ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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

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Contents

Aims and Scope of Acta Pharmaceutica Sciencia

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

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

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

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK

Editor

INSTRUCTIONS FOR AUTHORS

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

Manuscripts should contain the following elements in the following order :

Title Page

Abstract

Keywords

Introduction (Without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

It is best to use the Times New Roman' font, 12 font size, and all kinds of articles must be double-spaced including text, references, tables, and legends.

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

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

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

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

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

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

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

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

References in the text should be identified using Arabic numerals. Years of the references should be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication. References should conform to APA style and be numbered consecutively in the order in which they are cited in the text.

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

1. Scope and Editorial Policy

1.1 Scope of the Journal

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

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

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

1.2 Manuscript Categories

Manuscripts can be submitted as Research Articles and Reviews.

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

1.2.2 Reviews integrate, correlate, and evaluate results from published literature on a particular subject. They expected to report new and up to date experimental findings. They have to have a well-defined theme, are usually critical, and may present novel theoretical interpretations. Up to date experimental procedures

may be included. Reviews are usually submitted at the invitation of the Editors. However, experts are welcome to contact the Editors to ensure that a topic is suitable. Approval is recommended prior to submission.

1.3 Prior Publication

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

1.4 Patents and Intellectual Property

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

1.5 Professional Ethics

Editors, reviewers, and authors are expected to adhere to internationally accepted criteria's for scientific publishing. Helsinki declaration is applied and accepted for the ethical standards of the journal.

World Medical Association. (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects.. *Bulletin of the World Health Organization*, 79 (4), 373 - 374.

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

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

1.5.3. Use of Human or Animal Subjects. For research involving biological samples obtained from animals or human subjects, editors reserve the right to request additional information from authors. Studies submitted for publication

approval must present evidence that the described experimental activities have undergone local institutional review assessing safety and humane usage of study subject animals. In the case of human subjects authors must also provide a statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the studies' Material and Methods.

World Medical Association. (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects.. *Bulletin of the World Health Organization*, 79 (4), 373 - 374.

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1 General Considerations

Manuscripts should be kept to a minimum length. Authors should write in clear, concise English, employing an editing service if necessary. For professional assistance with improving the English, figures, or formatting in the manuscript before submission please contact to editorial office by e-mail for suggestions. Authors are required to subject their manuscript for

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

It is best to use the 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 200 words, organized in a findings-oriented format in which the most important results and conclusions are summarized. Code numbers may be used once in the abstract. After the abstract, a section of Keywords not more than five has to be given. Be aware that the keywords, chosen according to the general concept, are very significant during searching and indexing of the manuscripts.

Key words: instructions for authors, template, journal

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

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

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

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

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

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

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

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

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

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

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

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

2.2.7 References and Notes. APA style is used in the reference list and citations. List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. APA style is used in the reference list. However, in-text citations should be given superscript numbers (e.g. 1) according to order in the manuscript. List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used.

Journal article examples

Article with two authors example:

Tinworth, C. P., & Young, R. J. (2020). Facts, Patterns and Principles in Drug Discovery: appraising the Rule of 5 with measured physicochemical data. *Journal of Medicinal Chemistry*. doi:10.1021/acs.jmedchem.9b01596

Sahin, Z., Ertas, M., Berk, B., Biltekin, S. N., Yurttas, L., & Demirayak, S. (2018). Studies on non-steroidal inhibitors of aromatase enzyme; 4-(aryl/heteroaryl)-2-(pyrimidin-2-yl)thiazole derivatives. *Bioorganic & Medicinal Chemistry*, 26(8), 1986–1995. doi:10.1016/j.bmc.2018.02.048.

Articles up to 6 authors should be given examples as above, and articles with more than 6 authors should be given as et al. after sixth author.

APA Style examples:

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

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

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

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

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

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

below for TIFF file production resolutions.

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

- Black & White line art: 1200 dpi

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

- Color art (RGB color mode): 300 dpi

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

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

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

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

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

2.3 Specialized Data

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

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds.

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

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

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

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

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

3. Submitting the Manuscript

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

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

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

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

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

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

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

3.2.2 Institutions Authors should give their institutional information during submission.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ORIGINAL ARTICLES

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Investigating the stress effects on Fexofenadine hydrochloride and Montelukast sodium in bulk and tablet dosage form using a validated LC-DAD method

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ABSTRACT

The main aim was to develop RP-HPLC method for the simultaneous estimation of Fexofenadine hydrochloride and Montelukast sodium in tablet dosage form. A mobile phase comprising solvent A as 0.1% v/v trifluoroacetic acid in water and solvent B as acetonitrile with methanol in the ratio of 2:3% v/v was used and the flow was set in a gradient mode with a stationary phase of phenyl-hexyl silica column (150 × 4.6 mm, 5µ i.d).The detection wavelength is 254 nm using PDA detector. The method was validated according to ICH parameters. The results denote that the method is linear and within the range of 60-180 µg/ml for Fexofenadine and 5-15 µg/ml for Montelukast with the r2 of 0.9998 and 0.9999 respectively. The degradants peaks non-interfered with main peaks. The developed method is acceptable for the determination of Fexofenadine and Montelukast in routine analysis of dosage form.

Keywords: FEX-Fexofenadine hydrochloride, MON- Montelukast sodium, Co-elution, forced degradation, Gradient elution.

INTRODUCTION

Fexofenadine hydrochloride (FEX) is chemically 2-[4-[1-hydroxy-4-[4-hydroxy (diphenyl) methyl] piperidin-1-yl] butyl] phenyl]-2-methylpropanoic acid (figure 1) with amolecular mass of 501.68 g/mol and log P value of 2.81.[1] It is an second generation antihistaminic agent used for the symptomatic relief of nasal

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congestion, itching, management and treatment of diseases like chronic urticaria and allergic rhinitis[2,3]

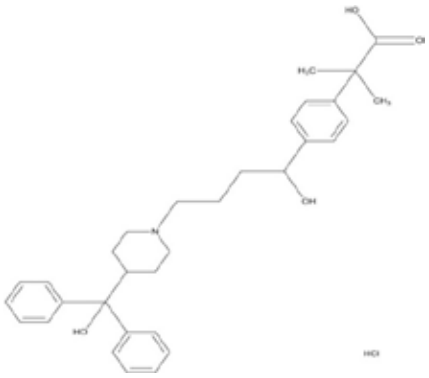

Figure 1. Structure of Fexofenadine hydrochloride

Montelukast sodium (MON) is chemically 2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl) ethenyl] phenyl]-3-[2-(2-hydroxypropan-2yl) phenyl] propyl] sulfanylmethyl] cyclopropyl] acetic acid (figure 2) with a molecular weight of 586.187 g/mol and log P value of 7.9[4]. It is an anti-asthmatic agent and cysteinyl leukotriene receptor antagonist which slackens the inflammation mediating action exerted by immune system and averts broncho constriction as well asinflammation in respiratory diseases[5]. FEX and MON in combined tablet dosage form areused for the treatment of tenacious allergic rhinitis, prurigo nodularis and pemphigoid nodularis. Fexofenadine is a non-sedating agent possessing inflammatory mediating action like MON. Both the drugs in concomitant dosage for mareused in the treatment of allergic reactions and inflammatory mediating actions[6].

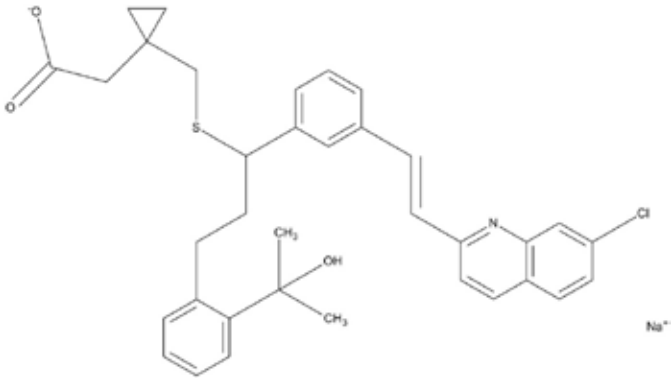

Figure 2. Structure of Montelukast sodium

A number of researchers have reported various analytical techniques for the determination of FEX and MON either alone or in combination from different matrices. These methods include electrometric titration[7,8],spectrometric methods such as colorimetry[8-13], UV-spectrometry[14-22],spectrofluorimetry[23-25],chromatographic techniques like Thin Layer Chromatography [26],High Performance Thin Layer Chromatography[27,28], High Performance Liquid Chromatography[22,29-56],Ultra Pressurized Liquid Chromatography[57,58],Hyphenated techniques such as Liquid chromatography- mass spectrometry[59-63]and capillary electrophoresis [62].Official HPLC methods are available for both the drugs individually in USP[63, 64]. Application of reported USP monograph method of Fexofenadine for the simultaneous estimation of FEX and MON fetched no peak for MON. Alternatively application of official USP method of MON for the determination of two drugs simultaneously resulted inpoor separation of two peaks. Reported stability indicating assay methods for simultaneous estimation of two drugs by HPLC[33-37]using C_{18} and C_8 analytical column, present the main peaks very near to the dead volume and the separation of degradants peaks were not clear due to co-elution with the main peaks.

In our present work, an ion pair agent was used in HPLC stability indicating assay to get good peak shapes. Reported stability indicating methods make use of C_{18}/C_8 column for the separation of two drugs where there are hydrogen bond interactions. Comparatively in our method phenyl-hexyls silica column[65]was used which exhibited both hydrogen bond as well as aromatic interactions. In order to obtain the complete separation of degradants peak from the significant main peaks the retention time values of both the analytes should be well separated so that there may not be overlapping of peaks. Also the gradient mode of separation prevents co-elution of degradants with the main peaks. Based on the log P values (non-polar) of both the drugs, it can be predicted that usage of more organic modifiers will result in poor resolution leading to the concurrent elution of the degradants along with the main peaks[66]. The developed method was validated according to ICH guidelines with the parameters like accuracy, linearity, precision, robustness, specificity, system suitability, etc.[67]

METHODOLOGY

Chemicals and Reagents.

Methanol (HPLC grade obtained from Finar, Mumbai, India), Acetonitrile (HPLC grade obtained from Finar, Mumbai, India), Trifluoroacetic acid, Sodium hydroxide, 30% Hydrogen peroxide, Hydrochloric acid (Analytical Reagent grade obtained from Rankem, Mumbai, India) and Milli Q water as double distilled water and membrane filters 0.45µm as membrane filters from Millipores Ltd, Banglore, India.

Instruments and Software

Chromatography study was performed in Empower software with Waters 2695 separations module with Photodiode array detector 2996 with a quaternary pump and autosampler injections. Separation was carried out using Phenonomenex, Phenyl-hexyl silyl column (150 × 4.6 mm, Luna 5µ i.d.) obtained from-Hyderabad, India. Analytical balance (semi-micro) Shimadzu AUW220D from Chennai, India. Ultrasonicator (LMUC-12) Spectrum tek from Chennai, India, Digital pH meter (PH12-5p-920) spectrum tek from, Hot air oven from Inlab equipment Pvt. Ltd. from Chennai, India.

Methods

Pure Standards

FEX and MON were obtained from Vital laboratories Pvt. Ltd, Gujarat, India with percentage purity of 99.07% (CAS No-**153439-40-8)** and 98.12% (CAS No 151767-02-1) respectively.

Pharmaceutical Formulation

Generic tablets of FEX (120 mg) and MON(10 mg) were obtained from the manufacturer Saimirra innopharm Pvt. Ltd., Chennai, India

Preparation of stock and working solutions

A stock solution of FEX (1.2 mg/ml) and MON (0.1 mg/ml) was prepared by accurately weighing 120.0 mg of FEX and 10.0 mg of MONusing Methanol: water (3:1) as diluent. Working standard solution was prepared in the concentration of FEX (120 µg/ml) and MON (10 µg/ml).

Chromatographic conditions

Chromatographic separation was carried out using (Phenomenex) Phenyl-hexyl silyl column (150 × 4.6 mm, Luna 5µ i.d.) in gradient mode. The mobile phase used was Solvent A: 0.1% v/v trifluoroacetic acid in water and solvent B: Acetonitrile: Methanol (2:3). Flow rate of 1.5 ml/min was maintained with an injection volume of 15 µl and thedetection was set at 254 nm using PDA detector. Prepared mobile phase was filtered using 0.45 µm Milli pore membrane filter. Total run time is 22.0 min, where the gradient elution is maintained at0-5mins solvent A is 48% and solvent B is 52%, at 10-17mins solvent A is 35% and solvent B is 65% and after 20 min the gradient comes back to initial condition. Chromatographic separation was carried out in ambient condition.

Calibration curve

From the stock solution, serial dilutions were made to prepare working standard solutions of FEX and MON in the concentration range of 60-180 µg/ml and 5-15 µg/ml respectively. A calibration curve is constructed with area versus concentration in microgram/ml.

Preparation of Sample Solution

Twenty tablets were finely powdered and sample solutions were prepared by accurately weighing a weight equivalent to 120 mg of FEX and 10 mg of MON and further dilutions were made to give concentration of 120 µg/ml and 10 µg/ml respectively.

Forced Degradation Studies

Forced degradation is stress testing and the dosage form is subjected to forced degradation using accelerated environmental conditions. Various impurities which arise during the storage of drug products in different environmental conditions can be studied using these stress studies. It also helps to calculate the inherent stability of the molecule, to determine the degradation pathways and endorses the stability indicating assay[67]. In this study the combined dosage form of FEX and MONwas exposed to degradation studies like acid hydrolysis, alkaline hydrolysis, thermal degradation, oxidative degradation and photolytic degradation

Acid hydrolysis

Acid hydrolysis was carried out in 807 mg of powdered tablet which is treated with 5 ml of 0.1N hydrochloric acid in a 100 ml volumetric flask. The volumetric flask was heated on a water bath at 60°c for 2 hours and allowed to cool. Neutralized the solution with 5 ml of 0.1N sodium hydroxide and diluted the volume with diluent. Final solution was made up to the concentration of FEX (120 µg/ml) and MON (10µg/ml) with diluent.

Alkaline hydrolysis

Accurately weighed 807 mg of powdered tablet and transferred to a 100 ml volumetric flask to which 5 ml of 0.1N sodium hydroxidewas added. The flask was heated on a water bath at 60°c for 2 hours and allowed to cool. Neutralization was carried out using 5 ml of 0.1N hydrochloric acid andmade up to volume with diluent to give a final concentration of FEX (120µg/ml) and MON (10µg/ml).

Thermal degradation

Accurately weighed 807 mg of powdered tablet and it was exposed to 105°c for

2 hours in hot air oven. Sample was allowed to cool and transferred to 100 ml of volumetric flask, the volume was made up with thediluent to give a concentration of FEX (120 µg/ml) and MON (10µg/ml).

Oxidative degradation

Oxidative degradation was carried out using 5 ml of 30% hydrogen peroxide (H_2O_2) to 807 mg of sample in 100 ml volumetric flask. Heated the flask on water bath for 2 hours at 60°c and allowed to cool. Final concentration of FEX (120µg/ml) and MON (10µg/ml) were prepared with diluent.

Photolytic degradation

Accurately weighed 807 mg of powdered tablet sampleand it wasexposed to sunlight for 2 hours, allowed to cool and transferred to volumetric flask. The final solution was made up with diluentto give a concentration of FEX (120 µg/ml) and MON (10µg/ml).

All the prepared solutions were injected in chromatographic system and the chromatograms were recorded for degradation studies.

RESULTS and DISCUSSION

Method development

Chromatographic conditions such as analytical column, mobile phase, flow rate, column temperature, sample temperature, detection wavelength were optimized for better separation of main peaks with degradants peaks.

Various mobile phase compositions, detection wavelength and analytical column from the reported methods[33-37]and official monograph methods[63,64] were tried during method development. While trying thefirst two reported methods with mobile phase composition of Phosphate buffer pH 6.0: Methanol (25:75) and 0.1% Triethylamine: Acetonitrile (30:70), good peak shape was obtained in both the methods butthe resolution factor and plate countwere less showing non-compliant intermediate precision. Third trial was with mobile phase ratio of ortho phosphoric acid and methanol (40:60) using C_8 analytical column, which yield eda poor resolution where there is a possibility of overlapping of degradants peak with that of analytes. After several trials, the chromatographic conditions were optimized as given and used in gradient mode: the mobile phase - 0.1% trifluoroacetic acid in water (Solvent A), acetonitrile with methanol (2:3) (Solvent B). FEX is less non-polar than the MON, so between 0-5 min Fexofenadine got eluted from the column and in5-17 min MONgot eluted due to increased non-polarity in mobile phase. Gradient mode was followed for the better separation of two peaks, so that the degradants peak can be well defined

by avoiding co-elution in forced degradation assay. The detection wavelength was set by isobestic point of two drug molecules in UV spectroscopy. The results of quantitative estimation are given in Table1 and %RSD was less than 2%. The standard chromatogram is shown in the figure 3.

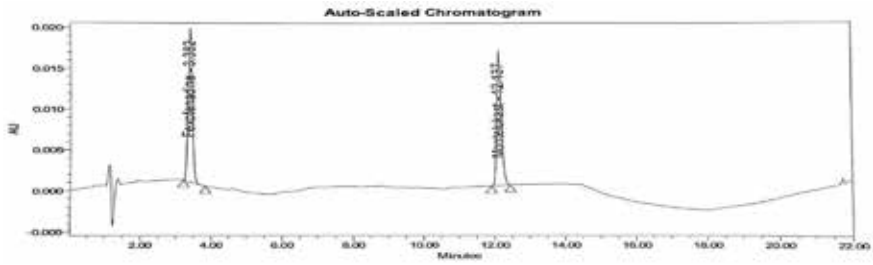

Figure 3. Chromatogram of 100% concentration of (120 µg/ml) Fexofenadine hydrochloride and (10 µg/ml) Montelukast sodium.

Table 1. Quantitative estimation of FEX and MON.

S.No	Sample Name	Retention Time		Amount present (%)	
		FEX	MON	FEX	MON
1	Standard	3.338	12.048	110.31	101.11
2	Sample 1	3.332	12.056	98.25	99.31
3	Sample 2	3.334	12.045	99.56	100.31

Validation of the Method

The developed method was validated according to ICH guidelines[68].

System suitability

System suitability was carried out by injecting six replicates of standard solution containing 120 µg/ml of FEX and 10 µg/ml of MON. The parameters like retention time (t_R), theoretical plates (N) and peak reproducibility are reported in Table 2.

Table 2. System suitability parameters for FEX and MON.

Parameter	Response	
	FEX	MON
t_R (min)& (RSD %)	3.290&(0.08)	12.006&(0.05)
Theoretical plates	3894	42733
Tailing factor	1.30	1.20

Linearity& Range

Determination of linearity of the method was executed by plotting the calibration curve of peak area versus the concentration range of 60-180 µg/ml FEX and 5-15 µg/ml MON. Result of linearity was statistically examined by correlation coefficient, slope of the regression line and y- intercept. The data are presented in Table 3.

Table 3. Calibration data for FEX and MON.

