinetic study of F1-F6 formulations

Formulations	Zero order	First order	Higuchi	KorsmeyerPeppas	
	R^2	R^2	R^2	N	R^2
F1	0.743	0.8323	0.902	0.25	0.9451
F2	0.725	0.8268	0.896	0.24	0.9512
F3	0.857	0.977	0.9648	0.26	0.9793
F4	0.639	0.673	0.8398	0.17	0.9231
F5	0.6177	0.6315	0.8024	0.14	0.8745
F6	0.7383	0.8846	0.9103	0.19	0.9554

Stability studies

As per the ICH guidelines, stability studies were performed on the promising formulation F3 under the condition of 40±2°C/75±5% RH for 90 days which disclosed that no remarkable variation in the colour, hardness, disintegration time, drug content and *in vitro* drug release were encountered. This shows the stability of the prepared optimized formulation. **Table 6** depicts the corresponding outcomes of the tests performed.

Table 6. Stability studies of F3 formulation at 40±2°C/75±5% RH.

Parameters	Days				
	0	15	30	60	90
Hardness (kg/cm^2)[b]	3.2±0.02	3.1±0.1	3.2±0.07	3.2±0.05	3.2±0.02
Disintegration Time (sec)[b]	25.2 ±0.03	25.2 ±0.03	25.2 ±0.03	25.2 ±0.03	25.2 ±0.03
Drug content[b]	99.5±0.08	99.0±0.01	99.2±0.06	99.2±0.06	99.5±0.03
In vitro release	98.12	98.0	98.12	98.0	98.3

[b]mean±SD, n=3

Salvia hispanica mucilage has a potential to be used as superdisintegrants in place of currently marketed synthetic superdisintegrating agent.

CONFLICTS OF INTEREST

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

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Development, Characterisation and Evaluation of PVP K-30/PEG Solid Dispersion Containing Ketoprofen

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ABSTRACT

The present study is designed with the objective to enhance the aqueous solubility of ketoprofen. For the said purpose solid dispersion employing PVP K-30 and PEG 6000 is prepared by hot melt method. The preparation of solid dispersion (SD) was optimized using 2-factor, 3 level central composite design. The preparation of SD was characterized by FTIR, DSC, XRD and SEM studies. Further, solubility, Gibb's energy and *in vitro* release study was determined for different batches of the formulation as suggested by the design expert. The solubility of different batches of SD was found to be between 36.2 to 53.0µg/ml whereas pure drug and physical mixture possess the solubility of 14.4 and 23.8 µg/mL, respectively. The *in vitro* release is found to be 76.74 to 95.32% and PM shows 75.4 % release and pure drug shows 23.5 % release. Thus, the ketoprofen-loaded solid dispersion would be useful for delivering poorly water-soluble ketoprofen with enhanced dissolution, solubility and no crystalline changes.

Keywords: Ketoprofen, solid dispersion, dissolution rate, solubility study.

INTRODUCTION

Poor aqueous solubility of pharmaceutically active ingredients has been a challenge to the researchers. More than 40 % of newly developed drugs candidates are poorly water soluble[1]. This low aqueous solubility further lowers the absorption in GI tract and thus reduced bioavailability is observed. Therefore, the development of new techniques to enhance drug solubility remains one of the major research areas in drug discovery and development process[2]. A scan of literature has revealed that numerous attempts has been made to improve the

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solubility and dissolution of drugs that include cyclodextrins complexes[3], drug micronization in to amorphous form[4], prodrug formation[5], solid dispersion[6,-12], salt formation[13], solubilization of drug in solvents, nanoparticles technology[14], physical modification, nanosuspension[15-19], modification of crystal habit such as polymorphus, pseudo polymorphous, self-emulsifying drug delivery systems (SEDDS)[20], liposomes[21-25] and use of surfactant " micelization" [26] etc. Out of numerous techniques explored so far solid dispersion have been proved to be the most promising strategy to improve the solubility of poorly water-soluble drugs. In solid dispersion compounds are mixed / dispersed in solid state in a suitable carrier or matrix to deliver the insoluble compounds[27]. Solid dispersions preparation by hot melt or fusion method is preferred because of it is simple and is useful for the compounds that do not undergo significant thermal degradation. Further toxicity and environmental issues that are associated with use of organic solvents make the technique more preferable[28].

Ketoprofen, categorized as BCS class II drug, is a safe propionic acid derivative non-steroidal anti-inflammatory drug. However, its poor water solubility (0.13 mg/ml at 25° C) limits its absorption and dissolution rate that results in delayed onset of action. Therefore, its incorporation in water soluble matrix solid dispersion have been considered as an effective method for improving its dissolution rate and solubility. A number of efforts have been made to incorporate ketoprofen into solid dispersion using mannitol, urea, PVP K-30[29], PVP K-30 and D-mannitol[30], bovine serum albumin[12], Macrogal and Kollagen[31] and PEG 6000[32]. On scouring the literature, it is observed that PVP and PEG are most popularly used polymers for the preparation of solid dispersion[33]. Low melting point of PEG (50-60°C) and rapid solidification offers it to be used in forming solid dispersion[25] while the PVP K-30 is high melting point (150-180°C) and small its molecular weight render it to be a suitable carrier in achieving high dissolution[34].

The present study is designed to obtain solid dispersion of ketoprofen with PEG 6000 and PVP K-30 using hot melt or fusion method with the objective to enhance solubility and dissolution of the drug. The preparation of solid dispersion was optimized by using 3 factor 2 level central composite experimental design. The optimized batch was characterized by FTIR, SEM, DSC, XRD spectra and evaluated for its in-*vitro* release behavior and solubility.

METHODOLOGY

Materials

Ketoprofen was obtained as a gift sample from Infinity Laboratories Pvt. Ltd

(Behra, India). Polyvinyl pyrrolidone (PVP) K30 (kollidon K30) and polyethylene glycol 6000(PEG 6000) (PEG 6000) was supplied by Symmetry laboratories, Faridabad. All other reagents and chemicals used in the study were of analytical grade and used as received.