Parameters	Response	
	FEX	MON
Range	60.00-180.00 µg/ml	5.00-15.00 µg/ml
Slope	12419.70	144000.00
Intercept	670.68	121.30
y-intercept	0.45	0.08
R^2	0.9998	0.9999

Accuracy

Accuracy of the method was determined by preparing three known concentrations of FEX and MON comprising60 & 5 µg/ml, 120 & 10 µg/ml and 180 & 15 µg/ml respectively representing 50%, 100% and 150% of working standard solution. Prepared solutions were injected into HPLC in triplicate manner and the results are reported in Table4. Results show that relative standard deviation (%RSD) was found to be less than 2.

Table 4. Accuracy of the proposed method at three different concentrations of FEX and MON.

Concentration %	Amount added (mg)		Amount found (mg)		Recovery (%)		Mean % Recovery (%RSD)	
	FEX	MON	FEX	MON	FEX	MON	FEX	MON
50%	0.060	0.005	0.059	0.005	98.33	100.23	98.32% (0.03%)	102.75% (1.73%)
	0.058	0.005	0.057	0.006	98.27	104.01		
	0.061	0.005	0.060	0.005	98.36	104.01		
100%	0.121	0.010	0.119	0.010	99.16	98.05	99.71% (1.41%)	98.71% (0.47%)
	0.120	0.011	0.122	0.010	101.66	99.09		
	0.120	0.010	0.118	0.009	98.33	99.00		
150%	0.183	0.015	0.185	0.015	101.09	102.03	100.36% (1.03%)	99.60% (1.73%)
	0.182	0.016	0.180	0.015	98.90	98.12		
	0.181	0.015	0.183	0.014	101.10	98.66		
Mean							99.46% (0.82%)	100.35% (1.31%)

Precision

The precision of the method was calculated by executing intra-day and inter-day precision at 100%concentration (120 µg/ml of FEX and 10 µg/ml of MON). Six injections of the above solutions were injected into HPLC system at some other time of the same day for intra-day precision. For inter-day precision, 100% solutions were injected six times by different analyst on different day. The results showed that the % relative standard deviation (%RSD) was less than 2% and are reported in Table 5.

Table 5. Precision of the proposed method for FEX and MON.

Parameters	Amount added (mg)		Amount found (mg)		Recovery (%)		Mean % Recovery (%RSD)	
	FEX	MON	FEX	MON	FEX	MON	FEX	MON
Intra-day precision	0.120	0.011	0.118	0.010	98.33	99.09	99.17 % (1.27%)	98.92 % (0.96%)
	0.122	0.010	0.119	0.009	97.54	98.01		
	0.118	0.012	0.117	0.012	99.15	100.82		
	0.123	0.011	0.124	0.011	100.81	99.14		
	0.122	0.010	0.120	0.010	98.36	98.14		
	0.120	0.012	0.121	0.011	100.83	98.34		
Inter-day precision	0.123	0.010	0.125	0.011	101.62	101.85	101.10 % (0.91%)	100.45% (1.27%)
	0.117	0.012	0.119	0.012	101.70	101.62		
	0.120	0.010	0.121	0.010	100.83	100.94		
	0.121	0.012	0.123	0.012	101.65	100.80		
	0.120	0.011	0.119	0.011	99.16	99.15		
	0.120	0.012	0.122	0.012	101.66	98.37		
Mean							100.14% (1.09%)	99.68% (1.12%)

Robustness

Robustness was calculated by making intentional small changes in the parameters of assay method. It was checked by changes in flow rate (±0.2 ml/min) and wavelength (±2 nm). The results are reported in Table 6 and it is shown that the intentional changes do not affect the method.

Table 6. Robustness of the proposed method for FEX and MON.

Variation parameters	Accuracy %[a] ±SD		Relative Standard Deviation (%RSD)	
	FEX	MON	FEX	MON
Flow rate (±0.2 ml/min)				
1.3 ml/min	101.82 ± 1.4	101.04 ± 0.8	0.86%	0.49%
1.7 ml/min	100.49 ± 1.6	99.57 ± 1.7	0.73%	0.74%
Wavelength (± 2nm)				
252 nm	103.34 ± 1.8	106.11 ± 1.5	0.87%	0.68%
256 nm	101.61 ± 1.2	101.17 ± 0.8	0.63%	0.66%

Specificity

Specificity is the capacity of the method to determine univocally the analyte in the sample in presence of excipients, impurities and degradation products. In present study specificity study was conducted by the forced degradation of the analyte molecule. All the samples were exposed to stress conditions such as acid hydrolysis, alkaline hydrolysis, thermal degradation, oxidative degradation and photolysis for about 2 hours. The results are reported in Table7 with % of degradation and recovery of analyte with peak purity. The forced degradation chromatograms are featured in Figure4-8. Though various unknown peaks, placebo peaks and a blank peak appeared in the chromatogram, the peak purity values show that the degraded products do not affect the purity of principle peaks of both analytes. Unknown peak 1 was found to be Fexofenadine related compound A with reference to the spectral index results of Radhakrishna and co-workers.[69]

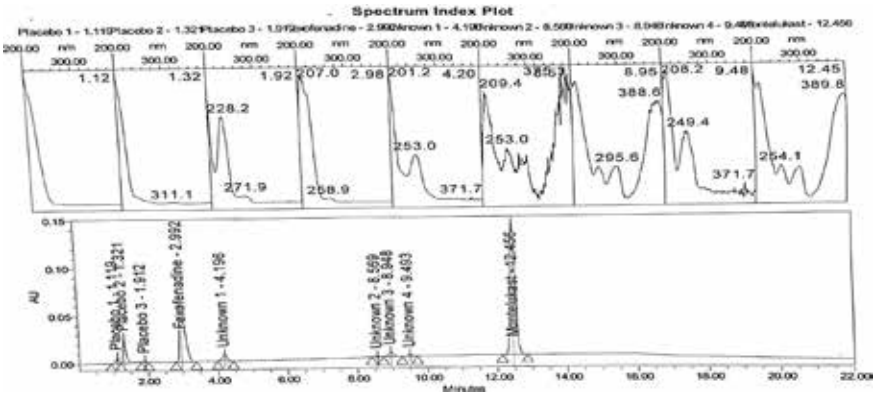

Figure 4. Chromatogram after acid degradation.

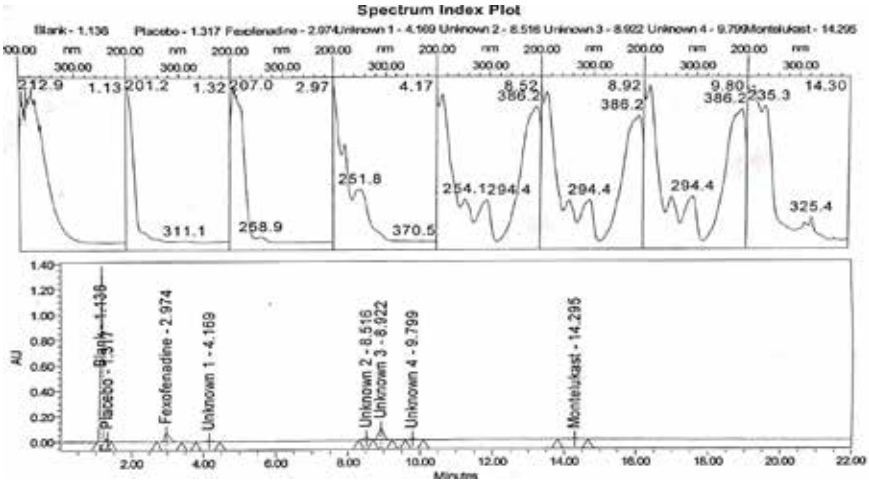

Figure 5. Chromatogram after alkaline degradation.

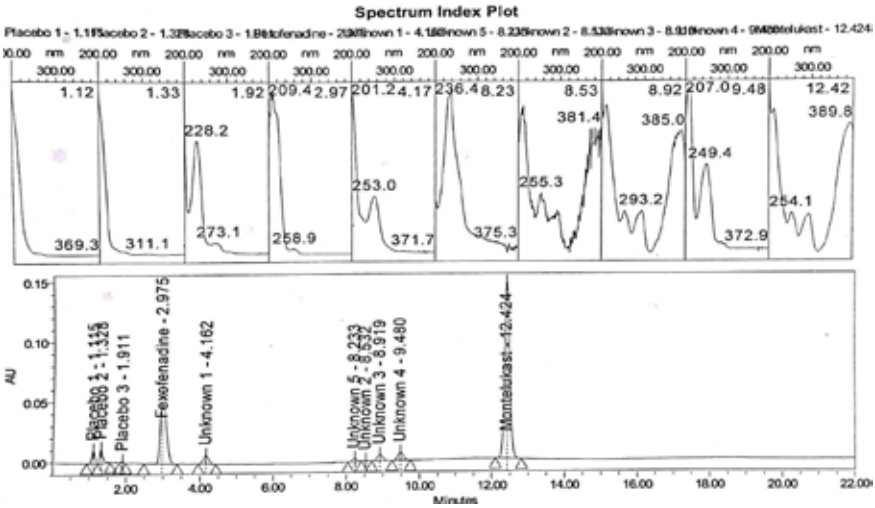

Figure 6. Chromatogram after Oxidative degradation.

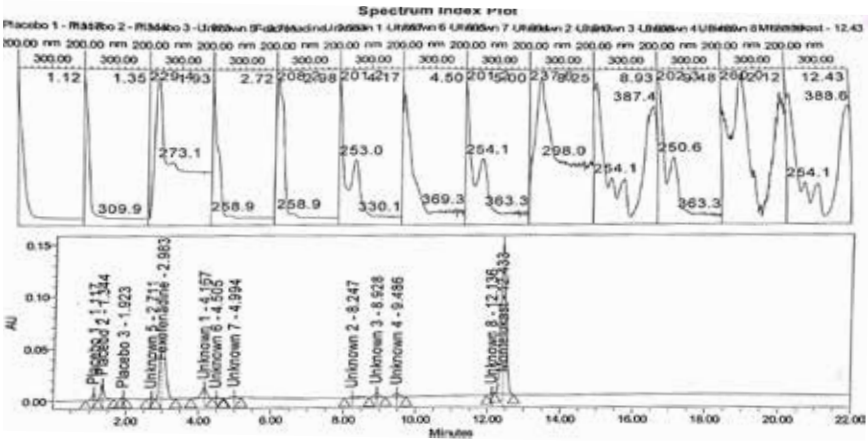

Figure 7. Chromatogram after Photolytic degradation.

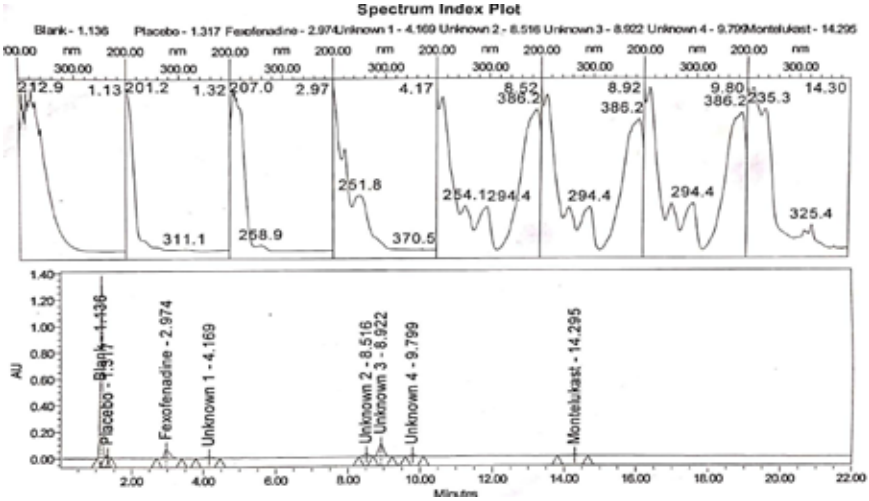

Figure 8. Chromatogram after Thermal degradation.

Table 7. Specifity of the proposed method FEX and MON after forced degradation.

Degradation condition	Recovery %		Degradation %[a]		Peak purity		Threshold purity	
	FEX	MON	FEX	MON	FEX	MON	FEX	MON
Acid	92.50	88.05	7.50	11.95	0.879	0.585	1.125	0.712
Alkali	94.70	96.20	5.30	3.80	0.778	0.545	1.058	0.705
Oxidative	86.50	91.30	13.50	8.70	0.895	0.515	1.180	0.762
Thermal	96.70	97.20	3.30	2.80	0.775	0.543	1.111	0.722
Photolysis	97.20	84.66	2.80	15.34	0.787	0.589	1.021	0.798

Gampa vijaya kumar and co-workers[33]did not observe any additional peaks in the chromatogram of degraded FEX and MON. In the present study FEXhighly degraded in oxidative degradation condition (30% hydrogen peroxide solution) as similar to Maher and Co-partner work[29]. Rameezuddin and collaborators[34]reported a very less oxidative degradation of 5.3% due to the usage of 3% hydrogen peroxide solution for FEX.Rajeev kumar and confederates[35]did not observe any supplementary peaks in the chromatogram of degradation whereasin the same degradation conditions unknown additional peaks were observed in the chromatogram of present study. Montelukast sodium is highly degraded in photolytic degradation study which is showing a similar response like the report of Juliana roman and co-partners [32]. The reason for high photo-degradation is that MON is a photosensitive compound [70]

Solution stability

Solution stability is the stability of standard and sample solutions which are injected into the HPLC system. The solvent used for sample solution has chances to decompose the chemical compound during sample preparation process. Normally, a degradation of 2% is allowed for solution stability from its initial condition [71]. Both the standard and sample solutions were found to be stable up to 25 hours.

Filter integrity

Filter integrity study is used to determine that the filters are used for the intended purpose and does not interfere in analysis. Filters such as nylon membrane, Teflon, Polyvinylidene fluoride, Polytetrafluoroethylene membrane filters were used during the sample preparation. None of the above filters used were found to interfere with study. Results are reported in Table8

A simple, rapid, precise stability indicating assay method using RP-HPLC for the determination of FEX and MONin tablet dosage form was developed. The proposed method was validated according to ICH guidelines with the validation parameters such as accuracy, precision, robustness, intra-day , inter-day precision and specificity. The percentage degradation was within the limit for commercial tablets and the degraded peaks do not interfere with significant main peaks. The proposed method can be used for routine analysis and quality control analysis of pharmaceutical preparations. The identification of unknown impurity peaks may be taken up as further research in this study.

Table 8. Filter integrity of the method.

S.No	Filter Integrity	% Deviation from the initial area	
		FEX	MON
1	Centrifuge	-	-
2	PVDF 0.45 µm (Polyvinylidene fluoride)	2.40	0.84
3	Nylon 0.45 µm	2.33	1.23
4	PTFE 0.45 µm (Polytetrafluoroethylene)	2.73	1.29

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Montvale, N.J. (**2006).** *Thomson PDR The Physicians' desk reference, 60th ed.* Florence.

2. Bachert, C. **(2009).** A review of the efficacy of desloratadine, fexofenadine, and levocetirizine in the treatment of nasal congestion in patients with allergic rhinitis. *Clinical therapeutics*, *31*(5), 921-944.

3. Compalati, E., Baena-Cagnani, R., Penagos, M., Badellino, H., Braido, F., Gómez, R. M., ... & Baena-Cagnani, C. E. **(2011)**. Systematic review on the efficacy of fexofenadine in seasonal allergic rhinitis: a meta-analysis of randomized, double-blind, placebo-controlled clinical trials. *International archives of allergy and immunology*, *156*(1), 1-15.

4. Sweetman, S. C. **(2009).** *Martindale: the complete drug reference*. Pharmaceutical press.

5. Scott, J. P., & Peters-Golden, M. **(2013).** Antileukotriene agents for the treatment of lung disease. *American journal of respiratory and critical care medicine*, *188*(5), 538-544.

6. Nalini, C. N., Vinoth, G., & Guganathan, P. Investigating **(2021).** the stress effects on Fexofenadine hydrochloride and Montelukast sodium in bulk and tablet dosage form using a validated LC-DAD method. *Unpublished work*.

7. Rele Rajan, V., Sawant Swapnil, A. **(2013).** A non-aqueous potentiometric titration method for quantitative determination of Fexofenadine form pharmaceutical preparation.*J. Chem. Pharm. Res. 5*, 286-289.

8. Safwan,A., Mouhammed, K., Ruba Mahrouesh. (**2013).** Extractive spectrophotometric and conductometric methods for determination of Fexofenadine Hydrochloride in pharmaceutical dosage forms. *Pharmaceutica Analytica Acta. 82,* 01-06.

9. Narayana, B., & Veena, K. **(2010)**. A new method for the spectrophotometric determination of fexofenadine hydrochloride.

10. Raghubabu, K., & Sanadhyarani, K. **(2014**). Assay of Fexofenadine hydrochloride in pharmaceutical preparation by visible spectrophotometry. *Der Pharma Chemica*, *6*(1), 436-439.

11. Polawar, P. V., Shivhare, U. D., Bhusari, K. P., & Mathur, V. B. **(2008)**. Development and validation of spectrophotometric method of analysis for fexofenadine HCl. *Research Journal of Pharmacy and Technology*, *1*(4), 539-540.

12. Kumar, K. S., Ravich, V., Raja, M. M. M., Thyagu, R., & Dharamsi, A. **(2006)**. Spectrophotometric determination of Fexofenadine hydrochloride. *Indian Journal of Pharmaceutical Sciences*, *68*(6).

13. Kumar, J. V., Ramachandran, D., Sushma, K., & Vijay, S. **(2010).** Visible spectrophotometric methods for estimation of montelukast sodium in bulk dosage forms and formulations. *Orient J Chem*, *26*, 293-6.

14. Breier, A. R., Steppe, M., & Schapoval, E. E. S. **(2007)**. Validation of UV spectrophotometric method for fexofenadine hydrochloride in pharmaceutical formulations and comparison with HPLC. *Analytical letters*, *40*(12), 2329-2337.

15. Dobariya Chandrika, T., Bhumika Patel, R., Zarna Dedania, R., & Vijendraswamy, S. M. **(2015).** UV Spectrophotometric method development and validation for Fexofenadine Hydrochloride in bulk and tablet dosage form. *J. Pharma Res*, *4*, 63-68.

16. Raghu, M. S., Shantharam, C. S., & Yogesh Kumar, K. **(2018).** Application of Bromate-Bromide Mixture as a Green Brominating Agent for the Determination of Fexofenadine Hydro-

chloride Pharmaceutical Dosage Form. *J. Anal. Pharm. Res*, *7*, 13-21.

17. Chaudhary, M., Kumar, P., & Thapliyal, D. **(2018).** Analytical method development and validation for determination of Montelukast by UV-spectroscopy in API and in pharmaceutical dosage forms. *Int. J. Pharm. Bio. Sci*, *4*, 482-487.

18. Pallavi, K., & Babu, S. **(2012).** Validated UV Spectroscopic method for estimation of Montelukast sodium from bulk and tablet formulations. *International Journal of Advances in Pharmacy, Biology and Chemistry*, *1*(4).

19. Kothapalli, P L., Mahajan, A. G., Thomas, A. B., Nanda, R. K.; Narnaware. S. **(2012).** Simultaneous spectrophotometric estimation of Fexofenadine Hydrochloride and Montelukast sodium in pharmaceutical dosage form. *Int. J. Institution Pharm. Life Sci. 2*, 72-80.

20. Patle, D., & Nagar, S. **(2017).** UV-visible spectrophotometric estimation of montelukast and fexofenadine by simultaneous equation method in bulk & combined tablet dosage form. *Curr Trends Biotechnol Pharm*, *11*(4), 382-388.

21. Vekaria, H. J., Muralikrsihna, K. S., Patel, G. F. **(2011).** Development and validation of spectrophotometric method for estimation of Fexofenadine hydrochloride and Montelukast sodium in combined dosage form. *Inventi Impact: Pharm Analysis & Quality Assurance. 15,* 2-9.

22. Kozan, I., Palabiyik, I. M., Karacan, E., & Onur, F. **(2008)**. Spectrophotometric and high performance liquid chromatographic determination of fexofenadine hydrochloride in pharmaceutical formulations. *Turkish Journal of Pharmaceutical Sciences*, *5*(3), 175-189.

23. Alothman, Z. A., Bukhari, N., Haider, S., Wabaidur, S. M., & Alwarthan, A. A. **(2010).** Spectrofluorimetric determination of fexofenadine hydrochloride in pharmaceutical preparation using silver nanoparticles. *Arabian Journal of Chemistry*, *3*(4), 251-255.

24. Alsarra, I., Khalil, N. Y., Sultan, M., Al-Ashban, R., & Belal, F. **(2005).** Spectrofluorometric determination of montelukast in dosage forms and spiked human plasma. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *60*(11), 823-826.

25. Al-Kindy Salma, M. Z., Khadija AI-Shamalani, Fakhr Eldin, Suliman, O., Haidar AI-Lawati, H. J. **(2011).** Terbium sensitized luminescence for the determination of Fexofenadine in pharmaceutical formulations.*Arabian J Chem. 8*, 1-15.

26. Pallavi, M., Deepali, A., Bansode & Suneela, Dhaneshwar, S. **(2015).** Stability indicating thin-layer chromatographic determination of Fexofenadine hydrochloride as bulk drug: application to forced degradation study. *Der Pharmacia Sinica. 6*, 30-39.

27. Athavia, B. A., Dedania, Z. R., Dedania, R. R., Vijayendra Swamy, S. M., Chandrika Dobariya, T. **(2017)**. Stability indicating HPTLC method development for Fexofenadine Hydrochloride.*European J Biomed Pharm Sci. 4*, 697-705.

28. Tandulwadkar, S. S., More, S. J., Rathore, A. S., Nikam, A. R., Sathiyanarayanan, L., & Mahadik, K. R. **(2012).** Method development and validation for the simultaneous determination of fexofenadine hydrochloride and montelukast sodium in drug formulation using normal phase high-performance thin-layer chromatography. *International Scholarly Research Notices*, *2012*.

29. Maher, H. M., Sultan, M. A., & Olah, I. V. **(2011).** Development of validated stability-indicating chromatographic method for the determination of fexofenadine hydrochloride and its related impurities in pharmaceutical tablets. *Chemistry Central Journal*, *5*(1), 1-10.

30. Nimje, H. M., Nimje, S. T., Oswal, R. J., & Bhamre, S. T. **(2012).** Stability indicating RP-

HPLC method for estimation of fexofenadine hydrochloride in pharmaceutical formulation. *E-Journal of Chemistry*, *9*(3), 1257-1265.

31. Patnaik, A., Panda, S. S., Sahoo, S., & Patro, V. J. **(2012)**. RP-HPLC method development and validation for the determination and stability indicative studies of Montelukast in bulk and its pharmaceutical formulations. *E-Journal of Chemistry*, *9*(1), 35-42.

32. Roman, J., Breier, A. R., & Steppe, M. **(2011)**. Stability indicating LC method to determination of sodium montelukast in pharmaceutical dosage form and its photodegradation kinetics. *Journal of chromatographic science*, *49*(7), 540-546.

33. Gampa Vijaya, K., Rajesh, T., Jaya Chandra, T. A. **(2018).** Stability indicating RP-HPLC method development and validation for simultaneous estimation of Montelukast sodium and Fexofenadine Hydrochloride in bulk and pharmaceutical dosage form. *Indo Am. J. P. Sci. 5*, 16756-16765.

34. Rameezuddin, M. D., Vasanth, P. M., Ramesh, T., Ramesh, M. **(2013).** Method development and stability indicating RP-HPLC method for the estimation of Montelukast and Fexofenadine for bulk and pharmaceutical dosage form.*Int.J.ChemTech Res. 5*, 2821-2829.

35. Rajeev Kumar, P., & Kumar, R. R. **(2017).** Validated stability-indicating isocratic RP-HPLC method of estimation of Montelukast sodium and Fexofenadine hydrochloride in bulk and in solid dosage by vierodt's method. *J. Chem. Pharm. Res*, *9*, 297-243.

36. Pankhaniya, M., Patel, P., & Shah, J. S. **(2013).** Stability-indicating HPLC method for simultaneous determination of montelukast and fexofenadine hydrochloride. *Indian journal of pharmaceutical sciences*, *75*(3), 284.

37. Padmavaathi, K., & Rao, S. **(2015).** M. A new stability-indicating RP-HPLC method for the simultaneous determination of Fexofenadine hydrochloride and Montelukast in combined dosage form. *Der Pharmacia Lettre*, *7*, 301-307.

38. Oliveira, D. C., Weich, A., & Rolim, C. M. B. **(2007).** Simple and reliable HPLC analysis of fexofenadine hydrochloride in tablets and its application to dissolution studies. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *62*(2), 96-100.

39. Breier, A. R., Paim, C. S., Steppe, M., & Schapoval, E. E. **(2005).** Development and validation of dissolution tests for fexofenadine hydrochloride capsules and coated tablets. *J Pharm Pharm Sci*, *8*(2), 289-298.

40. Rele, R. V. **(2016).** Determination of fexofenadine hydrochloride in pharmaceutical dosage form by reverse phase high performance liquid chromatography method. *Der Pharma Chem*, *8*(6), 224-228.

41. Sakalgaonkar, A. A., Mirgane, S. R., & Pawar, R. P. **(2008).** Validated LC method, with a chiral mobile phase, for separation of the isomers of Fexofenadine hydrochloride. *Chromatographia*, *68*(1), 143-146.

42. Wang, D., Zhou, C., Cong, R., Li, Y., & Wang, X. **(2017).** Simultaneous determination of Montelukast sodium S-enantiomer and A5 enantiomers in Montelukast sodium bulk drug by normal-phase chiral HPLC. *Indian Journal of Pharmaceutical Sciences*, *79*(1), 139-148.

43. Singh, R. M., Saini, P. K., Mathur, S. C., Singh, G. N., & Lal, B. **(2010).** Development and validation of a RP-HPLC method for estimation of montelukast sodium in bulk and in tablet dosage form. *Indian journal of pharmaceutical sciences*, *72*(2), 235.

44. Devi, K. K. D., Rani, A. P., Madhavi, B. R., & Mrudula, B. S. **(2010).** New RP-HPLC meth-

od for the analysis of montelukast sodium in pharmaceutical dosage form. *Int. J. Chem Tech Res*, *2*(1), 471-75.

45. Phadtare, D. G., Pawar, A. R., Kulkarni, R. R., & Patil, G. K. **(2016).** Method development and validation of Montelukast sodium in bulk and tablet formulation by HPLC. *Tablet*, *2*(80), 99-14.

46. Priyanka, D., Sireesha, M., Akiful Haque, S., & Harshini, V. B. **(2014).** Method development and validation of Fexofenadine hydrochloride and Montelukast sodium in a combined dosage form by RP-HPLC method. *Int J Med Nanotechnolo*, *1*, 174-178.

47. Tamilselvi, N., & Sruthi, K. **(2012).** Development of validated HPLC method for simultaneous estimation of Fexofenadine hydrochloride and Montelukast sodium in tablet dosage form. *International Journal of Pharmaceutical Sciences and Research*, *3*(12), 4876.

48. Lakshmi, Y. M., Srinivasa, R., Meghana, R. **(2013).** Method development and validation for simultaneous estimation of Fexofenadine HCl and Montelukast Sodium by RP-HPLC in pure and combined tablet dosage form. *World J Pharm Pharm Sci. 2*, 5948-5965.

49. Ravisankar, M., Uthirapathy, S., Thangadurai, A., & Dhanapal, K. **(2012)**. simultaneous estimation of fexofenadine hydrocloride and montelukast sodium in bulk drug and marketed formulation by RP-HPLC method. *Int Res J Pharm*, *3*, 356-9.

50. Tukaram, M. K., Risha, R. W., & Rajendra, B. K. **(2013).** Development and validation of RP-HPLC method for estimation of montelukast sodium and fexofenadine hydrochloride in pharmaceutical preparations. *Chemical Science Transactions*, *2*(3), 889-899.

51. Swarnalatha, G., Vijayakumar, B., Jothieswari, D., Poojitha, M., Ravi Chandrakumar, A., Gobala Krishnan, P. (**2016**). RP-HPLC Method development and validation for simultaneous estimation of Montelukast sodium and Fexofenadine HCl in a pharmaceutical dosage form. *Int J Med Chem Anal. 6*, 44-51.

52. Arayne, M. S., Sultana, N., Shehnaz, H., & Haider, A. **(2011).** RP-HPLC method for the quantitative determination of fexofenadine hydrochloride in coated tablets and human serum. *Medicinal chemistry research*, *20*(1), 55-61.

53. Uno, T., Yasui-Furukori, N., Takahata, T., Sugawara, K., & Tateishi, T. **(2004).** Liquid chromatographic determination of fexofenadine in human plasma with fluorescence detection. *Journal of pharmaceutical and biomedical analysis*, *35*(4), 937-942.

54. Al-Rawithi, S., Al-Gazlan, S., Al-Ahmadi, W., Alshowaier, I. A., Yusuf, A., & Raines, D. A. **(2001).** Expedient liquid chromatographic method with fluorescence detection for montelukast sodium in micro-samples of plasma. *Journal of Chromatography B: Biomedical Sciences and Applications*, *754*(2), 527-531.

55. Ochiai, H., Uchiyama, N., Takano, T., Hara, K. I., & Kamei, T. **(1998).** Determination of montelukast sodium in human plasma by column-switching high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, *713*(2), 409-414.

56. Liu, L., Cheng, H., Zhao, J. J., & Rogers, J. D. **(1997).** Determination of montelukast (MK-0476) and its S-enantiomer in human plasma by stereoselective high-performance liquid chromatography with column-switching. *Journal of pharmaceutical and biomedical analysis*, *15*(5), 631-638.

57. Vaghela, B., Rao, S. S., Reddy, A. M., Venkatesh, P., & Kumar, N. **(2012)**. Identification and

characterization of an oxidative degradation product of fexofenadine, development and validation of a stability-indicating RP-UPLC method for the estimation of process related impurities and degradation products of fexofenadine in pharmaceutical formulations. *Scientia pharmaceutica*, *80*(2), 295-310.

58. Mangamma, K., & Dannana, G. S. **(2017).** Stability-indicating UPLC method for estimation of Montelukast and Fexofenadine simultaneously in the presence of stress degradation products. *J Global Trends Pharm Sci*, *8*, 4542-4553.

59. Stanton, M. L., Joy, M. S., Frye, R. F. **(2010).** Validation and application of a liquid chromatography-tandem mass spectrometric method for quantification of the drug transport probe Fexofenadine in human plasma using 96-well filter plates. *J Chromatogr B. 878* 1-11.

60. Hofmann, U., Seiler, M., Drescher, S., & Fromm, M. F. **(2002).** Determination of fexofenadine in human plasma and urine by liquid chromatography–mass spectrometry. *Journal of Chromatography B*, *766*(2), 227-233.

61. Jo, M. H., Park, M. S., Seo, J. H., Shim, W. S., Yim, S. V., & Lee, K. T. **(2011)**. Pharmacokinetic analysis of montelukast in healthy Korean volunteers by high performance liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical Investigation*, *41*(5), 289-294.

62. Mikuš, P., Valášková, I., & Havránek, E. **(2005).** Determination of fexofenadine in tablets by capillary electrophoresis in free solution and in solution with cyclodextrins as analyte carriers. *Drug development and industrial pharmacy*, *31*(8), 795-801.

63. United States Pharmacopeia. **(2009)**. Monograph on Fexofenadine hydrochloride Tablets, America.

64. United States Pharmacopeia. **(2010).** Monograph on Montelukast sodium Tablets, America.

65. Long, W., Henderson, J. W., & Joseph, M. **(2008).** Comparing selectivity of phenylhexyl and other types of phenyl bonded phases. *LC GC North America*, (JUN), 49-49.

66. Bhal, S. K. **(2007).** LogP—making sense of the value. *Advanced chemistry development, Toronto, ON, Canada*, 1-4.

67. ICH. (**2003).** Stability Testing of New drug substances and Products, International Conferenceon Harmonization, Geneva.

68. ICH. **(2005).** Q2 (R1) Validation of Analytical Procedures: Text and Methodology International Conferenceon Harmonization of Technical Requirements for Registration ofPharmaceuticals for Human Use, Geneva.

69. Radhakrishna, T., & Reddy, G. O. **(2002).** Simultaneous determination of fexofenadine and its related compounds by HPLC. *Journal of pharmaceutical and biomedical analysis*, *29*(4), 681-690.

70. Al Omari, M. M., Zoubi, R. M., Hasan, E. I., Khader, T. Z., & Badwan, A. A. **(2007).** Effect of light and heat on the stability of montelukast in solution and in its solid state. *Journal of pharmaceutical and biomedical analysis*, *45*(3), 465-471.

71. Ahuja, S., & Dong, M. (Eds.). **(2005).** *Handbook of pharmaceutical analysis by HPLC*. Elsevier.

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Assessment of Salmonella spp. and Mold-Yeast Contamination in Packaged and Unpackaged Spices

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ABSTRACT

In this study packaged (n=15) and unpackaged (n=15) spices including black pepper, oregano, peppermint, red pepper, cinnamon were collected and analyzed microbiologically. Mold-yeast contents of packaged samples were 1.07×10^4 cfu/g in red pepper, 1.75×10^4 cfu/g in black pepper, 7.72×10^4 cfu/g in peppermint, 8.21×10^3 cfu/g in cinnamon, 4.37×10^4 cfu/g in oregano, whereas unpackaged samples were 7.82×10^3 cfu/g in red pepper, 7.62×10^3 cfu/g in black pepper, 4.35×10^4 cfu/g in peppermint, 5.54×10^3 cfu/g in cinnamon, 4.21×10^4 cfu/g in oregano. *Salmonella* spp. was not found in any of the samples. When comparing the mold-yeast content of packaged and unpackaged samples, the difference in the level of contamination between the two groups did not show statistical significance ($p>0.01$). Mold-yeast results of unpackaged red pepper, black pepper, peppermint, oregano, and packaged peppermint and oregano were above the maximum limits of Turkish Food Codex (TFC). Therefore, controlling mold and yeast content at the selling point is recommended.

Keywords: spice, contamination, mold, yeast, *Salmonella* spp.

INTRODUCTION

Spices are used in many areas in the food industry. They may undergo microbial contamination that may threaten human health due to inadequate or unhygienic conditions at the stages of collection, processing, and storage[1,2]. The contamination may occur from the soil, air, and water during collection and drying or due to animal waste in the environment, as well as using contaminated equip-

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ment during production [3]. These can lead to contamination of spices with microorganisms like *Salmonella* sp. mold and yeast [1]. Especially some types of mold and yeast can produce mycotoxins that cannot be degraded by cooking and threaten human health[5].

Molds are capable to produce mycotoxins easily at room temperatures ranging from 25°C to 30°C and moisture contents of above 16% [5]. These toxins, which are generally effective even in trace amounts, can cause chronic diseases or acute deaths [6]. Inappropriate storage directly affects the risk of mycotoxin proliferation, particularly, if spices were formerly contaminated with molds. Thus, monitoring of mold-yeast content at the selling point is critical to prevent from unhygienic storage. In former regulation of Turkish Food Codex mold-yeast content of spices has been limited as $1x10^4$ cfu/g [7], whereas this limit is discarded in current regulation[8]. However, the International Food Standards of Codex Alimentarius Commission still suggests a maximum limit of $1x10^3$ cfu/g for mold-yeast in spices [9].

Besides the risk of mycotoxin related to mold-yeast contamination, *Salmonella* sp. contaminations, and related outbreaks were reported in spices, e.g. in black pepper[10]. *Salmonella* sp. is an important foodborne pathogen and most of the regulations suggest its absence in a food sample. According to the current microbiological criteria for spices in Turkish Food Codex [8], *Salmonella* should not exist in 25 g of sample, as well as in the International Food Standards, Codex Alimentarius Commission suggests the absence of *Salmonella* in spice[9].

Traditionally, spices are added in most of the Turkish foods. It has been determined that approximately 95% of people in Turkey use spices to increase flavor, to enhance the smell and appearance, and to make the dishes appetizing [11]. Three most commonly used spices in the country were red pepper, black pepper, and peppermint, respectively [12]. However, the humidity of the climate in Turkey promotes the growth of mold and yeast in spices. Therefore, monitoring of the safety of spices seems to be important for the country.

As a consequence, the risk of microbial contamination in spices, this study aimed to investigate *Salmonella* spp. and the mold-yeast contents of five commonly used spices including; red pepper, black pepper, peppermint, cinnamon, oregano and compare the results of packaged and unpackaged ones. Limits set in the Turkish Food Codex (TFC) and Codex Alimentarius (CA) were also considered for the assessment of results.

METHODOLOGY

Sampling collection

A total of 30 spice samples in unpacked (n=15) and packaged (n=15) form including red pepper (dried-ground), black pepper (powder), peppermint (dried-leaves), cinnamon (powder), and oregano (dried-leaves) were obtained from markets and stores locating in İstanbul, in April 2019. All spices were collected as three samples from each spice and with a minimum amount of 50 grams. The analysis of the samples was carried out in the Basic Health Sciences Laboratory of Faculty of Health Sciences, Marmara University.

Microbiological analyses

To prepare the samples for analysis, five grams from each of three spices were mixed in sterile plastic bags. Hence, a total of a 15-gram sample mixture was obtained for each type of spice. Prepared sample mixture was diluted with 135 ml (1:9 ratio) of Peptone Water (PW, OXOID CM 009) for yeast-mold analyses and with Buffered Peptone Water (BPW, OXOID CM0509) for *Salmonella* spp. enrichment. Tenfold serial dilutions were prepared from the homogenized samples with PW for yeast-mold analyses. 0.1 ml of each sample was taken to the petri dishes containing Dichloran Rose Bengal Chloramphenicol Agar (DRBC, OXOID CM 727) and cultivated with the spread plate method. Samples incubated for 5 days at 25°C, aerobically. Yeast and mold colonies were enumerated after incubation.