Methods

Preparation of solid dispersion

SDs of ketoprofen were prepared by using hot melt technique by mixing required quantities of ketoprofen(100 mg) ,PVP K-30 and PEG 6000 as displayed in Table 1. The ingredients were added in decreasing order of their melting point and the resulting homogeneous preparation was cooled and stored in a desiccator for 24 h. Subsequently, the dispersion was ground using a mortar and sieved through a #100 sieve.

Physical mixtures (PM) was prepared by thoroughly mixing presieved equal weight ratio of ketoprofen, PEG 6000 and PVP K-30 using pestle and mortar until a homogeneous mixture was obtained.

Experimental design

The preparation of solid dispersion using PVP K-30 and PEG 6000 was optimized using 2-factor, 3 level central composite design. The concentration of PEG 6000 (X_1) and concentration of PVP K-30 (X_2) were selected as the formulation variables while the % drug release, solubility (µg/ml) and Gibb's energy (kg/mol) were chosen as response variables. Each independent variable was investigated at three levels (i.e. -1, 0, and 1). The experimental design and statistical analysis of the data was done by using Design Expert software (version 11.0).

Determination of drug content

The different batches of solid dispersions and physical mixture (PM) containing ketoprofen equivalent to 10mg were dissolved separately in 25 ml of phosphate buffer (pH 7.4). Samples were filtered through 0.45µm milipore filters and after appropriate dilution were analyzed by UV visible spectrophotometer at 260nm. The drug content was determined using calibration curve.

Characterization of solid dispersion

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy is employed to characterize the possible interactions between the drug and the carrier in the solid state. FTIR spectra of powder samples of ketoprofen, PEG 6000, physical mixture and solid dispersion were ob-

tained using a spectrophotometer (FTIR-8300, Shimadzu co., Kyoto, Japan) by using potassium bromide (KBr) pellet method in the scanning range of 400-4000 cm^{-1}.

Powder X-ray diffraction (PXRD)

PXRD is the most important tool to determine the structure of the compound. Powder XRD patterns were traced employing X-ray diffractometer (Philips PW 1830, the Netherlands) for the samples of ketoprofen, PVP K-30, PEG 6000, physical mixture and solid dispersion using Ni filtered Cukα radiation of wavelength 1.5404 A^o,a voltage of 35 kV, a current of 30 mA and receiving slit of 0.1 mm. The sample were analyzed over 2θ range of 5-50^o.

Scanning electron microscopy (SEM)

The particle shape and morphology of the sample was investigated using SEM (JEPL, JSM-6100). The samples for SEM were mounted on sample holder with double-sided adhesive tape after vacuum coated with gold.

Differential scanning Calorimetry (DSC)

DSC analysis of samples ketoprofen, PVP K-30, PEG 6000, physical mixture and solid dispersion was carried out using DSC (Q10 V9.9 Build 303, US). Each sample was heated at the scanning rate of 10^{o}C /min, from 30 to 200^{o}C.

Solubility studies

Ketoprofen pure drug, PM and solid dispersion containing ketoprofen equivalent to 10 mg was dispersed in 10 ml of distilled water and kept on shaker for 48hrs at room temperature (25°C) to determine the solubility of ketoprofen. The obtained solution was filtered by 0.45µm milipore filter paper and the drug content was determined by taking absorbance at 260 nm using *uv-vis* spectrophotometer. The amount of drug was calculated using the calibration curve in water. The Gibbs free energy of transfer (ΔG) of drug from water to the aqueous solution of carrier was calculated as equation 1.

$$\Delta G = -2.303RT \log S_o/S_s \qquad (1)$$

where S_o/S_s is the ratio of solubility of ketoprofen in aqueous solution of carrier to that of the same medium without carrier.

***In vitro* drug release**

In vitro dissolution studies were performed using the USP type II dissolution apparatus. Dissolution studies of pure drug (ketoprofen), PM and solid dispersions containing ketoprofen equivalent to 10mg were conducted in 300ml

phosphate buffer (pH 7.4) at 37±0.5°C with constant stirring rate of 50 rpm. The powders were dispersed over the dissolution medium. Aliquots of sample (5ml) was withdrawn at different time intervals and replaced with an equal amount of the dissolution medium to maintain a constant volume. Samples were filtered through 0.45µm milipore filters and analyzed by uv-vis spectrophotometer at 260nm. The mechanism of drug release from the solid dispersion was determined by fitting the release data to several models like zero order, first- order, Higuchi and Korsmeyer–Peppas model.

RESULTS AND DISCUSSION

Drug content of formulations

The content of ketoprofen in different batches of SD was determined by carrying out the assay of drug by *uv-vis* spectroscopy is displayed in Table-1. The drug content was found to be between 82.8 to 97.9 % whereas for PM containing equal amount of ingredients has drug content of 84.4%. From the data it is clearly indicated that the drug content in the formulated batches of SD and PM is quite handsome indicating that drug is for entrapped/present in a sufficient amount.

Fourier Transform Infrared Spectroscopy (FT-IR)

Characteristic IR absorption peaks of ketoprofen were detected at 2979.23 cm^{-1} due to –CH stretching, 1694 cm^{-1} (-C=O stretching of acid), 1654 cm^{-1} (-C=O stretching of ketone), 1598, 1583, 1457 cm^{-1} (-C=O stretching of aromatic ring), 1420 cm^{-1} (-C-H deformation of CH_3 asymmetrical) and 1370 cm^{-1} (-C-H deformation of CH_3 symmetrical) respectively[12].

Important vibrations detected in the spectrum of PEG are the -C-H stretching at 2889 cm^{-1} and the –C-O (ether) stretching at 1112 cm^{-1}. The –OH group is indicated by a strong band around 2890 cm^{-1}. The region from 2700-3000 cm^{-1} in the spectra of pure ketoprofen, PEG 6000, PM and SD exhibited peaks assigned to aliphatic –C-H stretching[35].

IR spectra of SD was found to be almost similar with that of PM because characteristic peaks representing specific functional group were detected. Especially the peak at 2888 cm^{-1} resulting from –C-H stretching vibration was observed in both SD and PM of ketoprofen[36]. So the characteristic peaks of ketoprofen also seen in SD and PM that indicate that ketoprofen is present in the SD and PM.