For the enrichment of *Salmonella* spp., homogenized samples were incubated at 37°C for 24 hours, aerobically. Thereafter, Xylose Lysine Deoxycholate Agar (XLD, Merck Nr.1.05287) was used for *Salmonella* analyses. 0.1 ml of homogenized samples were cultivated with the spread plate method. Petri dishes were incubated at 37°C for 24 hours, aerobically. *Salmonella* spp. determination was based on the formation of typical black centered red colonies in petri dishes.

The number of colonies counted as a result of microbiological analysis and calculated as the colony forming unit (cfu)/g and compared with the limits specified in Turkish Food Codex[7,8] and Codex Alimentarius[9].

Statistical analyses

Tenfold transformations were applied to all counts and statistical analyses were conducted using Microsoft Excel. A possible relationship between the colony count found in the packaged and unpackaged spices was analyzed by using the t-test.

RESULTS and DISCUSSION

According to the results of this study, all of the samples (100%) were contaminated with mold and yeast at different levels. *Salmonella* spp. was not detected in any of the samples. Mold and yeast growth in packaged spices were slightly lower than that of unpackaged ones, as shown in Table 1. However, this difference in the level of mold-yeast contamination between the two groups did not show statistical significance ($p>0.05$).

Table 1. Microbiological content of spice samples

Spices	Mold-yeast count (cfu/g)		Salmonella spp. (+ / -)*	
	Packaged (n=15)	Unpackaged (n=15)	Packaged (n=15)	Unpackaged (n=15)
Red pepper (n=6)	7.82×10^3	1.07×10^4	-	-
Black pepper (n=6)	7.62×10^3	1.75×10^4	-	-
Peppermint (n=6)	4.35×10^4	7.72×10^4	-	-
Cinnamon (n=6)	5.54×10^3	8.21×10^3	-	-
Oregano (n=6)	4.21×10^4	4.37×10^4	-	-
Frequency	100%	100%	-	-
p value	0.076			

**-.. no visible colony was found*

When the results are compared with the reliable limits in current microbiological criteria for spices of Turkish Food Codex (TFC, 2013), the absence of *Salmonella* is required, which is compatible with our results. This finding indicates that *Salmonella* contents of the samples in our study were within acceptable limits according to TFC-2013[8].

Mold-yeast content among the packaged spices, red pepper, black pepper, and cinnamon were below the former Turkish Food Codex (remarked as $1x10^4$ cfu/g in TFC-2009) limits [7]. It was determined that the mold-yeast content in packaged and unpackaged peppermint and oregano samples were above the maximum limits according to both of the TFC-2009 [7] and the CA [9]. Both the packaged and unpackaged cinnamon samples were above the maximum limit of CA [9] but below the maximum limit of TFC-2009[7] which among the unpackaged samples only cinnamon was lower than this.

In a study conducted by Kızıl and colleagues with the unpackaged black peppers taken from several cities of Turkey including İstanbul, *Salmonella* spp. was found only in black pepper samples taken from İstanbul and İzmir province, whereas found in red pepper samples taken from Samsun province[13]. In a study conducted in Brazil, the highest level of *Salmonella* sp. contamination

was found in black pepper [14], whereas in the United States, basil, black pepper, coriander, curry powder, garlic powder, oregano, paprika, and red pepper were contaminated with *Salmonella*[15]. On the other hand, *Salmonella* was not found in packaged and in unpackaged red peppers in different countries, such as in Turkey[16,17], in Iran[18], as well as in other packaged and unpackaged spices including black pepper, cinnamon, basil, turmeric, berbere in Ethiopia [19], and also in any of the spices and herbs, including black pepper, oregano, and cinnamon in Jordan[20]. Similar to these studies, none of the spice samples were contaminated with *Salmonella* spp. in our study.

Studies in Turkey show similarities in mold-yeast results of spices, which are mostly contaminated at least half of the collected samples. Hampikyan and colleagues examined microbiological contamination of 420 spice samples from İstanbul markets, used in the meat industry in Turkey, and found around the 85% samples contaminated with yeast and/or mold[21]. In a study conducted by Coşkun, black pepper, red pepper, and cinnamon in Tekirdağ markets were examined, molds were detected in 55% of samples, and whereas only 27% of the samples were below the mold-yeast limit of TFC [22]. In Diyarbakır, 70% of black pepper and 60% of red pepper samples were contaminated with mold, whereas yeast contamination was 10% in black pepper and 50% in red pepper samples [2]. In Kahramanmaraş, 91.3% of the red pepper samples were positive in terms of mold and yeast, and the average colony number of mold and yeast was 4.8×10^5 cfu/g [23]. In Şanlıurfa, red pepper obtained from supermarkets, open markets and retail stores, was examined and the samples contaminated with mold were also consist of a high amount of mycotoxins (up to 86.01 g/kg), which exceeded the legal mycotoxin limit of 10 μg/kg set by the Turkish Food Codex [24].

There is a high frequency of mold-yeast contamination of spices all around the world. In a study of Banerjee and Sarkar (2003), 97% of the spices, including black pepper, red pepper, and cinnamon were contaminated with mold, whereas yeast was found in only one of cumin sample [25]. In a recent study conducted in Argentina, mold-yeast counts of Argentinian paprika was between 2×10^2 and 1.9×10^5 cfu/g, in which some samples exceeded the reliable limits of international food regulations [26]. Fogele and colleagues analyzed several spices including curry, black pepper, cumin, clove, cinnamon, thyme, basil, rosemary, and found mold contents of spices sold in small sellers significantly higher than those of sold in supermarkets. Although in our study difference of packaged and unpackaged samples did not show statistical significance, the result of the study of Fogele and colleagues indicated that the environmental factors, production process, and place where spices sold are effective on the mold growth [27]. Current food security

criteria in TFC-2013 recommend analyzing *Salmonella*, *B.cereus,* and coagulase-positive *Staphylococci* in spice samples*,* i.e. mold-yeast discarded [8]. Although mold and yeast might be destroyed by the cooking of spice, their toxins might be still present in food. On the other hand, mold and yeast content can be considered as a kind of indicator of their proper storage condition and their contamination at the market level is still important. Especially when consumers do not take care of appropriate storage conditions at home, previously contaminated molds can grow easily and mycotoxins might be produced even at home. Therefore, at least controlling mold and yeast content at the selling point is a critical step to minimize the risk of mycotoxin exposure via consumption of spices.

In this study *Salmonella* sp. and mold-yeast content of packaged and unpackaged spices were investigated. Although *Salmonella* sp. was not detected in any of the samples, all of the samples were contaminated with mold-yeast. It has been observed that mold-yeast contamination in red pepper and black pepper samples is close to the maximum level of TFC, and both packaged and unpackaged peppermint and oregano samples were above the reliable limits. These findings are conforming with other studies in Turkey and also in other countries. Nevertheless, mold-yeast contamination in this study highlights particularly the necessity of monitoring frequently consumed spices and related mycotoxin analyses for estimation of the risk of exposure to mycotoxin via consumption in a portion.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Schweiggert, U., Reinhold, C., Schieber, A. **(2007).** Conventional and alternative processes for spice production – a review. *Trends Food Sci. Tech. 18*, 260-268.

2. Vural, A., Arserim Kaya, N. B., Mete, M. **(2004).** Bazı öğütülmüş baharatlarda küf ve maya florasının incelenmesi. *Dicle Univ. Tip Fakul. Derg. 31*, 15-19.

3. Yılmaz, H., Şanlıer, N. **(2014).** Baharat Işınlama. *Gıda. 39*, 111-118.

4. Milani, J., Maleki, G. **(2014).** Effects of processing on mycotoxin stability in cereals. *J. Sci. Food Agric. 94*, 2372–2375.

5. Thanushree, M. P., Sailendri, D., Yoha, K. S., Moses, J. A., Anandharamakrishnan, C. **(2019).** Mycotoxin contamination in food: an exposition on spices. *Trends Food Sci. Tech. 93*, 69-80

6. Yentür, G., Er, B. **(2011).** Gıdalarda aflatoksin varlığının değerlendirilmesi. *Turk Hij. Den. Biyol. Derg. 69*, 41-52

7. Turkish Food Codex (TFC). **(2009).** Türk Gıda Kodeksi Mikrobiyolojik Kriterler Tebliği. Turkish Republic, Official Gazette, Nr. 27133

8. Turkish Food Codex (TFC). **(2013).** Türk Gıda Kodeksi Baharat Tebliği. Turkish Republic, Official Gazette, Nr. 28614.

9. Codex Alimentarius Commission (CA). **(2003).** Joint FAO/WHO, food standards programme FAO/WHO coordinating committee for the Near East. Second Session. Cairo, Egypt, *11*.

10. Codex Alimentarius Commission (CA). **(2003).** Joint FAO/WHO, food standards programme FAO/WHO coordinating committee for the Near East. Second Session. Cairo, Egypt, *11*.

11. Demircioğlu, Y., Yaman, M., Şimşek, I. **(2007).** Kadınların baharat kullanım alışkanlıkları üzerine bir araştırma. *TAF Preventive Medicine Bulletin. 6*, 161-168.

12. Kılıçhan, R., Çalhan, H. **(2015).** Mutfakların sihri baharat: Kayseri ilinde baharat tüketim alışkanlıklarının belirlenmesine yönelik bir çalışma. *Journal of Tourism and Gastronomy Studies. 3*, 40-47.

13. Kızıl, M., Wassouf, N., Metin, M. M. T., Gümüş, D., Dikmen, D., Uyar, M. F. **(2015).** Açıkta satılan baharatlarda *Escherichia coli* O157:H7 ve *Salmonella* spp. varlığının moleküler mikrobiyojik analiz yöntemi ile belirlenmesi. *Beslenme ve Diyet Dergisi. 43*, 228-236.

14. Moreira, P. L., Lourencao, T. B., Pinto, J. P., Rall, V. L. **(2009).** Microbiological quality of spices marketed in the city of Botucatu, Sao Paulo, Brazil. *J. Food Prot. 2*, 421-424.

15. Zhang, G., Hu, L., Pouillot, R., Tatavarthy, A., Van Doren, J. M., Kleinmeier, D., Ziobro, G. ….& Hammack, T. S. **(2017).** Prevalence of *Salmonella* in 11 spices offered for sale from retail establishments and in imported shipments offered for entry to the United States. *J. Food Prot. 80*, 1791–805.

16. Kimiran-Erdem, A., Arslan-Aydoğdu E. Ö., Gürün, S. **(2013).** Bacteriological analysis of the red pepper spices marketed as packaged and unpackaged in Istanbul. *IUFS J. Biol. 72*, 23-30

17. Çalışkan, B. **(2008).** İstanbul ilinde tüketime sunulan kırmızı biberlerin bazı patojenler, kimyasal ve toksikolojik maddeler yönünden incelenmesi üzerine bir araştırma, Yüksek Lisans Tezi, Gıda Mühendisliği Ana Bilim Dalı, Namık Kemal Üniversitesi, Tekirdağ.

18. Salari, R., Najafi, M. H., Boroushaki, M. T., Mortazavi, S. A., Najafi, M. F. **(2012).** Assessment of the microbiological quality and mycotoxin contamination of Iranian red pepper spice. *J. Agr. Sci. Tech. 14*, 1511-1521.

19. Bedada, T. L., Derra, F. A., Gebre, S. G., Sima, W. G., Edicho, R. M., Maheder, R. F., Negassi T. Y., Asefa, Y. B. **(2018).** Microbial Evaluation of Spices in Ethiopia. *Open Microbiol. J. 12*, 422-429.

20. Dababneh, B. F. **(2013).** An innovative microwave process for microbial decontamination of spices and herbs. *Afr. J. Microbiol. Res. 7*, 636-645.

21. Hampikyan, H., Bingol, E. B., Colak, H., Aydin, A. **(2009).** The evaluation of microbiological profile of some spices used in Turkish meat industry. *J. Food Agric. Environ. 7*, 111-115.

22. Coşkun, F. **(2010).** Microbiological Characteristics of Some Spices Sold in Tekirdağ Markets. *J. Tekirdag Agric. Fac. 7*, 85-93.

23. Erdoğrul, Ö. T. **(2000).** Kahramanmaraş'ta satılan acı kırmızı pul biberin bazı mikrobiyolojik özellikleri. *KSÜ Fen ve Mühendislik Dergisi. 3*, 108-113.

24. Karaaslan, M., Arslanğray, Y. **(2015).** Aflatoxins B1, B2, G1, and G2 contamination in ground red peppers commercialized in Sanliurfa, Turkey. *Environ. Monit. Assess.* 187, 184.

25. Banerjee, M., Sarkar, P. K. **(2003).** Microbiological quality of some retail spices in India. *Food Res. Int. 36*, 469-474.

26. González, M. G. M., Romero, S. M., Arjona, M., Larumbe, A. G., Vaamonde, G. **(2017).** Microbiological quality of Argentinian paprika. *Rev. Argent Microbiol. 49*, 339-346.

27. Fogele, B., Granta, R., Valcin, O., Berzin A. **(2018)**. Occurrence and diversity of *Bacillus cereus* and moulds in spices and herbs. *Food Control. 83*, 69–74

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Synthesis of New 1,2,4-Triazole Derivatives and Investigation of Their Matrix Metalloproteinase-9 (MMP-9) Inhibition Properties

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ABSTRACT

Nine new 2-[[5-((4-acetamidophenoxy)methyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl] thio]-*N*-(aryl/heteroaryl)acetamide (**5a-5i**) derivatives were synthesized and tested for their matrix metalloproteinase-9 (MMP-9) inhibition potency which is associated with antiproliferative activity. None of the compounds exhibited MMP-9 inhibition activity close to standard drug however, two compounds **5e** bearing 6-methylbenzothiazole and **5g** bearing 6-ethoxybenzothiazole moieties showed the highest MMP-9 inhibition which were determined over than 50% percentage at 100 μg/mL concentration. Some physicochemical properties of all final compounds were calculated via SwissADME and the results were modest to be able to an oral drug. Molecular docking studies were realized with MMP-9 enzyme (PDB ID: 5I12) and **5d, 5e**, and **5g** compounds for comparing the active and non-active/low effective structures.

Keywords: 1,2,4-Triazole, matrix metalloproteinase-9 (MMP-9), antiproliferative activity, molecular docking

INTRODUCTION

Cancer is a common and tough disease which its many types still have no definite and radical treatment. In this regard, the disease has been tried to be controlled by understanding the damages it causes and focusing on targeted path-

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ways related to the emergence or metastasis of the disease.[1,2] Target theraphy is mostly includes a specific site, such as tumour vasculature or tumoral intracellular organelles which provides protection from the side effects of treatment and leaving the surrounding tissue unaffected. Additionally, gene therapy and expression of genes triggering apoptosis with(out) some enzymes and wild type tumour suppressors or the targeted silencing mediated by siRNAs are the other ways of targeted chemotheraphy.[3,4] Matrix metalloproteinase enzymes MMPs are zinc- and calcium-dependent endopeptidases involved in physiological and pathological processes such as embryo implantation, wound healing, tissue remodeling and formation of some malfunctions like cancer, arthritis, genetic disorders, chronic kidney, aging and cardiovascular diseases.

The MMP family consists of 25 secreted and membrane-bound members, among them MMP-2 (gelatinase A) and MMP-9 (gelatinase B) serve in inflammatory response and angiogenesis. It has been determined that gelatinases show tumorigenic activity by the way of degradation of the extracellular matrix (ECM) and non-ECM functions that activates pro-TNF-α and TGF-β (transforming growth factor beta) molecules in tumor development stages, such as tumor cell growth, migration, invasion, and metastasis. The regulatory role of MMPs in cancer progression is a current strategy for treatment cancer disease.[5-7]

Nitrogen containing heterocycles are valuable drug designing skeletons, among these 1,2,4 triazoles carrying three nitrogen atoms have considerable interest due to their synthetic easibility, electron-rich chemical properties and wide spectrum of biological applications such as antioxidant, anticonvulsant, anti-inflammatory, antimicrobial and antiviral activities. 1,2,4-triazole nucleus is also placed in drug candidates including H_1/H_2 histamine receptor blockers, cholinesterase active agents, central nervous system (CNS) stimulants, antianxiety and sedatives along with a lot of clinically used drugs eg. alprazolam (tranquilizer), benatradin (diuretic), trapidil (hypotensive), trazodon (antidepressant, anxiolytic), ribavirin (antiviral), fluconazole, itraconazole, terconazole (antifungal), anastrozole, letrozole and vorozole (antineoplastics).[8-12] The antiproliferative activity of 1,2,4-triazole derivative has been reported to associated with cell cycle arrest in the G_2/M phase via binding property to variable receptors and enzymes due to the ring's polar nature. Additionally, other triazole including molecules have been determined to exhibit their anticancer potentcy via tubulin polymerization inhibition and modulation, mitochondrial depolarization, and induction of apoptosis through activation of caspase-3, inhibition of kinase, aromatase and steroid sulfatase, methionine aminopeptidase inhibitors and tankyrase enzymes.[13,14]

Based on the reported data and as an expansion work of our previous study,[15] novel nine 1,2,4-triazole derivatives were synthesized, inhibition of MMP-9 enzyme was investigated and structure-activity relationships of the molecules was discussed.

METHODOLOGY

Chemistry

All chemicals used in the syntheses were purchased either from Merck Chemicals (Merck KGaA, Darmstadt, Germany) or Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO, USA). The reactions and the purity of the compounds were observed by thin-layer chromatography (TLC) on silica gel 60 F254 aluminum sheets obtained from Merck (Darmstadt, Germany). Melting points of the synthesized compounds were recorded by the MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) and were presented as uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded by a Bruker 300 MHz and 75 MHz digital FT-NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6, respectively. In the NMR spectra, splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet. Coupling constants (*J*) were reported as Hertz. High resolution mass spectrometric (HRMS) studies were performed using an LC/MS-IT-TOF system (Shimadzu, Kyoto, Japan). Elemental analyses were performed on a Leco 932 CHNS analyzer (Leco, Michigan, USA).

Ethyl 2-(4-acetamidophenoxy)acetate (1)

N-(4-Hydroxyphenyl)acetamide (0.033 mol, 5g), ethyl chloroacetate (0.04 mol, 4.88 g) and potassium carbonate were refluxed in acetone for 6h. After checking the reaction with TLC, the solvent was evaporated, and the material was filtered and washed with water. The raw product was crystallised from ethanol.

N-[4-(2-Hydrazinyl-2-oxoethoxy)phenyl]acetamide (2)

Ethyl 2-(4-acetamidophenoxy)acetate (**1**, 1 eq.) and hydrazine monohydrate (1.2 eq) were stirred in ethanol at room temperature overnight. When the reaction was over, the mixture was separated from the solvent. The precipitated material was filtered and crystallised from ethanol.

N-(4-(2-Oxo-2-(2-(phenylcarbamothioyl)hydrazinyl)ethoxy)phenyl)acetamide (3)

N-(4-(2-hydrazinyl-2-oxoethoxy)phenyl)acetamide (**2**) and phenylisothiocyanate were refluxed in EtOH for 3h. After TLC, the solvent was evaporated, and the solid was collected.

N-[4-((5-Mercapto-4-phenyl-4H-1,2,4-triazol-3-yl)methoxy)phenyl] acetamide (4)

N-(4-(2-oxo-2-(2-(phenylcarbamothioyl)hydrazineyl)ethoxy)phenyl)acetamide (**3**) was refluxed with 2N potassium hydroxide prepared in ethanol for 2h. After TLC, the pH was adjusted to 7 in a cold environment to precipitate the material.

General procedure for the synthesis N-substituted-2-[[5-((4-acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]acetamide derivatives (5a-5i)

N-(4-((5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)methoxy)phenyl)acetamide (**4**) (0.0015 mol, 0.5 g) and equivalent 2-chloro-*N*-substituted acetamide derivatives were reacted in the presence of potassium carbonate in acetone. After 4-5 h, it was finished, the solvent was evaporated and treated with water, then it was crystallised from ethanol to yield the final product.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-y]thio]-N-(6-nitrobenzothiazol-2-yl)acetamide (5a)

m. p. 240 °C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.98 (s, 3H, CH_3), 4.33 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (d, J=8.98 Hz, 2H, Ar-H), 7.40 (d, J=8.98 Hz, 2H, Ar-H), 7.48-7.64 (m, 5H, Ar-H), 7.54 (d, J=8.95 Hz, 1H, Ar-H), 8.27 (dd, J_1=2.43 Hz, J_2= 8.94 Hz, 1H, Ar-H), 9.03 (m, 1H, Ar-H), 9.79 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.26 (C-CH_3), 36.54 (S-CH_2), 60.55 (O-CH_2), 115.45, 119.40, 120.76, 121.11, 122.25, 126.66, 127.43, 130.29, 130.64, 133.86, 143.21, 151.76, 152.38, 153.46, 154.22, 164.81, 168.27 (acetamide C=O), 168.85 (C=O). For $C_{26}H_{21}N_7O_5S_2$ calculated: 54.25 % C, 3.68 % H, 17.03 % N; found: 54.27 % C, 3.69 % H, 17.05 % N. HRMS [M-H]$^-$: calculated: 574.0973, experimental: 574.1010.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(benzothiazol-2-yl)acetamide (5b)

m. p. 270 °C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.99 (s, 3H, CH_3), 4.31 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (d, J=9.02 Hz, 2H, Ar-H), 7.30-7.60 (m, 9H, Ar-H), 7.77 (d, J=8.03 Hz, 1H, Ar-H), 7.80 (d, J=7.86 Hz, 1H, Ar-H), 9.78 (brs, 1H, NH), 12.70 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.28 (C-CH_3), 36.08 (S-CH_2), 60.56 (O-CH_2), 115.45, 120.76, 120.89, 122.17, 127.43, 130.29, 130.64, 130.91, 133.00, 133.87, 151.66, 152.40, 153.47, 167.45 (acetamide C=O), 168.24 (C=O). For $C_{26}H_{22}N_6O_3S_2$ calculated: 58.85 % C, 4.18 % H, 15.84 % N; found: 58.82 % C, 4.19 % H, 15.85 % N. HRMS [M-H]$^-$: calculated: 529.1122, experimental: 529.1153.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-y]thio]-N-(6-fluorobenzothiazol-2-yl)acetamide (5c)

m. p. 276°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 2.00 (s, 3H, CH_3), 4.31 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (dd, J_1=1.97 Hz, J_2=7.08 Hz, 2H, Ar-H), 7.27-7.33 (m, 1H, Ar-H), 7.40 (dd, J_1=2.01 Hz, J_2=7.08 Hz, 2H, Ar-H), 7.50-7.58 (m, 5H, Ar-H), 7.78 (dd, J_1=4.84 Hz, J_2= 8.88 Hz, 1H, Ar-H), 7.89 (dd, J_1=2.65 Hz, J_2= 8.7 Hz, 1H, Ar-H), 9.79 (brs, 1H, NH), 12.73 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.24 (CH_3), 36.02 (S-CH_2), 60.56 (O-CH_2), 108.53,108.89, 114.63, 114.96, 115.45, 120.76, 122.32, 127.43, 130.29, 130.64, 133.00, 133.09, 133.23, 133.88, 145.69, 151.64, 152.42, 153.47, 157.57, 158.19, 160.75, 167.54 (acetamide C=O), 168.26 (C=O). For $C_{26}H_{21}FN_6O_3S_2$ calculated: 56.92 % C, 3.86 % H, 15.32 % N; found: 56.94 % C, 3.85 % H, 15.35 % N. HRMS [M-H]$^-$: calculated: 547.1028, experimental: 547.1075.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-chlorobenzothiazol-2-yl)acetamide (5d)

m. p. 258°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.98 (s, 3H, CH_3), 4.31 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (d, J=9.03 Hz, 2H, Ar-H), 7.40 (d, J=9.01 Hz, 2H, Ar-H), 7.46 (dd, J_1=2.23 Hz, J_2= 8.61 Hz, 1H, Ar-H), 7.50-7.58 (m, 5H, Ar-H), 7.76 (d, J= 8.66 Hz, 1H, Ar-H), 8.13 (d, J=2.13 Hz, 1H, Ar-H), 9.79 (brs, 1H, NH), 12.80 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.27 (C-CH_3), 36.06 (S-CH_2), 60.55 (O-CH_2), 115.45, 120.77, 121.97, 122.33, 127.00, 127.42, 128.16, 130.29, 130.65, 132.99, 133.62, 133.87, 147.88, 151.63, 152.41, 153.46, 159.14, 167.40 (acetamide C=O), 168.25 (C=O). For $C_{26}H_{21}ClN_6O_3S_2$ calculated: 55.27 % C, 3.75 % H, 14.87 % N; found: 55.28 % C, 3.72 % H, 14.86 % N. HRMS [M-H]$^-$: calculated: 563.0732, experimental: 563.0768.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-methylbenzothiazol-2-yl)acetamide (5e)

m. p. 280°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.98 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 4.30 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.78 (d, J=9.04 Hz, 2H, Ar-H), 7.26 (dd, J_1=1.25 Hz, J_2= 7.30 Hz, 1H, Ar-H), 7.41 (d, J= 9.10 Hz, 2H, Ar-H), 7.49-7.59 (m, 5H, Ar-H), 7.65 (d, J= 8.28 Hz, 1H, Ar-H), 7.76 (s, 1H, Ar-H), 9.79 (brs, 1H, NH), 12.63 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 21.46 (CH_3), 24.27 (C-CH_3), 36.08 (S-CH_2), 60.55 (O-CH_2), 115.44, 120.76, 121.82, 127.43, 127.98, 130.28, 130.63, 132.05, 133.00, 133.60, 133.87, 146.92, 151.68, 152.40, 153.47, 157.39, 167.30 (acetamide C=O), 168.25 (C=O). For $C_{27}H_{24}N_6O_3S_2$ calculated: 59.54 % C, 4.44 % H, 15.43 % N; found: 59.50 % C, 4.42 % H, 15.45 % N. HRMS [M-H]$^-$: calculated: 543.1279, experimental: 543.1326.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-y]thio]-N-(6-methoxybenzothiazol-2-yl)acetamide (5f)

m. p. 256°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.98 (s, 3H, CH_3), 3.80 (s, 3H, O-CH_3), 4.29 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (d, J=9.03 Hz, 2H, Ar-H), 7.04 (dd, J_1=2.58 Hz, J_2= 8.85 Hz, 1H, Ar-H), 7.40 (d, J= 8.98 Hz, 2H, Ar-H), 7.50-7.58 (m, 5H, Ar-H), 7.66 (d, J= 8.85 Hz, 1H, Ar-H), 9.79 (brs, 1H, NH), 12.57 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.27 (C-CH_3), 36.04 (S-CH_2), 56.08 (O-CH_3), 60.56 (O-CH_2), 105.18, 115.45, 120.76, 121.72, 127.43, 127.98, 130.28, 130.64, 133.00, 133.24, 133.87, 143.04, 151.69, 152.39, 153.47, 156.21, 156.65, 167.13 (acetamide C=O), 168.25 (C=O). For $C_{27}H_{24}N_6O_4S_2$ calculated: 57.84 % C, 4.32 % H, 14.99 % N; found: 57.85 % C, 4.35 % H, 14.95 % N. HRMS [M-H]$^-$: calculated: 559.1228, experimental: 559.1269.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-ethoxybenzothiazol-2-yl)acetamide (5g)

m. p. 226°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.34 (t, J=6.95 Hz, 3H, O-CH_2-CH_3), 1.97 (s, 3H, CH_3), 4.06 (q, J_1=6.93 Hz, J_2=13.85 Hz, 2H, O-CH_2-CH_3), 4.29 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.77 (d, J=8.97 Hz, 2H, Ar-H), 7.02 (dd, J_1=2.51 Hz, J_2=8.82 Hz, 1H, Ar-H), 7.40 (d, J=8.96 Hz, 2H, Ar-H), 7.50-7.58 (m, 6H, Ar-H), 7.65 (d, J= 8.85 Hz, 1H, Ar-H), 9.79 (s, 1H, NH), 12.56 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 15.17 (O-CH_2-CH_3), 24.25 (C-CH_3), 36.00 (S-CH_2), 60.56 (O-CH_2), 64.04 (O-CH_2-CH_3), 105.81, 115.45, 115.82, 120.73, 127.43, 130.28, 130.64, 133.00, 133.87, 142.93, 152.39, 153.47, 155.90, 167.08 (acetamide C=O), 168.26 (C=O). For $C_{28}H_{26}N_6O_4S_2$ calculated: 58.52 % C, 4.56 % H, 14.62 % N; found: 58.53 % C, 4.55 % H, 14.65 % N. HRMS [M-H]$^-$: calculated: 573.1384, experimental: 573.1436.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(4-nitrophenyl)acetamide (5h)

m. p. 250°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.98 (s, 3H, CH_3), 4.25 (s, 2H, S-CH_2), 5.03 (s, 2H, O-CH_2), 6.76-6.77 (m, 2H, Ar-H), 7.38-7.41 (m, 2H, Ar-H), 7.49-7.58 (m, 5H, Ar-H), 7.80-7.83 (m, 2H, Ar-H), 8.23-8.26 (m, 2H, Ar-H), 9.80 (brs, 1H, NH), 10.98 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.27 (C-CH_3), 37.26 (S-CH_2), 60.51 (O-CH_2), 115.44, 120.74, 125.58, 130.29, 130.63, 133.00, 133.86, 142.81, 145.35, 152.30, 153.43, 167.00 (acetamide C=O), 168.26 (C=O). For $C_{25}H_{22}N_6O_5S$ calculated: 57.91 % C, 4.28 % H, 16.21 % N; found: 57.93 % C, 4.25 % H, 16.15 % N. HRMS [M-H]$^-$: calculated: 517.1300, experimental: 517.1325.

2-[[5-((4-Acetamidophenoxy)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(4-fluorophenyl)acetamide (5i)

m. p. 196°C, ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 1.99 (s, 3H, CH_3), 4.17 (s, 2H, S-CH_2), 5.03 (s, 2H, O- CH_2), 6.77 (d, *J*=8.99 Hz, 2H, Ar-H), 7.13-7.19 (m, 2H, Ar-H), 7.41 (d, *J*= 8.98 Hz, 2H, Ar-H), 7.48-7.51 (m, 2H, Ar-H), 7.56-7.58 (m, 5H, Ar-H), 9.80 (brs, 1H, NH), 10.42 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 24.25 (C-CH_3), 37.16 (S-CH_2), 60.53 (O-CH_2), 115.45, 115.73, 116.36, 127.44, 130.26, 130.59, 133.05, 133.86, 135.63, 151.98, 152.23, 153.45, 160.13, 165.79 (acetamide C=O), 168.26 (C=O). For $C_{25}H_{22}FN_5O_3S$ calculated: 61.09 % C, 4.51 % H, 14.25 % N; found: 61.11 % C, 4.52 % H, 14.21 % N. HRMS [M-H]$^-$: calculated: 490.1355, experimental: 490.1401.

Activity Studies

Matrix Metalloproteinase (MMP) Inhibition Assays

MMP-9, colorimetric kits were purchased from Enzo Life Sciences Inc. (Farmingdale, New York, NY, USA). The MMP Colorimetric Drug Discovery Kits are a complete assay system designed to screen MMP inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5). The MMP cleavage site peptide bond is replaced by a thioester bond in the thiopeptide. Hydrolysis of this bond by an MMP produces a sulfhydryl group, which reacts with DTNB [5,50-dithiobis(2-nitrobenzoic acid), Ellman's reagent] to form 2-nitro-5-thiobenzoic acid, which can be detected by its absorbance at 412 nm. The assays were conducted in triplicate. The UV absorbance was read at 412 nm using a microplate reader (BioTek, PowerWave, Gen5 software, Winooski, VT, USA) at room temperature. NNGH was used as a control inhibitor.[16] Data were expressed as Mean±SD.

The inhibitor% remaining activity of MMPs was calculated using the following equation:

Inhibitor% activity remaining = (V inhibitor/V control) × 100.

The inhibition (percent) of MMPs was calculated using the following equation:

I (%) = 100 – Inhibitor% activity remaining

Docking Studies

The crystal structure of MMP-9 was retrieved from the Protein Data Bank server (PDB code: 5I12). The protein preparation process, ligand preparation process, grid generation, docking and visualization studies were performed on Schrodinger's Maestro molecular modeling package.[17]

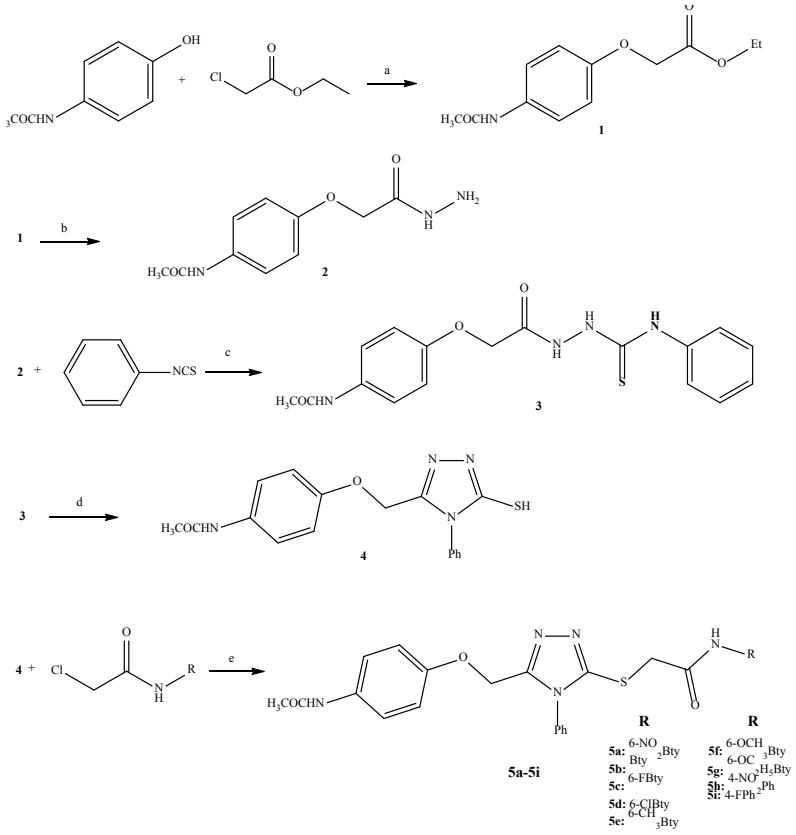
The water molecules were removed from the crystal structure. Ligands were set to the physiological pH (pH = 7.4) at the protonation step. In molecular docking simulations: Glide/XP docking protocols were applied for the prediction of topologies of **5f**, **5g** and **5h** at the active site of target structure[4], then they were docked to the active site of 5I12.

RESULTS and DISCUSSION

Chemistry

In this study, we aimed to synthesize novel 3,4,5-trisubstituted triazole derivatives. In the first of five steps, *N*-(4-hydroxyphenyl)acetamide and ethyl chloroacetate were refluxed in acetone.

After the reaction was completed, the acetone was evaporated, and the material was washed with water to acquire the intermediate. The obtained product, ethyl 2-(4-acetamidophenoxy)acetate (**1**) was reacted with hydrazine monohydrate in ethanol. The end of the reaction was checked by TLC, hydrazide compound was gained by filtration. *N*-(4-(2-oxo-2-(2-(phenylcarbamothioyl)hydrazineyl) ethoxy)phenyl)acetamide (**2**) was dissolved in ethanol and boiled with phenylisothiocyanate to synthesize *N*-phenyl-2-(2-(quinolin-8-yloxy)acetyl)hydrazine-1-carbothioamide (**3**) and then in next step this compound was refluxed with 2N potassium hydroxide prepared in ethanol. The reaction was terminated by controlling the TLC. The pH was set to 7 to allow the material to settle in a cold environment. At last, the resulting triazole molecules (**4**) were acquired with the reaction of appropriate 2-chloro-*N*-substituted acetamide derivatives to get the final nine molecules (**5a-i) (Scheme 1)**. The target compounds (**5a-i**) were obtained purely and the structures of the compounds were elucidated with spectroscopic methods.



Scheme 1. Synthesis of the target compounds. *Reaction conditions*. a) Acetone, K_2CO_3, reflux, 6h; b) EtOH, $H_2NNH_2.H_2O$, rt, 12h; c) EtOH, reflux, 3h; d) 2N KOH (ethanolic), reflux, 2h; e) Acetone, K_2CO_3, r.t, 4-5h.

The structures of the synthesized materials were elucidated by ^{1}H-NMR, ^{13}C-NMR, HRMS and elemental analysis. The ppm values of the peaks of the -NH proton were detected at 9.78-12.80 ppm. The values of the proton peaks of O-CH_2 were seen at 5.03 ppm exactly, and values of S-CH_2 protons were assigned at 4.17-4.33 ppm. The peak value of the CH_3 for all molecules was singlet at 1.97- 2.00 ppm. For compound **5e**, the substituent CH_3 peaks were observed at 1.98 and 2.40 ppm.

The O-CH_3 peak at 3.80 ppm in compound **5f** was an expected value due to the substituent. The CH_3, O-CH_2-CH_3 and O-CH_2-CH_3 peaks attributed to the substituents in the compound **5g** were observed at 1.97 ppm, 4.05 ppm, and 1.34

ppm, respectively. Also, multiple peaks were observed in the aromatic region due to the phenyl ring in the substituent. In ^{13}C-NMR spectra of the compounds, signals belong to C-CH_3 S-CH_2 and O-CH_2 were detected at 24.24-24.28 ppm, 36.00-36.54 ppm, and 60.51-60.56 ppm, respectively. The signal of carbonyl carbon was detected at 165.79-168.85 ppm in common with all final compounds. HRMS results of the compounds were processed in negative ion mode and peaks were detected following the molecular weights of the compounds.