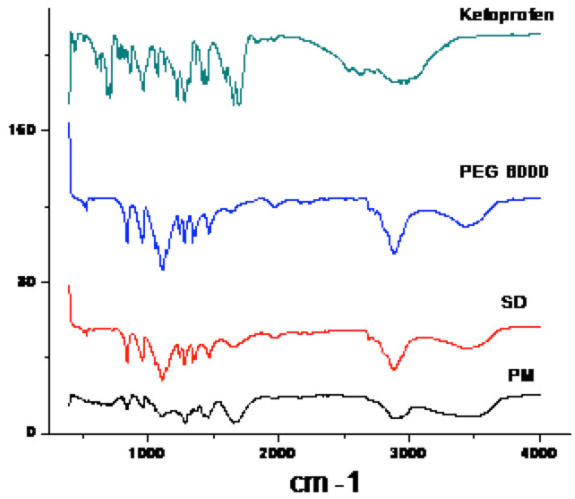

Figure 1. FTIR spectra of pure ketoprofen, PEG 6000, SD and physical mixture.

Power X-ray diffraction (PXRD)

The powder XRD of pure ketoprofen, physical mixtures, polymers PEG 6000, PVP K-30 and solid dispersion are shown in figure 2. The presence of numerous distinct peaks in the X-ray-diffraction spectrum of ketoprofen indicates that ketoprofen is present as a crystalline material with characteristics sharp peaks appearing at a diffraction angle of 2θ at 18.608, 22.976 and 27.23 etc. XRD spectra of PVP K-30 showed no intrinsic peaks at the diffraction angles examined, which is typical of amorphous sample. PEG 6000 exhibited a distinct pattern with diffraction peaks at 2θ of 19.12, 23.504, revealing its crystalline nature. As expected, all major characteristic crystalline peaks for the drug were also observed in the SD and PM. These results indicated that ketoprofen was present in an unchanged crystalline state in the SD[37-39].

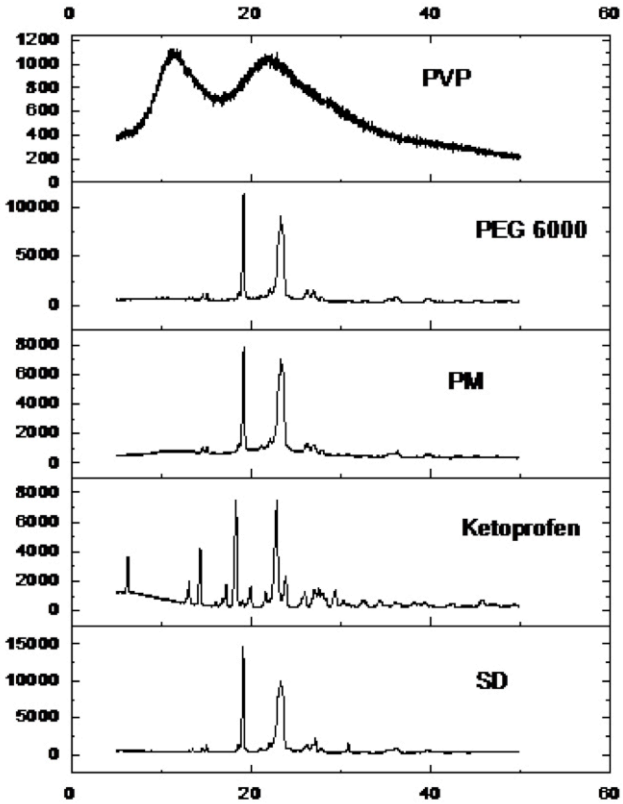

Figure2. Powder X-ray-diffraction of PVP K30, PEG 6000, physical mixture (PM), pure ketoprofen and solid dispersion (SD).

Scanning electron microscopy (SEM)

SEM photomicrographs of solid dispersion shown in figure 3. On observing the SEM image of solid dispersion, the surface is observed as rough and flaky.

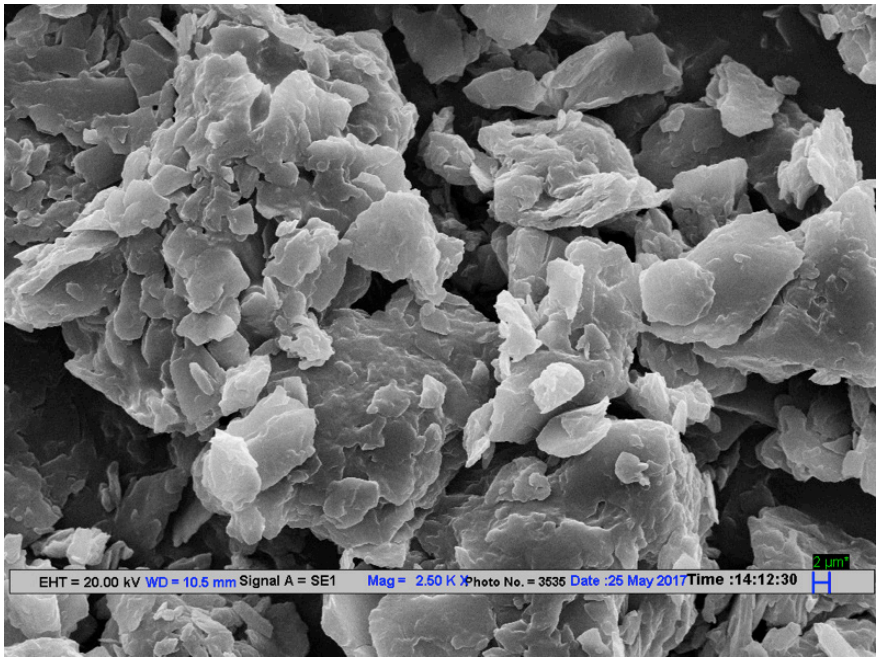

Figure 3. SEM photomicrograph of solid dispersion.