ADME Parameters

Some properties of the synthesized compounds were displayed in **Table 1**, which were calculated by SwissADME.[18-20] The number of HBA was calculated minimum 6 and maximum 8, and also number of HBD was 2. Log P values were estimated between 2.23 and 3.65. All compounds have violations of RoF equal to or less than 2. Therefore, the final compounds may be applied for oral use according to forecast.[21] These findings were also in harmony with the activity results. Especially, compound **5e** stand out via TPSA (161.10 Å^2) and Log P (3.36). These differences between **5e** and other derivatives may be the reasons which explain the incoherent in the activity tests due the solubility.

Table 1. Some properties of synthesized compounds

	HBA	HBD	TPSA	Log $P_{o/w}$	Log S (Ali)	RoF (V)
5a	8	2	206.92	2.23	-7.18	No (2)
5b	6	2	161.10	3.05	-6.69	Yes (1)
5c	7	2	161.10	3.34	-6.50	Yes (1)
5d	6	2	161.10	3.48	-7.04	Yes (1)
5e	6	2	161.10	3.36	-6.60	Yes (1)
5f	7	2	170.33	2.92	-6.56	Yes (1)
5g	7	2	170.33	3.11	-6.94	Yes (1)
5h	7	2	169.26	2.65	-6.57	No (2)
5i	6	2	123.44	3.65	-5.89	Yes (0)
SD-1	7	1	110.39	1.37	-3.76	Yes (0)

HBA: H-bond acceptor, **HBD:** H-bond acceptor, **TPSA:** Topologic polar surface area (Å^2) **Log $P_{o/w}$:** *Consensus* Log $P_{o/w}$ (Average of all five predictions), **Log S:** Water Solubility, **RoF (V)**: Rule of Five (violation number), **SD-1**: Standard Drug (*N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid).

Activity Results

All compounds (**5a-5i)** and the standard drug were investigated for the MMP-9 inhibition activity were shown in **Table 2**.

Table 2. Inhibition % on MMP-9

	Inhibition %
5a	40.86 ± 1.62
5b	48.21 ± 1.27
5c	23.22 ± 3.57
5d	9.52 ± 2.06
5e	62.5 ± 2.53
5f	27.50 ± 1.52
5g	51.19 ± 2.06
5h	15.18 ± 1.26
5i	41.07 ± 2.52
NNGH	91.10 ± 2.50

100 μg/mL of each synthesized compounds was used to determine the inhibition % on MMP-9. **NNGH**: *N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid, and 1.3 μM/mL of it was used to determine the inhibition% on MMP-9.

Enzyme Studies

According to the MMP-9 inhibition results, the most active compound was compound **5e** with methyl substitution on benzothiazole ring. Also, compound **5g** (6-ethoxybenzothiazole) showed moderate inhibition activity. These results were worthy of note that the development of new treatments against cancers like lung carcinoma. To better understand the structure-activity relationship, in our investigation we proceeded to the next step: molecular docking study.

Molecular Docking and Structure-Activity Relationship (SAR) Studies

For comparing the active and non-active/low effective structures, **5d, 5e,** and **5g** were docked to MMP-9 enzyme (PDB ID: 5I12). The docking poses collected as shown in **Figure 1-6**.

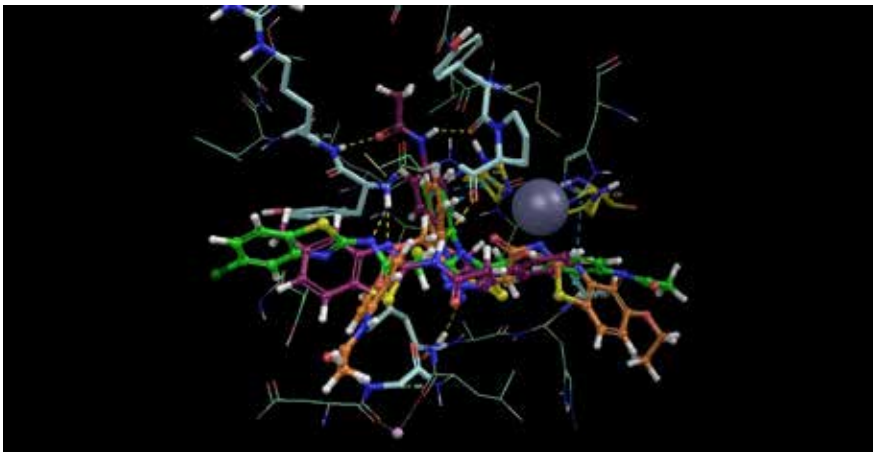


Figure 1. 3D best docking poses of compounds. [5d(green carbons), 5e (maroon carbons) and 5g (orange carbons) with Zn^{+2} (violet ball) in the active site of 5I12. The binding site amino acids were colored with turquoise blue carbons.]

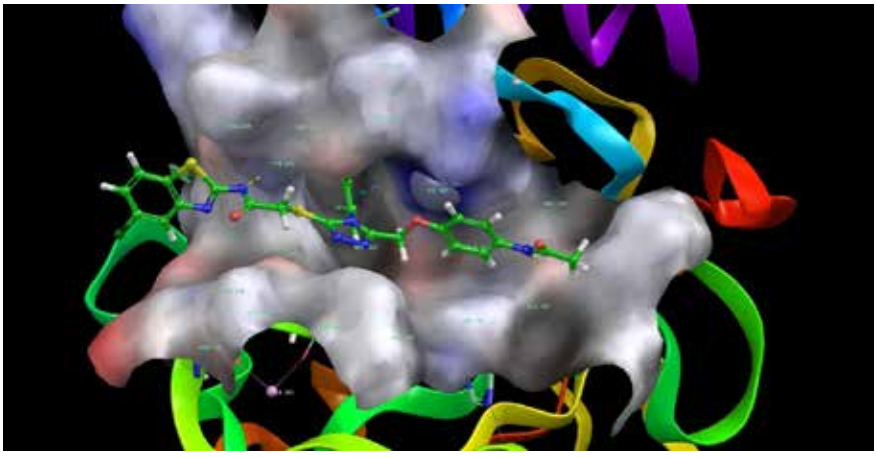


Figure 2. 3D best docking pose of compounds 5d in active site of 5I12

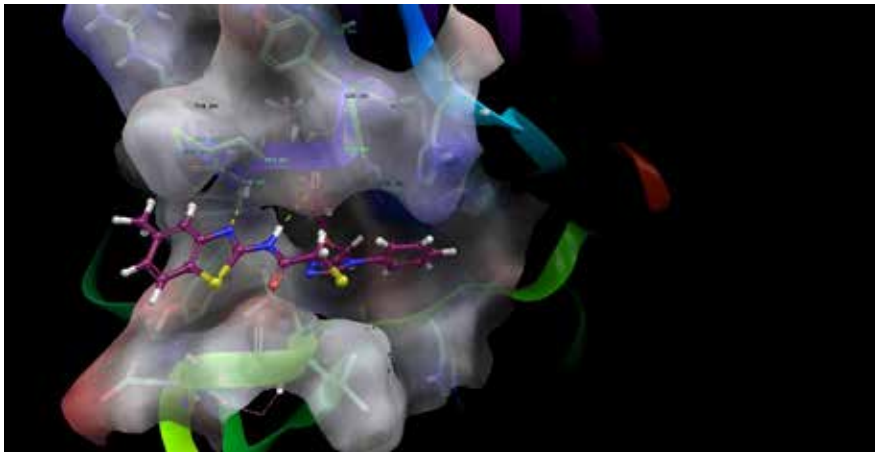


Figure 3. 3D best docking pose of compound 5e in active site of 5I12

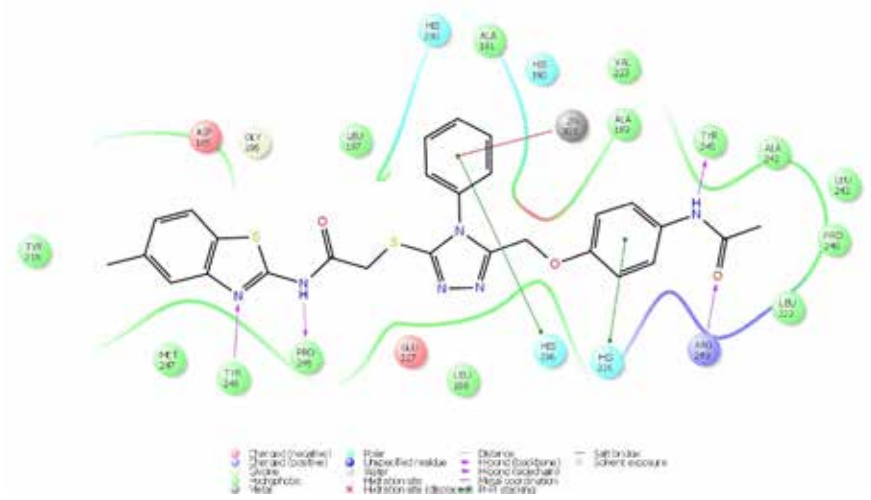


Figure 4. 2D interaction diagram of compound 5e in active site of 5I12

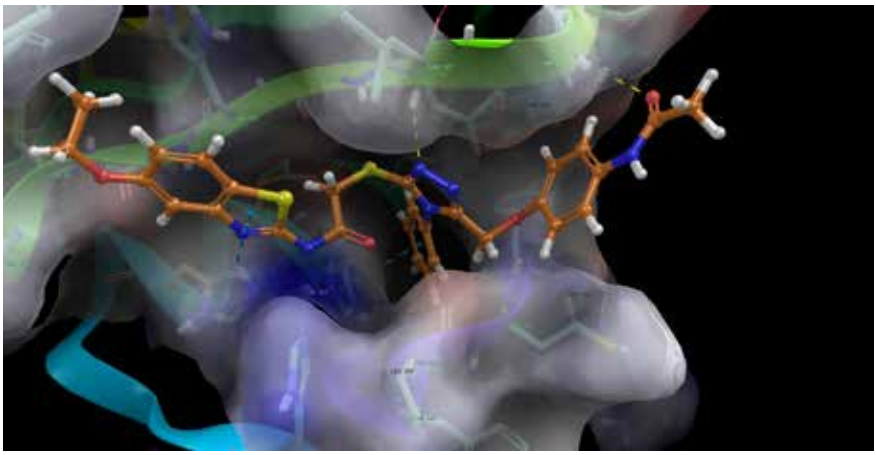


Figure 5. 3D best docking pose of compound 5g (green carbons) in active site of 5I12

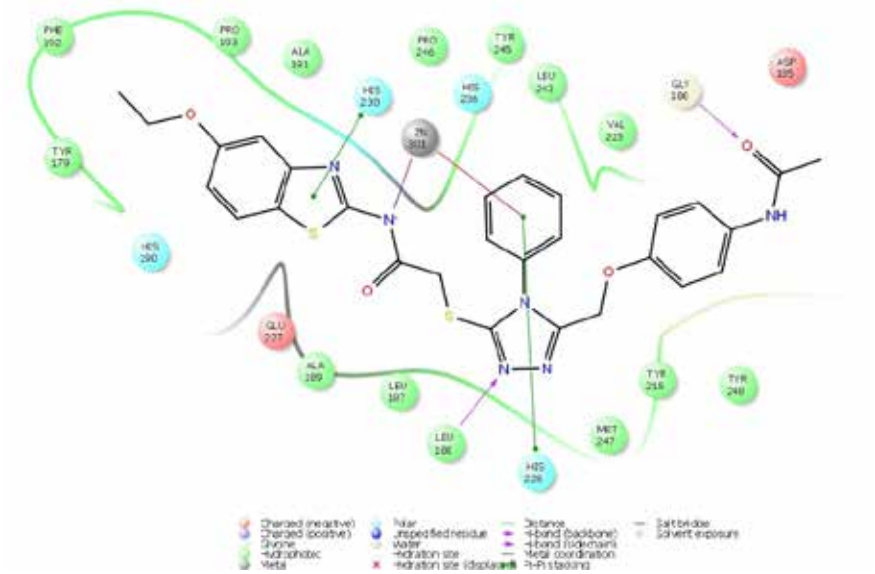


Figure 6. 2D interaction diagram of compound 5g in active site of 5I12

According to docking results, compound **5e** interacted with His226, His236, Tyr245, Pro246, Tyr248, and Arg249 active site amino acids and Zn301 metal ion. Between the phenyl rings and the histamine amino acids occurred π-π stacking, and four H-bonds were viewed with the benzothiazole nitrogen, acetamide nitrogen, acetanilide nitrogen and oxygen. These bonds interacted with hydrophobic (Tyr and Pro) and positively charged amino acid (Arg). There was a π-cation interaction between triazole's phenyl and Zn301.

Compound **5g** interacted with Gly186, Leu188, His226, His230 amino acids, and Zn301 metal ion. Between phenyl and His226, and benzothiazole and His230 occurred π-π stacking. There was two H-bond displayed between the triazole nitrogen (3rd) and leucine, and acetanilide oxygen and glycine. Also, there was observed the salt bridge between Zn301 and acetamide nitrogen. Also, the π-cation interaction between the metal ion and triazole phenyl was observed.

Compound **5d** interacted with Ala191, His226, His236, and Try248 amino acids and Zn301 metal ion. Between the phenyl rings and histamine amino acids (226, 236) occurred π-π stacking. There were two H-bonds which were between acetanilide nitrogen and Ala191, and acetamide nitrogen and Tyr248. There was observed π-cation (with phenyl of triazole) and metal coordination interactions.

Previous studies mentioned that zinc ion at the active site of the catalytic domain is closely related to the hydrolytic activity of MMP families.[22,23] Thus, the effectiveness depends not only on the number of hydrogen bond but also on the bonds that the ligand forms with histamine and zinc. Our docking results point out that zinc ion, and its bonding amino acids (His226, His230, and His336) are the important residues as much as H-bonds in MMP-9 activity cavity. However, the relationship between the differences in inhibition% was found related to hydrogen bonds. The results of the docking study and in vitro enzyme study were in good harmony.

For less or non-active compounds, the electron-withdrawing groups at the benzothiazole ring decreased the inhibition activity. It may be caused by bulky atoms and/or non-hydrophobic affinity. Additionally, although there was not enough data to compare the electron-withdrawing or donating groups at phenyl, the enzyme study proved that the activities of the electron-withdrawing groups (-NO_2 and -F) showed that less than 50% inhibition. Therefore, for future studies, the electron-donating group and/or hydrophilic moiety may be thought. Also, as a new perspective, *N* substitution on the triazole ring may have a short carbon chain with composing π interactions for hydrophobicity and easy placement. The importance of π–π stacking between the ligand and the imidazole ring of histamine was underlined.22 On the other hand, the docking results showed that the substitutions at benzothiazole should comprise of carbon chain without heteroatoms. Although the ethoxy derivative (**5g**) showed the inhibition activity, it was seen that the oxygen atom didn't show H-bond interaction. The methoxy (**5f**) derivative also didn't display the inhibition activity, hence the group at benzothiazole may be constituted of the carbon chain without heteroatoms.

Briefly, we concluded the results that although final compounds have good pharmacokinetic profiles, only two compounds (**5e** and **5g**) showed an attractive activity. The distinctness between the active compounds and others were related to the hydrophobic properties. Also, the interactions between the ligand and enzyme were discussed for future studies. Hopefully, these modifications especially will be increased the anticancer activity through MMP-9 inhibition or at least give an option. In the next studies, these are the main data in the designing strategy.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

REFERENCES

1. Atlihan-Gundogdu, E., Ilem-Ozdemir, D., Ekinci, M., Ozgenc, E., Demir, E. S., Sánchez-Dengra, B., & González-Alvárez, I. **(2020)**. Recent developments in cancer therapy and diagnosis. *Journal of Pharmaceutical Investigation*, 1-13.

2. Boumahdi, S., & de Sauvage, F. J. **(2020).** The great escape: tumour cell plasticity in resistance to targeted therapy. *Nature reviews Drug discovery*, *19*(1), 39-56.

3. Pucci, C., Martinelli, C., Ciofani, G., **(2019)**. Innovative Approaches for Cancer Treatment: Current Perspectives and New Challenges. *Ecancermedsci. 13*, 961.

4. Sabari, J. K., Offin, M., Stephens, D., Ni, A., Lee, A., Pavlakis, N., ... & Li, B. T. **(2019).** A prospective study of circulating tumor DNA to guide matched targeted therapy in lung cancers. *JNCI: Journal of the National Cancer Institute*, *111*(6), 575-583.

5. Alaseem, A., Alhazzani, K., Dondapati, P., Alobid, S., Bishayee, A., & Rathinavelu, A. **(2019).** Matrix Metalloproteinases: A challenging paradigm of cancer management. In *Seminars in cancer biology* (Vol. 56, pp. 100-115). Academic Press.

6. Fischer, T., Senn, N., & Riedl, R. **(2019).** Design and structural evolution of matrix metalloproteinase inhibitors. *Chemistry–A European Journal*, *25*(34), 7960-7980.

7. Fields, G. B. **(2019).** The rebirth of matrix metalloproteinase inhibitors: moving beyond the dogma. *Cells*, *8*(9), 984.

8. Yüksek, H., Özdemir, G., Manap, S., Yılmaz, Y., Kotan, G., Gürsoy-Kol, Ö., & Alkan, M. **(2020).** 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. *ACTA Pharmaceutica Sciencia*, *58*(1).

9. Holla, B. S., Veerendra, B., Shivananda, M. K., & Poojary, B. **(2003).** Synthesis characterization and anticancer activity studies on some Mannich bases derived from 1, 2, 4-triazoles. *European Journal of Medicinal Chemistry*, *38*(7-8), 759-767.

10. Sztanke, K., Tuzimski, T., Rzymowska, J., Pasternak, K., & Kandefer-Szerszeń, M. **(2008).** Synthesis, determination of the lipophilicity, anticancer and antimicrobial properties of some fused 1, 2, 4-triazole derivatives. *European journal of medicinal chemistry*, *43*(2), 404-419.

11. El-Sherief, H. A., Youssif, B. G., Bukhari, S. N. A., Abdel-Aziz, M., & Abdel-Rahman, H. M. **(2018).** Novel 1, 2, 4-triazole derivatives as potential anticancer agents: Design, synthesis, molecular docking and mechanistic studies. *Bioorganic chemistry*, *76*, 314-325.

12. Bhat, K. S., Poojary, B., Prasad, D. J., Naik, P., & Holla, B. S. **(2009)**. Synthesis and antitumor activity studies of some new fused 1, 2, 4-triazole derivatives carrying 2, 4-dichloro-5-fluorophenyl moiety. *European Journal of Medicinal Chemistry*, *44*(12), 5066-5070.

13.. Ahmed, F. F., Abd El-Hafeez, A. A., Abbas, S. H., Abdelhamid, D., & Abdel-Aziz, M. **(2018)**. New 1, 2, 4-triazole-Chalcone hybrids induce Caspase-3 dependent apoptosis in A549 human lung adenocarcinoma cells. *European journal of medicinal chemistry*, *151*, 705-722.