Differential scanning calorimetry (DSC)

DSC curves obtained for pure ketoprofen, physical mixture and solid dispersion are shown in figure.4. The thermogram of ketoprofen showed an endotherm onset of heating at 91.97 °C and end of melting point is 97.59 °C with heat of fusion (ΔH) 93.0185 J/g, corresponding to its melting point and indicating its crystalline nature. Interestingly, a sharp peak corresponding to the drug was also observed in both the PM and the SD, which in contrast to the thermogram seen with most SDs. It indicates the absence of strong interactions between the drug and the carriers during preparation of the SD. It also suggests that ketoprofen was present in an unchanged crystalline state in the SD. Thus, similar to the XRD findings, these result indicated that ketoprofen was present in an unchanged crystalline state in the SD[40]. Our results suggest that the enhanced solubility of ketoprofen was not due to the transformation of the crystalline form into an amorphous state, but instead were due to the attachment of the carriers to the surface of poorly water-soluble ketoprofen, converting the hydrophobic drug to hydrophilic form in this solid dispersion.

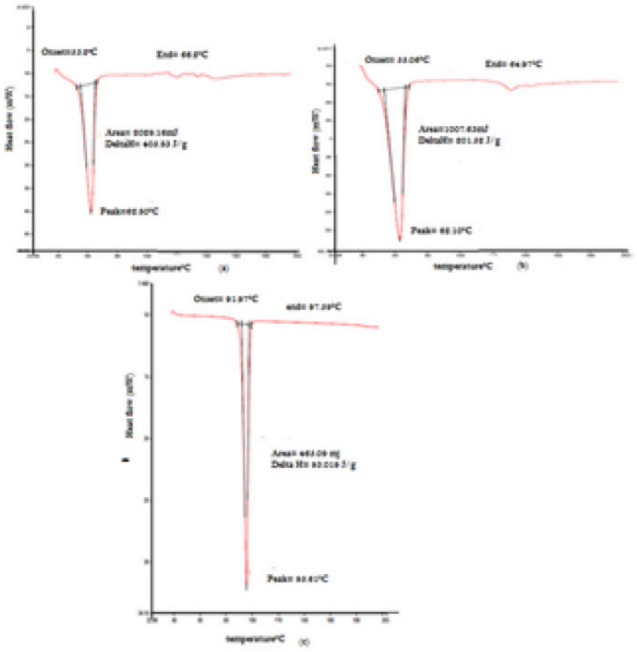

Figure 4. DSC Thermogram of SD (a), PM (b) and ketoprofen (c).

Solubility studies

The different batches of SD were prepared containing PEG 6000, PVP K-30 and ketoprofen as per the design protocol (Table 1). The pure ketoprofen dispensed a solubility of 14.4 µg/ml in water at room temperature whereas the physical mixture showed solubility of 23.8 µg/ml. The physical mixture presented solubility higher than the pure drug because of the polymers (PEG 6000 and PVP K-30) are present. However, the solid dispersion conferred solubility values ranging from 36.2-53.0 µg/ml. This increase may be due to formation of soluble complex between drug and polymers whereas the lower solubility in case of physical mixture as compared to SD may be explained on the basis of results obtained from DSC and XRD studies revealing that ketoprofen is still present in crystalline state.

Table 1. Solubility, in-vitro release, drug content and Gibb's free energy of different batches.

Batches	Conc. Of PEG 6000 (mg) (X_1)	Conc. Of PVP K-30 (mg) (X_2)	Solubility (µg/ml) (Y_1)	% release in 60 min. (Y_2)	Drug content (%)	Gibb's energy (KJ/mol)
SD1	1000	1000	36.2	77.89	86.4	-3
SD2	4000	1000	47.9	89.2	82.8	-4.3
SD3	1000	2000	36.9	78.76	95.3	-3.4
SD4	4000	2000	53.0	95.32	97.9	-5.7
SD5	1000	1500	35.7	76.74	88.9	-2.9
SD6	4000	1500	51.6	90.98	86.4	-4.2
SD7	2500	1000	40.8	81.34	86.4	-3.6
SD8	2500	2000	52.8	92.36	96.8	-4.9
SD9	2500	1500	45.8	87.4	86.2	-3.9
SD10	2500	1500	42.5	84.39	87.6	-3.1
SD11	2500	1500	42.9	86.74	87.8	-3.34
SD12	2500	1500	43.5	84.5	86.5	-3.2
SD13	2500	1500	44.7	86.12	88.0	-3.8
Ketoprofen			14.4	23.5		

Table 1 shows the results of solubility of different batches of solid dispersions prepared as per the design protocol. The responses generated were fitted into various polynomial models using experimental design. The response solubility was fitted best into quadratic model with square root transformation of the data. The adjusted polynomial equation obtained for the solubility (Y_1) shown in equation (1) with determination correlation (R^2) of 0.842.

$$Y_1 = 6.63+0.5330\ X_1+0.2160X_2 \qquad (1)$$

Table 2 summarizes the results of ANOVA on the response surface model. The polynomial model was found to be significant ($p < 0.05$) with non-significant lack of fit ($p > 0.05$). The good correlation between the experimental and predicted response is indicated by higher value of R^2 (> 0.8). Adequate precision that measures the signal to noise ratio (17.58) was much above the required value of 4, indicating adequate signal and model fit to navigate the design space. Fig. 5 (a) display the combined effect of concentration of PEG 6000

& PVP K-30 on solubility. It can be inferred from the plots that there exists a curvilinear relationship between independent and dependent variables. It is inferred from the plot that higher level of polymers & ketoprofen results in increase in solubility.

To attain stability a natural tendency to acquire minimum Gibbs energy is always there. The plot of Gibbs free energy against varying concentration of polymers (PEG 6000 & PVP K-30) and ketoprofen (Fig. 5c) construed that the process is more favorable at higher level of polymers possessing minimum value of ΔG. Further, all the values of ΔG are negative (Table 1) at all levels of carrier demonstrating spontaneity of drug solubilization process.

$$\Delta G = -3.51 - 0.8167X_1 - 0.5083X_2 - 0.2500X_1X_2 + 0.0431X_1^2 - 0.6819X_2^2$$

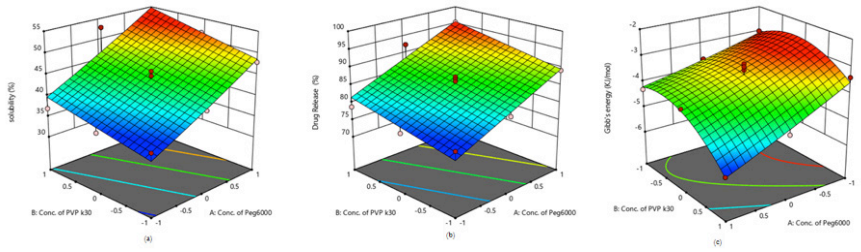

Figure 5. (a,b,c) Response surface plots showing effect of concencentration of PEG 6000 & PVP

K-30 on solubility (Y_1), *in-vitro* release (Y_2) and Gibb's free energy.