14. Kaur, R., Ranjan Dwivedi, A., Kumar, B., & Kumar, V. **(2016)**. Recent developments on 1, 2, 4-triazole nucleus in anticancer compounds: a review. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *16*(4), 465-489.

15. Yurttaş, L., Evren, A. E., Kubilay, A., Temel, H. E., & Çiftçi, G. A. **(2020)**. 3, 4, 5-Trisubstituted-1, 2, 4-triazole Derivatives as Antiproliferative Agents: Synthesis, In vitro Evaluation and Molecular Modelling. *Letters in Drug Design & Discovery*, *17*(12), 1502-1515.

16. Kaplancikli, Z. A., Altıntop, M.D., Atli, O., Sever, B., Baysal, M., Temel, H. E., ...& Oz-

demir, A. (**2017).** Synthesis and Evaluation of A New Series of Thiazole Derivatives As Potential Antitumor Agents and MMP Inhibitors. *Anticancer Agents Med Chem*. *17*(5), 674-681.

17. Maestro, S. In LLC, New York, 2016.

18. Daina, A., Michielin, O., & Zoete, V. **(2017).** SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific reports*, *7*(1), 1-13.

19. Daina, A., Michielin, O., & Zoete, V. **(2014)**. iLOGP: a simple, robust, and efficient description of n-octanol/water partition coefficient for drug design using the GB/SA approach. *Journal of chemical information and modeling*, *54*(12), 3284-3301.

20. Daina, A., & Zoete, V. **(2016).** A boiled-egg to predict gastrointestinal absorption and brain penetration of small molecules. *ChemMedChem*, *11*(11), 1117.

21. Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. **(1997)**. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*, *23*(1-3), 3-25.

22. Nuti, E., Cuffaro, D., D'Andrea, F., Rosalia, L., Tepshi, L., Fabbi, M., ... & Rossello, A. **(2016).** Sugar-Based Arylsulfonamide Carboxylates as Selective and Water-Soluble Matrix Metalloproteinase-12 Inhibitors. *ChemMedChem*, *11*(15), 1626-1637.

23. Gao, Q., Wang, Y., Hou, J., Yao, Q., & Zhang, J. **(2017).** Multiple receptor-ligand based pharmacophore modeling and molecular docking to screen the selective inhibitors of matrix metalloproteinase-9 from natural products. *Journal of computer-aided molecular design*, *31*(7), 625-641.

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Biomarkers in Parkinson's disease - An effective tool for diagnosis of PD

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ABSTRACT

Parkinson's disease (PD) ranks the second position of neurodegenerative disorder mainly arises due to depleted levels of neurotransmitter dopamine in substantia nigra. PD is characterized by decreased levels of dopamine due to dopaminergic cell death and manifested by tremor, rigidity, bradykinesia and postural abnormalities. Considerable latency is observed in dopaminergic cell death and appearance of clinical symptoms in PD, so it is necessary to diagnose the disease in early stages for better treatment. Establishment of reliable biomarkers which indicates the progression of the disease and will aid in better clinical management of PD patients. Biomarkers when coupled with system biology tools help in discovering transcriptomic, proteomic, genomic and metabolic molecules and other signaling pathways responsible for differential diagnosis of parkinsonism disorder and serves as tool in monitoring disease progression. Identifying the subjects at earlier stage of disease would pave the way to novel drug therapies to decrease the progression of disease.

Keywords: Parkinson's disease, LRRK2, DJ-1biomarker, neuroimaging biomarkers, reflex tears, α-synuclein

INTRODUCTION

Parkinson's disease (PD) is a complex syndrome with different clinical subtypes characterized by a large motor and non-motor features which intern effect the

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function and quality of life of affected individuals. Pathological evidences suggest that the pathological process of PD begins much earlier prior to the appearance of typical motor symptoms. About 70-80% of striatal dopamine and one third of substantia nigra neurons and striatal dopaminergic neurons have already been lost before the diagnosis is made. [1, 2]

Pathology of PD is complicated. Recent studies have reported the evaluation of brain samples of PD patients showing the presence of Lewy bodies (LB) which is the hallmark of PD to trace the damaged neurons throughout the nervous system. Braak et al conducted a study and reported that there are six neuropathological stages of PD [3], which are shown in Fig 1.

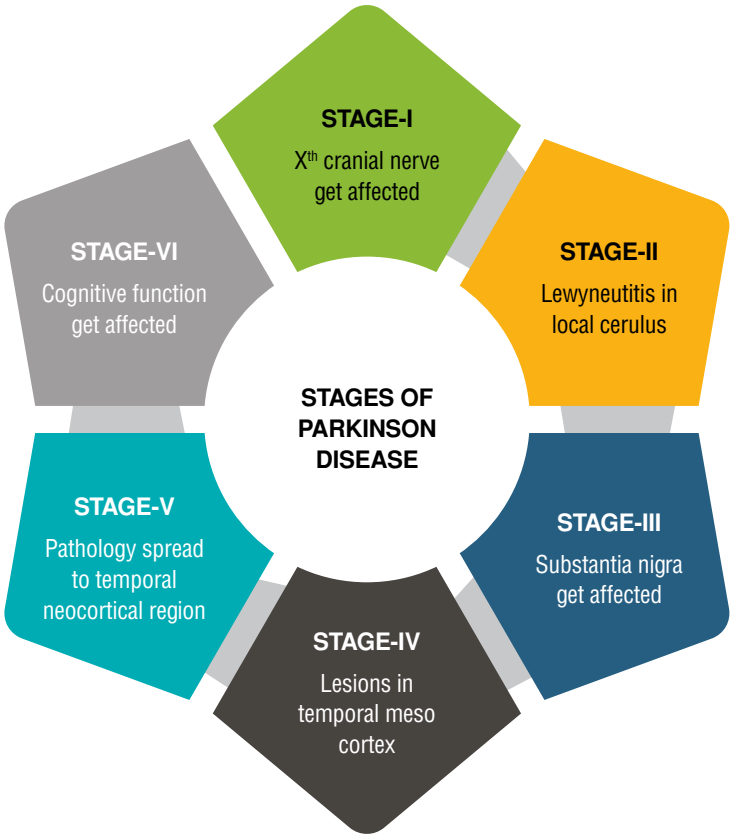

Figure 1. Neuropathological stages of Parkinson disease

STAGE-I: In this stage beginning of pathology is traced in lower medulla oblongata in the dorsal motor nucleus of the tenth cranial nerve and the olfactory structures specifically in anterior part.

STAGE-II: Lewy neuritis is observed in locus coeruleus, exacerbation of lesions in dorsal motor nucleus.

STAGE-III: Damage to the substantia nigra is observed.

STAGE-IV: Presence of lesions in the temporal mesocortex.

STAGE-V: Pathology is seen in the adjoining temporal neocortical fields.

STAGE-VI: Cognitive status correlates with the neuropathological stage.

According to National Institute of Health (NIH), a biomarker is a tool which assists in detection and diagnosis of disease and indicates the pharmacological response to a therapeutic intervention. [4] Development of reliable and sensitive biomarkers will help in detection of individual at risk before the progression of classical symptoms in a stage where neuroprotective therapies have higher efficacy. Establishment of a reliable biomarker will help in discriminating PD from other neurodegenerative disorders. Biomarkers are the tools which assist the physicians in developing pathogenesis-targeted therapeutics of PD. [5]

Biomarkers in PD

Parkinsonism is manifested by motor features including bradykinesia, tremors, rigidity and postural abnormalities along with the non-motor features. Myriad of biomarkers are used at different stages of PD. Different phases of PD are shown in Figure 2.

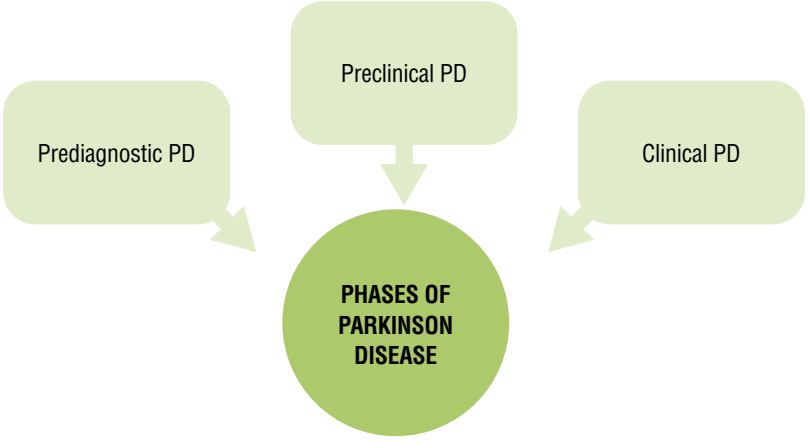

Figure 2. Different phases of Parkinson's disease

Prediagnostic phase of PD

In established PD, patients experience non-motor symptoms which are as troublesome as motor features, and treatment is often difficult. Non-motor symptoms are experienced early and there is substantial evidence which suggests that they also aid in early diagnosis of the disease several years prior to the appearance of clinical symptoms. A number of studies have demonstrated the association of PD with earlier diagnosis such as depression, anxiety, constipation and erectile dysfunction. [6, 7, 8]

Biomarkers in prediagnostic stage

The clinical diagnosis of PD requires multiple motor features to be established, and while subtle motor signs may be present, a clinical diagnosis of PD is not possible until motor symptoms become more definite. [9] Non-motor features potentially serves as biomarkers in early diagnosis of PD. Environmental factors also contribute to the occurrence of PD. Exposure to pesticides in farmers and people living in rural areas are associated with increased risk of PD. Rotenone and paraquat which are chemically relative to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine specifically increase the risk of PD. [10] Mendelian randomisation (MR) study is a powerful technique which correlates between the risk factors and progression of the disease. This technique was used by Simon and colleagues, they used polymorphism of single nucleotide in SLC2A9 gene to estimate the genetic variability of serum urate levels. Outcome of this study suggested elevated levels of urate in serum which delayed progression of the disease. Findings of this MR study emerged as novel therapeutic strategy to treat the disease progression by modulating therapeutic urate levels. [11]

Biomarkers in preclinical phase of PD

Preclinical phase is that stage of PD which precedes the diagnostic phase of PD which is devoid of motor symptoms of the disorder characterised by subtle non-motor features of the disease which can be detected by physiological examination. Various biomarkers are available for determining preclinical stage which may develop PD in future. [12] Biomarkers in preclinical phase of PD are represented in Figure 3.

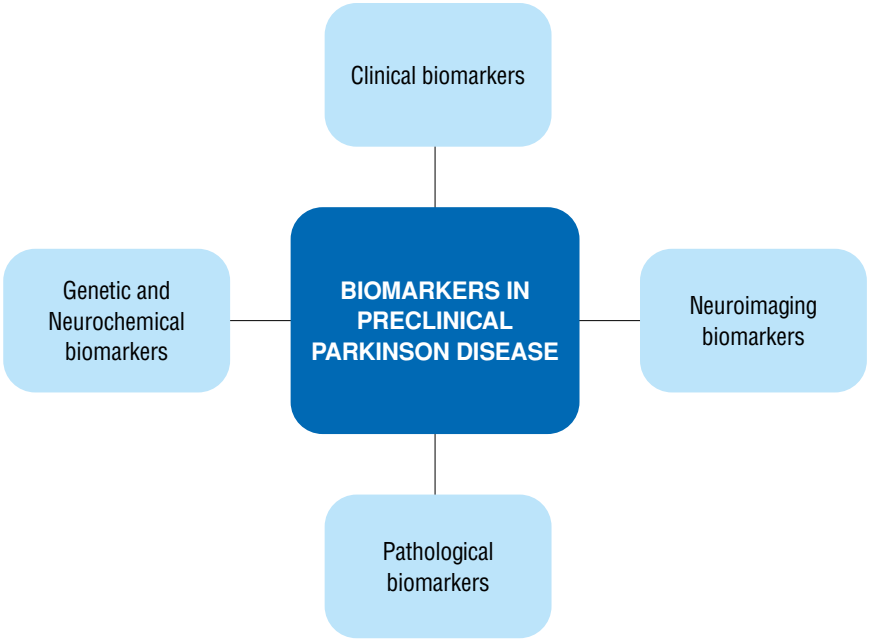

Figure 3. Biomarkers in preclinical stage of Parkinson's disease

Clinical biomarkers of preclinical pd

Evidences suggest that prior to the appearance of motor features of PD various premonitory sign and symptoms are seen [13, 14] which are as follows,

Sleep Disturbances

Patients with PD have disturbed sleep pattern characterised by rapid behavioural disorder (RBD) which is seen much prior to the onset of motor symptoms. [15, 16] In a long-term clinical study conducted with 93 subjects with RBD, 14 patients developed Parkinsonism with high degree of cognitive impairment. [17]

Essential tremors

Neuropathological studies and epidemiological studies have suggested that patients with essential tremors have co-existent PD. [18] Cohort study conducted on 3813 geriatric patients in central Spain reported that patients with Essential tremors are 4 times more prevalent to develop PD than controls. [19]

Olfactory Dysfunction

Population based prospective studies revealed the association between olfactory loss and future development of PD. [20] About 90% of the patients suffer-

ing from PD exhibits anosmia. [21, 22] Odour separation measures indicate the severity of disease and further studies support that olfactory dysfunction is the early sign of idiopathic PD. Neuroimaging and neurochemical marker studies revealed that olfactory loss in PD can be seen 2 to 7 years prior to the clinical development of PD. [23]

Constipation

Dysautonomia is generally traced at the advanced stages of PD. According to epidemiological studies conducted among the various symptoms of autonomic dysfunction constipation appears earliest and much prior to motor symptoms. [24]

Pathological biomarkers in preclinical pd

Degeneration of dopaminergic neurons in substantia nigra pars compacta, associated with proteinaceous inclusions within the cytoplasm known as Lewy Bodies (LB) is the crucial pathological hallmark of PD.

Braak hypothesis however suggested that neurodegeneration of non-dopaminergic neurons in the caudal regions of brainstem particularly in dorsal motor nucleus of vagus and olfactory regions precedes the dopaminergic degeneration. Braak hypothesis states that α-synuclein pathological changes in PD begin in the lower brainstem and progress in caudal-rostral pattern. This promotes the notion that non-motor features reflecting this prenigral involvement can precede the classic motor features of PD.

Based on the study conducted by implanting embryonic dopaminergic cell in the striatum of patients with PD have developed pathological features including formation of LB has led to the hypothesis that α-synuclein can be secreted into extracellular space of brain and spread PD in a prion like fashion. [25]

Olfactory features

According to Hawkes et al synucleinopathy in PD starts in olfactory bulb and enteric plexuses this paved the way to dual-hit hypothesis according to this an unknown neurotropic pathogen initiates the pathogenesis underlying sporadic PD in anterograde and retrograde fashion. [26]

Skin biopsy

By performing punch skin biopsy using immune staining and panneuronal marker for protein gene product, has shown the involvement of peripheral autonomic nervous system in early stages of PD. Presence of α-synuclein deposits in dermis of PD patients concludes it as variant synucleinopathy. [27, 28]

NEUROIMAGING BIOMARKERS IN PRECLINICAL PD

Presymptomatic stages of PD can be detected by various imaging techniques. [29] Brain abnormalities in PD patients using various techniques such as dopamine transporter-single photon emission computed tomography (DAT-SPECT), positron emission tomography (PET), transcranial B-mode sonography (TCS), diffusion weighted imaging (DWI), magnetic resonance imaging (MRI), sonography.

Positron Emission Tomography

PET scan involves emission of positrons which are positively charged. DAT-SPECT at the straiatal regions of substantia nigra can be assessed employing radiolabeled dopamine analogs such as ^{18}F,^{11}C,^{18}F-dopamine[30], ^{18}F-FE-PE2I, ^{18}F-LBT999 [31], ^{11}C-methylphenidate, ^{11}C, ^{18}F radiolabelled dihydrotetrabenazine (DTBZ) are the radioligands used for quantification of vesicular monoamine transporter type 2 (VMAT2).

Both DAT and VMAT2 ligands trace the early signs of dopaminergic damage. [32] DAT ligands estimate the uptake and decarboxylation of levodopa coupled with the amount of radiolabeled dopamine stored in vesicles. Reduced DAT binding is the earliest indication of dopaminergic dysfunction in individuals and serves as potential biomarker of preclinical PD. [33] Another radiolabelled ^{11}C-dihydrotetrabenazine detects nigrostriatal deficits in preclinical PD indicating the severity of PD.

DAT SPECT

Single photon emission computed tomography (SPECT) imaging technique aids in visualising the dopamine transporter (DAT) levels in the brain. Studies revealed reduced binding of DAT in patients with PD which further indicates the severity and asymmetry of motor clinical scores. [34] Binding of N-fluoropropyl-2b-carbomethoxy-3b-4-[^{123}I] iodophenyl-nortropane ([^{123}I]-FP-CIT) to the striatum is helpful in detecting the severity even at preclinical stage in subjects with de novo PD, thus any abnormality in imaging serves as fruitful biomarker of early PD. [35]

Trans Cranial B-Mode Sonography (TCS)

Most reliable method used to measure the velocity of blood flowing through brain vessels by measuring the echoes and frequency of ultrasound waves. When patients with PD are subjected to TCS increased echogenicity of substantia nigra is traced in the brains of PD patients. According to Skoloudik et al., higher echogenicity is mainly due to elevated levels of iron, gliosis due to alteration or malfunction of blood brain barrier. [36]

Magnetic Resonance Imaging (MRI)

MRI is a form of DWI (Diffused Weighted Imaging) which is based on diffusion principle. It measures the amount of water diffused through the tissue thereby determines the structural defects. Increased diffusivity of water indicates the necrosis of cells or reduced volume of the tissue region. This technique also distinguishes PD from multiple system atrophy (MSA). Patients with MSA exhibits more diffusivity of water in the middle cerebella peduncles compared to PD patients. [37] High resolution 3-Tesla MRI can also trace the diminished volume of caudate and putamen in PD patients. [38]

Genetic Biomarkers in preclinical PD

Alpha-Synuclein – Levels of α-synuclein in PD patients's CSF have been found to be decreased which is due to syucleinopathy. [39] Thus α-synuclein is employed as a potential biomarker in clinical diagnosis of PD. [40]

LRRK2 (OMIM*609007) – Mutations in LRKK2 is responsible for 10-15% of familial cases with autosomal dominant PD, [41] 30-40% of familial cases are mainly due to mutations in LRRK2 such as G2019S which was evolved as a potential screening tool in Ashkenazi Jews and North African Arabs. [42, 43]

Multi functional protein DJ-1(OMIM*602533) - Decreased levels of this protein is responsible for familial, Autosomal recessive PD. [44]

Parkin gene (OMIM*601828) - Mutation of parkin gene is major cause of several tumors and autosomal recessive PD. [45]

Transcription factor NURR1 (NR4A2) (OMIM*601828) - It is a member of nuclear receptor family essential for the development, survival, functional maintenance of dopaminergic neurons in midbrain. [46] A study conducted on 278 subjects suffering from PD, 166 healthy subjects and 256 controls with neurological disease and the results revealed that there is decreased expression of NURR1 gene in PD patients. [47]

Cocaperone st_{13} (omim*606796) - It stabilizes Heat shock protein-70 (HSP-70) which has the ability to modify α-synuclein misfolding and toxicity. Levels of ST13 m-RNA was significantly reduced in PD patients. [48]

Biomarkers in clinical phase of pd

Clinical phase of PD is associated with motor symptoms such as bradykinesia, rigidity and resting tremors. Detection of motor signs and symptoms serves as potential markers in progression of disease at different stages. NIH sponsored a clinical trial study named DATATOP for determining motor scores to identify

disease progression. Several non-motor features precede the classical motor features. Such as olfactory dysfunction, REM sleep disorder, bowel dysfunction and depression serve as markers of disease. [49]

Neurochemical biomarkers in parkinson's disease

Orexin

Lateral and posterior neurons of hypothalamic region possess a neuropeptide hormone named orexin also known as hypocretin. It has a key role in regulating sleep-wake cycle, [50] cardiovascular response, hypertension [51] and heart rate. Narcolepsy seen in PD patients is mainly due to degeneration of hypocretin neurons in hypothalamus. Severity of disease is directly related to the concentration of orexin. [52] Orexin levels are decreased in PD patients than healthy subjects.