In vitro drug release

Table 1 shows the in vitro drug release data at 60 min. From these results, it is observed that only 23.5% of ketoprofen was released in 1 hour from drug solution and 75.4% from the PM whereas different batches of SD show 76.74 to 95.32% release of ketoprofen in 1hr study. The polynomial equation obtained for the dependent variable Y_2 (*in vitro* release) is shown in equation (2).

$$Y_2 = 85.52 + 7.02X_1 + 3.00X_2 \qquad (2)$$

Table 2 summarizing the results of ANOVA on response surface model (fitted best in cubic model after none transformation of the data). The responses generated were fitted into various polynomials models using the experimental design. It was observed that response *in vitro* release (Y2) fitted best into cubic response surface model.

Table 2. Model summary statistic

Model						Lack of Fit	
Response factor (Y)	**F-value**	**Prob.>F**	**R^2**	**Adeq. Prec.**	**C.V (%)**	**F-value**	**Prob.>F**
Y1	33.27	<0.0001	0.84	17.95	2.69	4.41	0.086
Y2	40.89	<0.0001	0.86	20.17	2.42	3.28	0.135

The polynomial model was found to be significant ($p < 0.05$) with non-significant lack of fit ($p > 0.05$). The higher value of R^2 (> 0.9) pertinent good correlation between the experimental and predicted response. Adequate precision that estimates the signal to noise ratio (20.17) was much above the required value of 4, indicating adequate signal and model fit to navigate the design space. Figure 5 (b) display the combined effect of concentration of PEG 6000 & PVP K-30 on % release that exhibited a linear relationship between independent and dependent variables, suggested that higher level of PEG 6000 & PVP K-30 favours the expedited release. The numerical optimization tool using desirability approach was employed to prepare solid dispersion. The optimization of independent variables was done with constraints of maximum solubility and maximum % release.

The optimization of independent variables was done with constraints of maximum solubility and maximum % release. The parameters suggested by the design were concentration of PEG 6000 (0.993%) & concentration of PVP K-30 (0.985%) that provide SD with solubility of 54 µg/ml (predicted value 54.23µg/ml) and % release 95.43% (predicted value 95.87 %). The closer agreement between predicted and observed values indicated the high prognostic ability of the model. Figure 6 displays the in vitro release profile of ketoprofen as pure drug and from the optimized batch of formulation.

A numerical optimization tool of design expert software was used further for calculating the optimal concentration of PVP K-30 and PEG 6000 for preparation of solid dispersion. The optimal calculates parameters were found to be concentration of PEG 6000(4000 mg) and concentration of PVP K-30(2000 mg) which provided us with the drug release is 95.32 %.

The optimized batch of ketoprofen containing solid dispersion of PVP K-30 and PEG 6000 was found to batch no. 4 (optimized batch) and drug release is 95.32 % of the drug.

It is clearly observed that the dissolution rate of pure ketoprofen is low because 23.5% of drug dissolved in 60 min. The resultant study showed that the dis-

solution of drug from physical mixture was improved (75.4 %) but the Solid dispersion from resulted in a marked increase of ketoprofen dissolution (95.32 %) compare to physical mixture at the end of 1 hr. Typical mechanism for improvement of dissolution characteristics of drug via solid dispersion are particle size reduction, and improved wettability and dispersibility of ketoprofen due to surface tension lower effect of PVP K-30.

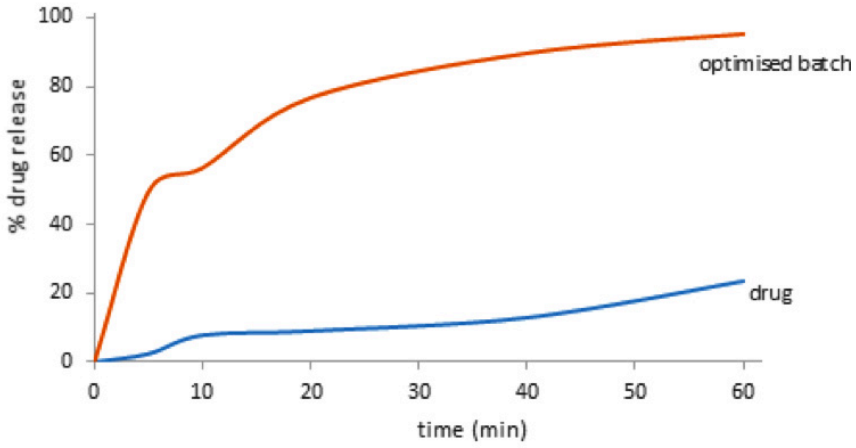

Figure 6. *In vitro* release profile of drug (pure ketoprofen) and solid dispersion (optimized batch).

The release of ketoprofen from solid dispersion was fitted into various kinetic models to estimate their release kinetics and mechanism of release. The results of release rate data for the formulation fitted best into Higuchi model (R^2 = 0.917, n=1.02) of release kinetic. Further, the value of 'n' >1.0 indicates that the release of ketoprofen from solid dispersion occurs by super-case-2 transport that is the release occurred by relaxtion or erosion of polymer after swelling in biological fluid.

Hot melt method is a fast and simple technique for preparing solid dispersion. The solubility and dissolution rate of ketoprofen from solid dispersion with PVP K-30 and PEG 6000 was markedly increased in comparison to pure ketoprofen and physical mixture. As it is revealed from the DSC and PXRD spectra that ketoprofen is in present in crystalline form but the dissolution rate has been increased. Our results suggest that the enhanced solubility of ketoprofen was not due to the transformation of the crystalline form into an amorphous state, but due to the attachment of the carriers to the surface of poorly water-

soluble ketoprofen, converting the hydrophobic drug to hydrophilic form in the solid dispersion. The higher dissolution rate of ketoprofen in SD was therefore attributed to improved wetting of crystal surface mainly due to presence of PEG 6000 and PVP K-30 particles, which provoked the solubilization effect. In conclusion, this surface-attached ketoprofen-loaded SD would be of use for delivering poorly water-soluble ketoprofen with enhanced solubility and dissolution, but without crystalline changes.

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Effect of Ginger on Hyperglycemia Induced by Streptozotocin in Pregnant Rats and Postnatal Neurodevelopment of their Offspring

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ABSTRACT

This study was performed to investigate the consequences of severe maternal hyperglycemia induced by streptozotocin in Wistar rats on postnatal development of offspring. Besides, the ability of a ginger extract to restore glycemic balance in dams and prevent the appearance of disorders in offspring. Diabetes was induced in dams before pregnancy by a single injection of streptozotocin at a dose of 50 mg / kg. Ginger was orally administered from the fourteenth (14th) to the nineteenth (19th) day of pregnancy. Two tests before and after weaning were performed to monitor the offspring development. Our study clearly showed that treatment of diabetic pregnant rats with a ginger extract lowers blood glucose levels and help prevent disorders in offspring.

Keywords: Maternal diabetes, Postnatal neurodevelopment, Streptozotocin, Wistar rats, Ginger

INTRODUCTION

Natural products include a variety of chemical compounds that have been evolutionarily selected for their ability to improve the survival of an organism.[1]

Because of the diversity of their biological activities, they have been widely used in the field of human health as a dietary supplement in traditional medicine for thousands of years.[2]

Ginger (*Zingiber officinale*) is a plant frequently used for thousands of years as a culinary seasoning, in particular in Asian cuisine, and is a common medicinal

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agent in the traditional medicine systems of China, India and other Eastern cultures. Ginger has several beneficial pharmacological effects (hypoglycemic, insulinotropic, hypolipidemic) in laboratory animals [3] and in humans.[4]

It has been demonstrated that ginger or its extracts possess certain pharmacological activities, including analgesic effects[5]. Herbal medicines are considered as a fundamental source of new compounds based on pharmacological active principle, where about 11% of basic drugs in the 21st century are derived exclusively from plant origin[6].

In our study, we investigate the effect of maternal diabetes induced by streptozotocin in Wistar rats on postnatal neuro-development of offspring. Moreover, the effect of a ginger extract on maternal hyperglycemia and its impact on the development of offspring.

METHODOLOGY

Experimental Animals

Wistar strain female and male (that will be used for mating) weighing 240 ±10 g were obtained from Pasteur Institute (Algiers, Algeria). The rats were housed in clean polyethylene cages (58 × 38 × 19 cm, 5 rats per cage for each sex) and maintained at standard facility conditions of temperature (25 ± 2 °C), humidity (65 ± 5%) with a photoperiod of 12 h light and 12 h dark cycle. The rats were fed with commercial chow and tap water ad libitum. Procedures for the care and handling of animals were in compliance with current international laws and policies (NIH Publication No. 85–23, 1985, revised 1996).

Study desing

After adaptation period, the female rats were divided into four (4) experimental groups, and treated as follows:

- Group 1: included control dams (C)

- Group 2: included dams treated with ginger extract (G)

- Group 3: included diabetic dams (D),

- Group 4: included diabetic dams treated with ginger extract (D + G)

Animal Treatments

Diabetes Induction

Streptozotocin (STZ) was obtained from Sigma – Aldrich chemicals (USA).

Diabetes was induced by a single intraperitoneal injection of a freshly prepared

solution of STZ (50mg/kg body weight) in 0.1 M citrate buffer (pH 4.5). Animals were considered as diabetic if their blood glucose values were above 250 mg/dl on the third day after STZ injection. The blood glucose levels were mesured from the rat tail vein[7] by using Accucheck verio glucometer (United Kingdom).

Ginger extract administration

The ginger extract used in our experiment is a product of a leading Life Science and High Technology company (Sigma-Aldrich, United States)

(Ginger extract-naturel, Sigma-Aldrich, code: W 252108, CAS Number: 84696-15-1).

Ginger extract was conducted to pregnant rats in groups (G) and (D + G) by oral gavage once daily in 80mg/kg body weight/ day at 08h :30 in 6 consecutive days, from the fourteenth (14th) to the nineteenth (19th) day of pregnancy.

Mating

The female rats of the different groups were individually housed in appropriate polyethylene cages and subjected to the first vaginal smears to determine estrus cycle phases based on a standard cytological analysis.[8] Each pro-estrus female was placed overnight in cage with one male of the same strain. Second vaginal smears were examined the next morning and the presence of spermatozoa was considered as day 1 of pregnancy (gestationel day 1 = GD 1).

Farrowing

Parturition day was designated as PND 0. (PND = Postnatal day).

For the experimental study of offspring, we used 12 male and 12 female rats from each group.

Postnatal development evaluation

In order to monitor the postnatal development, the offspring were weighed in PND 1, PND7, PND 14 and PND 21.

Tests applied in male and female pups (Before and after weaning)

Negative geotaxis (PND8)

The negative geotaxis test is supposed to evaluate motor coordination and the cerebellar integration. The test consists in placing the rattons at PND 8 on an inclined plane at an angle of 25 degrees to the horizontal and their heads pointed down the slope. The measured variable is the latency time for a rat to make a complete half-lap of 180 degrees, ending up at the top of the slope. (measurement of equilibration, maturation of the cerebellum and semicircular

canals of the inner ear).[9]

Object recognition test (PND 45)

The test is performed in order to evaluate the recognition memory in rodents because it uses their natural preference for a new object compared to a familiar object. [10]

At postnatal 45, the offspring were placed in an open arena (50 × 50 × 40) and allowed to freely explore two identical objects during a pretest of 5 minutes (exploration phase) and then returned to their cages. After a one-hour retention phase, the pups were resubmitted for 5 minutes at the test session (recognition phase) during which one of the two familiar objects previously explored was replaced by a new object.[11] The recognition memory was evaluated during the test session and expressed by the recognition index which indicates the ratio of the exploration time of the new object on the exploration time of the two objects.

Statistical analysis

All results were expressed as the means ± standard deviation (M±SD).

Statistical calculations were made using Minitab software (version 18.1.0.0, Minitab Inc., USA). The comparison between the different groups was performed using Student's t test.

RESULTS

Maternal glycemia

Table 1. Variation in maternal blood glucose during the experiment (g/l). Results are expressed as the mean ± SD (n = 7).

Groups	Before gestation		During gestation			After gestation	
	Before induction	After induction	GD 1	GD 14	GD 19	PND 1	PND 21
C	**0.94±0.10**	0.89±0.05	0.91±0.04	0.92±0.07	0.90±0.11	0.93±0.03	0.88±0.12
G	**0.90±0.11**	0.92±0.07	0.93±0.09	0.94±0.04	0.89±0.03	0.90±0.08	0.89±0.07
D	**0.92±0.03**	3.04±0.13	3.20±0.12	4.10±0.15	4.32±0.17	4.41±0.10	4.71±0.23
D + G	**0.88±0.05**	3.08±0.09	3.17±0.05	4.13±0.12	2.57±0.21	2.53±0.14	2.52±0.17

On the third day after the induction of diabetes, the blood glucose levels in STZ injected diabetic rats were significantly (P<0.001) increased than of normal control rats. However, we found that the elevated blood glucose levels in diabetic rats (D+G) were significantly (P<0.001) decreased after 6-days ginger administration compared to diabetic rats (D). (Table 1)

Offspring body weight evolution

Pups of diabetic dams (D) gained significantly less weight than those from control dams during the postnatal development ($p < 0.001$). However, pups of diabetic dams treated with ginger (D + G) gained significantly more weight than those from diabetic dams during the postnatal development ($p < 0.001$). The difference at PND 21 is very highly significant (p <0.001), (Fig 1)

Figure 1. Offspring body weight change (g) during the development from PND 1 to 21. Results are expressed as the mean ± SD; n = 24 (12 males, 12 females). (β: comparison vs C), (γ: comparison vs D).

Body weight of male and female pups

Groups	PND 1		PND 7		PND 14		PND 21	
	M	F	M	F	M	F	M	F
C	5.83±0.55	5.66±0.62	13.08±1.11	12.66±1.02	25.50±0.86	24.08±1.32	42.08±1.38	40.58±1.18
G	5.94±0.44	5.76±0.40	14.25±0.59	13.33±1.02	26.75±0.72	24.83±1.14	44.41±0.49	41.50±1.11
D	4.91±0.64	5.16±0.37	8.25±0.92	8.75±1.09	11.25±1.16	11.91±1.03	18.83±0.89	19.66±0.94
D + G	5.33±0.47	5.41±0.49	9.75±0.59	10.16±0.79	15.08±0.64	16.25±0.82	24.50±0.86	26.25±0.92

Table 2. Body weight of male and female offspring (g). Results are expressed as the mean ± SD; n = 24 (12 males, 12 females).

Table 2 shows the body weight (g) of male and female offspring at day PND1, PND7, PND14 and PND21. We found a delay in weight growth in males of diabetic dams (D) compared to females. In PND 1 and PND 7 our results show no significant difference in body weight between males and females from diabetic (D) and diabetic ginger-treated dams (D + G) (P> 0.05). PND 14 and PND 21 showed a very highly significant increase in body weight of females from ginger-treated diabetic dams (D + G) compared with males (p <0.001). In PND 21, our results show a significant increase in body weight of females from diabetic dams (D) compared with males (P <0.05), (Table 2).

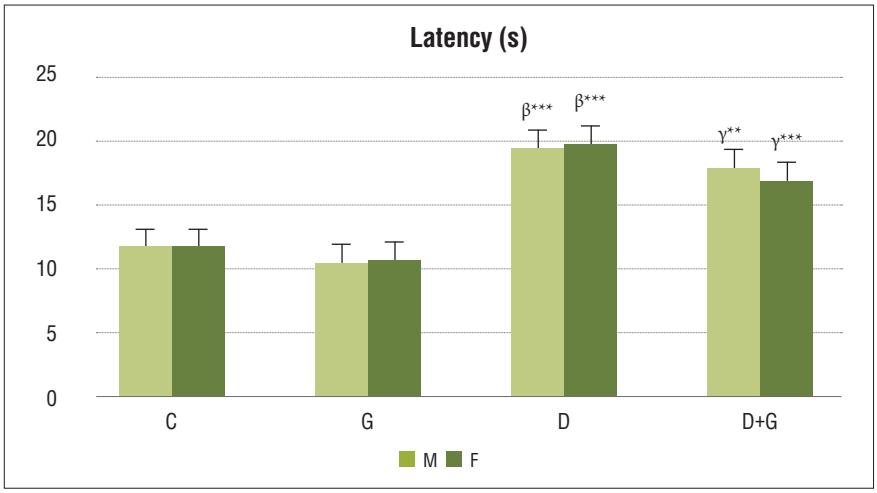

Figure 2. Latency time (s) in the negative geotaxis test at day PND 8. Results are expressed as the mean ± SD ; n = 24 (12 males, 12 females). (β: comparison vs C), (γ: comparison vs D)

Variation in postnatal neurodevelopment parameters of pups:

Negative geotaxis

According to this test, latency significantly increased (p <0.001) in male and female pups of diabetic dams (D) compared to pups of control dams. In contrast, highly significant decrease (p <0.01) in latency in male offspring of diabetic dams treated with ginger (D + G) compared to male offspring from diabetic dams (D), while in female offspring, significant decrease (p <0.001). Latency significantly (P<0.05) decreased in female of diabetic dams treated with ginger (D + G) compared to males (Fig 2).

Object recognition test

As depicted in Fig 3, recognition index significantly (P<0.001) decreased in males and females of diabetic dams as compared to pups of control dams. Significantly increase (P<0.001) of recognition index in offspring (male and female) of diabetic dams treated with ginger (D + G) compared to offspring of diabetic dams (D), (Fig 3).

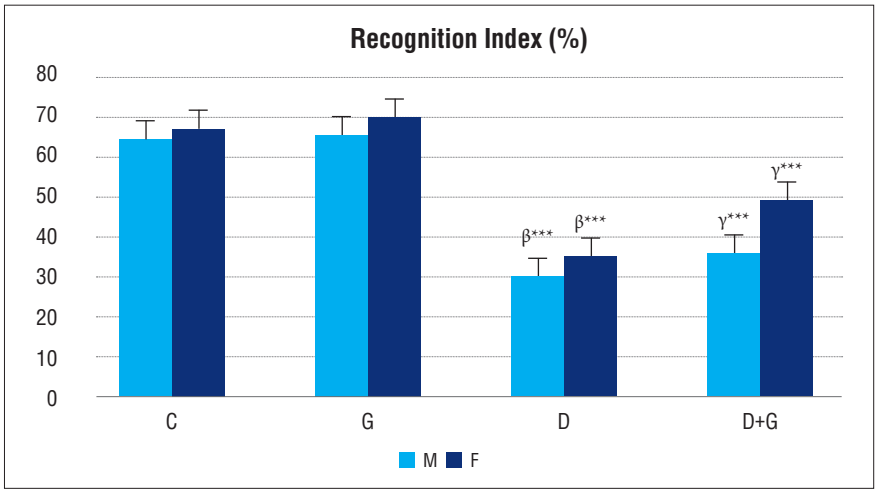

Figure 3. Recognition index in offspring at PND 45. Results are expressed as the mean ± SD; n = 24 (12 males, 12 females). (β: comparison vs C), (β: comparison vs D).

Diabetes mellitus is a group of chronic metabolic disorder characterized by hyperglycemia that causes lesions affecting several devices or systems especially vessels and nerves.[12]

In this study we reported the effects of severe maternal hyperglycemia induced by streptozotocin on development of offspring and the ability of a ginger extract to restore glycemic balance in dams and prevent the onset of disorders in offspring.

Elevated maternal blood glucose in diabetic groups testifies the installation of the diabetic disease.[13] According to the conclusions of several studies, this hyperglycemia is due to the effects of Streptozotocin by its action on pancreatic β cells, leading to a reduction of the cell mass.[14] Streptozotocin activates the expression of protein kinase C, responsible for the dephosphorylation of the insulin receptor.[15]

Ginger has been used traditionally to treat a large number of conditions such as fever and infectious diseases, abdominal pain, abdominal spasms, nausea and vomiting, motion sickness, arthritis, rheumatism, ulcerative colitis, gin-

givitis hypertension and diabetes.[16] The bioactive components of ginger were characterized by spectroscopic analysis in the form of zingerone, gingerdione, dehydrozingerones which had a potent antioxidant, shogaols, gingerols and a volatile oil.[17]

Despite the introduction of hypoglycaemic agents from natural and synthetic sources, diabetes and its complications remain a major medical problem in the world's population.

Currently, some medicinal plants are used to treat diabetes, and ginger is one of the most potent herbs traditionally used to treat diabetes mellitus.[18]

Treatment of diabetic pregnant rats (D + G) with ginger extract for six consecutive days reduced glycemia. This is in agreement with previous reports.[3]

According to the literature, two models have been proposed to study diabetes in rodents during pregnancy and its implications for offspring. Moderate diabetes model causes mild hyperglycemia in mothers and macrosomia in the offspring, being similar to repercussions of diabetes type 2 and gestational diabetes mellitus. On the other hand, severe diabetes model causes high hyperglycemia in mothers and microsomia in the offspring.[19,20] Both macrosomic and microsomic infants may have long-term consequences at childhood, adolescence, and adulthood.

Our results showed that the body weight of the offspring of diabetic dams is lower than that observed in offspring of control dams as previously reported by Piazza *et al.*[21].

Compelling evidence have shown that this maternal type 1 diabetes model can induce microsomia in the offspring, possibly as a result of intrauterine growth restriction with placental commitment, [20,22,23] leading to long-term effects, especially in metabolic and neurologic parameters in the infants.[24,25]

The continuation of the hyperglycemic status in diabetic dams (D) after parturition seemed to contribute to the reduced growth during suckle, and pups remained small until weaning age (PND 21) as previously described by Chandna *et al.* [26] The observed effects in offspring may not be only due to the gestational diabetes, but also to the malnutrition at early stages of offspring development, since that maternal type 1 diabetes can delay lactogenesis onset by affecting prolactin secretion and lactose, citrate, and total nitrogen concentrations in the milk.[27]

On the other hand, in the offspring of diabetic dams treated with ginger (D + G), our results showed an increase in body weight, which may be due to the decrease in maternal blood glucose after treatment with ginger extract and

stimulation of lactation in these dams.

Developmental milestones play an important role in assessing the maturation of neonatal neurologic reflexes and serve as predictors of behavioral changes in adults.[28]

In our study, the offspring of diabetic dams presented neurodevelopmental delay in the negative geotaxis test according to and complementing the cognitive impairments reported by the clinic.[29,24] Furthermore, a decrease in the object recognition index this is in agreement with the results of Kim *et al.* [30] who have demonstrated, with the same experimental model, impaired memory in step-down avoidance task on PND42.

The administration of ginger in diabetic dams during pregnancy had a beneficial effect on the neurological development of the pups, latency time in the negative geotaxis test was improved especially in females suggesting selective action on one sex. Also, there has been an increase in recognition memory in offspring of diabetic dams treated with ginger.

In addition, this plant extract and its active component, 6-gingerol, also inhibited cholinesterase activity, which increased acetylcholine, a neurotransmitter that plays an important role in learning and memory.[31]

There is considerable recent experimental evidence that ginger treatment has significantly improved word recognition, numerical alertness, choice reaction, digital working memory and spatial working memory scores in middle-aged women.[32]

These studies have shown that ginger can improve cognitive decline in the early stage of dementia in old age. Ginger intake during critical moments of fetal development is associated with an interaction with the neurophysiological processes of postnatal behavior.

Maternal diabetes is an intrauterine environment that is detrimental to the development of offspring, can cause brain damage and increases the risk of neurological disorders.

Our results indicate that severe maternal hyperglycemia during the fetal and perinatal period predisposes offspring to delayed physical development and neurodevelopmental disorders.

Our study clearly showed that treatment of diabetic pregnant rats with a ginger extract lowers blood glucose levels and can help prevent disorders in offspring.

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