Glial fibrillary acidic protein (GFAP) in CSF is expressed by astrocytes. Increased GFAP levels mainly responsible for decreased levels of orexin, thus GFAP is considered as potential biomarker. [53]

8-Hydroxy-2'-Deoxyguanosine (8-OHdG)

It is a reactive oxygen species (ROS) of 8-hydroxyguanine responsible for DNA lesions. [54] Increased serum levels of 8-OHdG are observed in PD. Hence, 8-OHdG serves as potential biomarker in PD. [55]

Dopamine transporter (DAT)

Increased dopamine levels are toxic to the neurons by undergoing oxidation to form reactive quinones. In order to maintain the levels of dopamine, dopamine transporters such as vesicular monoamine transporter 2 (VMAT 2) store excess dopamine in vesicles. Any change in dopamine transporter levels is an indicator of PD. [56]

Dopamine metabolites

Decreased levels of 3, 4-dihydroxy phenyl acetic acid (DOPAL) and DOPA in CSF are helpful in diagnosing preclinical PD. DOPAL is responsible for oligomerization and aggregation of α-synuclein in PD. [57]

Dopamine Receptor

Among the five different types of dopamine receptors (D1-D5), D3 type of receptor is significantly reduced due to reduced dopamine signals [55]. Therefore, D3R is used as a potential biomarker for PD. [58]

Apolipoprotein A1 (APO A1)

Liver and small intestine are the major sources of APO A1 which is an essential constituent of high-density lipoprotein (HDL). APOA1 along with APOE are in-

volved in lipid transportation in the brain. [59] CSF of PD patients show reduced levels of APOA1and tetranectin have been observed, thus act as a potential biomarker for PD. [60, 61]

RNA-Based PD Biomarkers

A novel diagnostic approach is developed to identify PD in early stages. MiRNA's are non-coding which consists of 21-24 nucleotides emerged as potential biomarkers of PD because of their presence in CSF as well as in exosome. A study conducted by Dos Santos et al analyzed expression of miRNA profile using NGS (next generation sequencing) in CSF of PD patients and identified miRNA based biomarker pannel of Let-7f-5p,miR-125a-5p,miR-151a-3p,miR-27-a-3p and miR-423-5p five best ranking variables. [62]

Peripheral Proteasome and Caspase Activity

PD is associated with accumulation of intracellular inclusions called Lewy's bodies (LB). Mutations in proteasomes lead to their dysfunction and potentiates accumulation of aggregated α-synuclein. [63] Increased ROS in case of mitochondrial deficiency increases oxidation of α-synuclein leading increased ATP-independent proteosomal activity and greater α-synuclein oligomerization. Any changes in ATP levels inhibit 26s proteasome. [64] Severity of the disease is associated with decreased proteosome 20s activity and increased caspase 3 activity. Raised levels of caspase 3 initiate apoptosis thereby decrease proteasome activity. Therefore, proteasome and caspase are used as potential biomarkers. [65]

Novel Biomarkers in PD

Proteomics, metabolomics and transcriptomics are novel tools capable of tracing any changes in protein, metabolites, RNA profiles and body fluids to distinguish healthy subjects and diseased subjects. [66]

CSF fluid metabolite profile

Pathological changes in brain are directly related to composition abnormalities of CSF. PD is associated with neurodegenerative changes of dopaminergic neurons in nigrostriatal pathway. Levels of dopamine and its metabolites determine the progression and severity of the disease and are considered to be potential biomarkers.

CSF fluid is analyzed by various analytical techniques, one of the analytical techniques used for analyzing CSF is LCECA (LC-electrochemistry array). Reduced levels of homovanillic acid (HVA), dihydoxyphenylaceticacid (DOPAC), L-Dopa and dihydroxyphenylglycol have been reported in PD patients. [67]

Another analytical technique used is gas chromatography-time of flight/ mass spectrometry (GC-TOF/MS), various metabolites such as tryptophan, creatinine, 3-hydroxyisobutyricacid; glucose mannose levels are reduced in PD patients. [68, 69]

Blood profile

Screening of blood of healthy and diseased subjects suffering from PD led to the discovery of novel biomarkers for diagnosis of the disease. Dysregulation of kynurenine pathway is seen in PD patients. [70, 71, 72] The changes in kynurenine metabolites in PD serves as biomarker and also pave the way to novel therapeutic strategy for treating PD by giving kynurenic acid supplement or by reducing quinolinic acid using kynurenine 3-monooxygenase inhibitors. Urate levels in blood have significant importance in determining the progression and severity of the disease. Urate is an important antioxidant present endogenously. [73] Attributing to its antioxidant property increased urate levels help in fighting against oxidative stress in PD patients. High urate levels indicate low risk and slow progression of the disease. [74, 75] These findings led to the longitudinal study of a nutritional supplement inosine which increases urate levels so used in treating PD.

Sometimes misdiagnosis of PD due to presence of co-existing neurodegenerative diseases such as progressive supra-nuclear palsy (PAP), primary progressive multiple sclerosis (PPMS), multiple system atrophy (MSA) may misled the diagnosis and treatment.

NMR (nuclear magnetic resonance) and LC-MS (liquid chromatography-mass spectrometry) study reports revealed that branched chain amino acids (BCAA'S) levels are high in patients with PD, PAP, MSA compared to healthy subjects. [76] N-8-acetyl sperm dine is effective in determining disease onset and progression of the disease. In addition to the biomarker activity it also plays significant role in delaying or slowing down the progression of the disease. [77]

Urine analysis

Biopyrrin is the oxidative product of bilirubin. Biopyrrin exacerbates the oxidative stress associated with PD and identified as novel biomarker for sporadic PD. [78] Urine being a rich source of metabolites is screened by GC-MS and LC-MS techniques. Results revealed that dysregulation of steroidogenesis, tryptophan, and phenylalanine pathways are related to the progress of the disease.

Fecal examination

Fecal short chain fatty acids (SCFAs) promote GI motility and regulate functions of ENS (enteric nervous system). GC-based analysis reported the decreased levels of SCFAs are responsible for development of gastrointestinal motility disorders in PD patients compared to controls. [79, 80]

Other Naive Biomarkers

Reflex tears

Reflex tears emerged as novel biomarker for diagnosis of PD. Reflex tearing involves the sensory and afferent nerve fibers which are damaged in PD. A study conducted involving 84 PD patients and 84 healthy controls. Oligomeric α-synuclein (α-syn oligo), total α-synuclein, lactoferrin and matrix metalloproteinase 9 levels are raised in tears of PD patients compared to healthy controls. Flow rate is significantly reduced in PD patients. [81, 82]

Saliva α-synuclein

A study conducted at Al-Mustansiriya University engaged 20 PD patients and 20 healthy subjects. Each subject is tested for salivary flow rate, salivary protein and α-synuclein using ELISA (Enzyme linked immune sorbent assay). Unstipulated saliva collected from each subject and it is screened through ELISA. Saliva α-synuclein levels is less in PD patients compared to healthy subjects. Therefore, saliva α-synuclein can be used as a potential biomarker in PD. [83]

Interleukins (IL-6, IL-10, IL-12)

High plasma concentration of proinflammatory agents like IL-6, IL-10 and IL-12 increases the risk of PD and thereby causing immunological disturbances in PD patients. [84, 85]

Low density lipoprotein

A study involving 236 PD patients and healthy controls reported that reduced LDL levels increase the risk of PD. This further promotes the novel therapeutic strategy of decreasing the risk of PD by increasing cholesterol levels. [86, 87]

Hair color

In PD patients the dark color of hair found to be decreased. Red hair colored and blond subjects are at high risk of PD and have a role of pigmentation in pathogenesis of PD. [88]

All the above-mentioned naive biomarkers are interesting and worth but need to be validated to consider as potent biomarkers. Figure 4 represents the various biomarkers of PD.

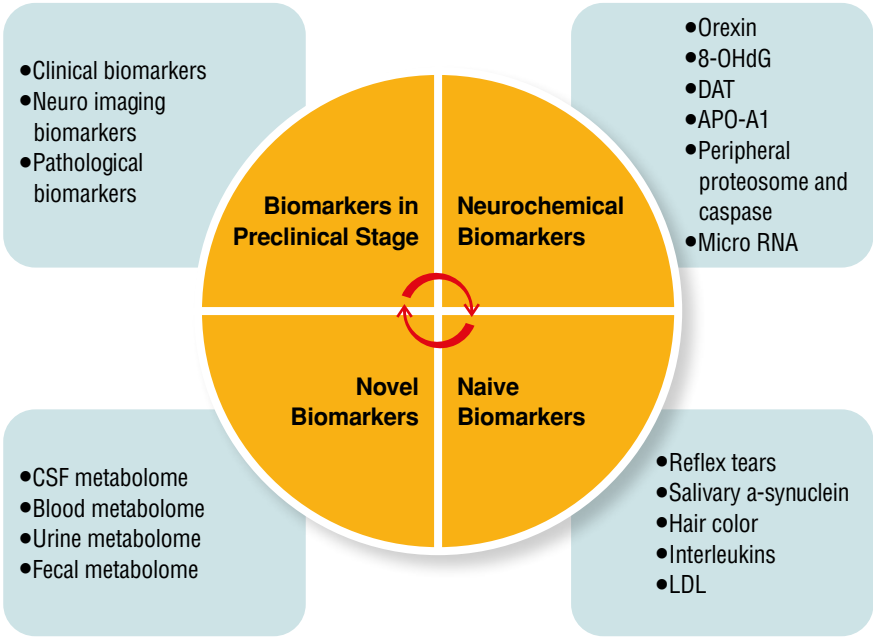

Figure 4. Biomarkers in Parkinson's disease

RESULTS and CONCLUSION

PD is the second most common neurodegenerative disorder affecting more than 10 million individuals worldwide. Because of considerable time lapse between neuronal damage and appearance of clinical symptoms, it is essential to discover and develop the biomarkers and diagnostic tools for prognosis of the disease and developing novel strategies in treatment. Quality of life improvement is the most urgent and unmet need in the field of science. Biomarkers are the unique tools which ease the process of diagnosis of the disease at early stages to identify at-risk individuals. Imaging techniques such as DAT-SPECT, MRI and PET are available but because of high cost and nonspecific target these techniques are not validated at preclinical stages. Many neurochemicals, non-motor features such as anosmia, olfactory dysfunction, constipation emerged as successful biomarkers for future diagnosis of PD pathology. Newly introduced biomarkers such as reflex tears, salivary levels of α-synuclein gathered considerable attention but they need to be validated to use them as potential biomarkers.

Single parameter is unlikely to become a fruitful biomarker. Intensive examination is to be done to introduce potent biomarkers which further potentiate the development of therapeutic strategies to increase the quality of life of patients

by prognosis of the disease. Studies which allow the assessment of combined data from various fields such as clinical, laboratory, imaging, and genetics must be preferred. Current review is presented with myriad of biomarkers and their ability to develop the novel therapeutic strategies in treating PD but these findings are just the "tip of the iceberg". In order to get access to the wider picture of PD etiology in-depth biological investigation has to be done. Discovery of miRNAs is a breakthrough in field which is a growing hope that screening these miRNAs greatly contribute in diagnosing the disease at early stages. Parkinson's Progression Markers Initiative (PPMI) is conducting an observational clinical study aimed to evaluate the data obtained through cohort studies employing patients with PD along with healthy subjects utilizing advanced imaging, sampling coupled with other laboratory biomarkers using body fluids to identify biomarkers of Parkinson disease progression. With the application of novel techniques such as proteomics, metabolomics, genomics and transcriptomics, it is likely to develop biomarkers which aid in detecting asymptomatic individuals with early disease so that they get benefit from early therapeutic intervention.

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CONFLICTS OF INTERESTS

There are no conflicts of interest among the authors.

REFERENCES

1. Bohnen, N.I.; Albin, R.L.; Koeppe, R.A.; Wernette, K.A.; Kilbourn, M.R.; Minoshima, S.; Frey, K.A. (**2006**) Positron emission tomography of mono-aminergic vesicular binding in aging and Parkinson disease. *J Cereb Blood Flow Metab*. 26(9):1198-1212.

2. Graford, S.; Very, M.; Bonnet, A.M.; Beinis, J.Y.; Gallinari, C.; Meaume, S.; Piette, F.; Hauw, J.J.; Duyckaerts, C. (**2006**). Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lowy body–associated neuronal loss in the substantial Ingra. *Arch Neurol*. 63(4):584-588.

3. Braak, H.; Del Tredici, K.; Rüb U.; De Vos R.A.; Jansen Steur, E.N.; Braak, E. (**2003**). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging*. 24(2):197-211.

4. Hiker, R.; Schweitzer, K.; Co burger, S.; Ghaemi, M.; Weisenbach, S.; Jacobs, A.H.; Rudolf, J.; Herholz, K.; Heiss, W.D. (**2005**). Nonlinear progression of Parkinson disease as determined by serial positron emission topographic imaging of striatal fluorodopa F 18 activity. *Arch Neurol*. 62(3):378-382.

5. Chaudhuri, K.R.; Schapira, A.H. (**2009**). Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol*. 8:464–474.

6. Noyce, A.J.; Bestwick, J.P.; Silveira-Moriyama, L.; Silveira-Moriyama, L.; Hawkes, C.H.; Giovannoni, G.; Lees, A.J.; Schrag, A. **(2012**). Meta-analysis of early nonmotor features and risk factors for Parkinson disease. *Ann Neurol*. 72:893–901.

7. Schrag, A.; Horsfall, L.; Walters, K.; Noyce, A.; Petersen, I. (**2015**). Prediagnostic presentations of Parkinson's disease in primary care: a case-control study. *Lancet Neurol*. 14:57– 64.

8. Adams-Carr, K.L.; Bestwick, J.P.; Shribman, S.; Lees A.; Schrag, A.; Noyce, A.J. (**2015**). Constipation preceding Parkinson's disease: a systematic review and meta-analysis. J *Neurol Neurosurg Psychiatry*. 87(7):710-716.

9. Gibb, W.R.; Lees, A.J. (**1988**). The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry*. 51:745–752.

10. Nicholson, J.K.; Connelly, J.; Lindon, J.C.; Holmes, E. (**2002**). Metabolomics: a platform for Studying drug toxicity and gene function. *Nat Rv Drug Discov*. 1:153-161.

11. Grimes, J.H.; O'Connell, T.M. (**2011**). The application of micro-coil NMR probe technology to metabolomics of urine and serum. *J Biomol NMR*. 49: 297-305.

12. Siderowf, A.; Stern, M.B. (**2006**). Preclinical diagnosis of Parkinson's disease: Are we there yet? *Curr Neurol Neurosci Rep*. 6(4):295–301.

13. Tolosa, E.; Gaig, C.; Santamarıa, J.; Compta, Y. (**2009**). Diagnosis and the premotor phase of Parkinson disease. *Neurology*. 72(7 Suppl):S12-S20.

14. Chaudhuri, K.R.; Healy, D.G.; Schapira, A.H. (**2006).** National Institute for Clinical Excellence. Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol*. 5(3):235-245.

15. Monderer, R.; Thorpy, M. (**2009**). Sleep disorders and daytime sleepiness in Parkinson's disease. *Curr Neurol Neurosci Rep*. 9(2):173-180.

16. Postuma, R.B.; Gagnon, J.F.; Vendette, M.; Fantini, M.L.; Massicotte-Marquez,J.; Montplaisir, J. (**2009**). Quantifying the risk of in idiopathic REM sleep behaviour disorder. Neurodegenerative disease *Neurology*. 72(15):1296-1300.

17. Gagnon, J.F., Vendette, M., Postuma, R.B.; Desjardins, C.; Massicotte-Marquez, J.; Panisset, M.; Montplaisir J. (**2009**). Mild cognitive impairment in rapid eye movement sleep behaviour disorder and Parkinson's disease. *Ann Neurol*. 66(1):39-47.

18. Louis, E.D.; Faust,P.L.; Vonsattel, J.P.; , Rajput, A.; Robinson, C.A.; Rajput, A....& Hernandez, N. (**2007**). Neuropathological changes in essential tremor: 33 cases compared with 21 controls. *Brain*. 130 (12):3297-3307.

19. Benito-Leo, N.J.; Louis, E.D.; Bermejo-Pareja, F. (**2009**). Neurological Disorders in Central Spain Study Group. Risk of incident Parkinson's disease and Parkinsonism in essential tremor: a population based study. *J Neurol Neurosurg Psychiatry*. 80(4): 423-425.

20. Ross, G.W.; Petrovitch, H.; Abbott, R.D.; Tanner, C.M.; Popper, J.; Masaki, K....& White, L.R. (**2008**). Association of olfactory dysfunction with risk for future Parkinson's disease. *Ann Neurol*. 63(2):167-173.

21. Haehner, A.; Hummel, T.; Hummel, C.; Sommer, U.; Junghanns. S.; Reichmann, H. (**2007**). Olfactory loss may be a first sign of idiopathic Parkinson's disease. *Mov Disord*. 22(6):839-842.

22.Silveira-Moriyama,L.;Petrie, A.; Williams,D.R.; Evans, A.; Katzenschlager, R.; Barbosa, E.R.; Lees, A.J. (**2009).** The use of a color-coded probability scale to interpret smell tests in suspected Parkinsonism. *Mov Disord*. 24(8):1144-1153.

23. Goldstein, D.S.; Holmes, C.; Bentho, O.; Sato, T.; Moak, J.; Sharabi, Y....& Eldadah, B. A. **(2008).** Biomarkers to detect central dopamine deficiency and distinguish Parkinson disease from multiple system atrophy. *Parkinsonism Relate Disord*. 14(8):600-607.

24. Mostile, G.; Jankovic, J. (**2009**). Treatment of dysautonomia associated with Parkinson's disease. *Parkinsonism Relat Disord*. 15(suppl 3):S224-232.

25. Olanow, C.W.; Prusiner, S.B. (**2009**). Is Parkinson's disease a prion disorder? *Proc Natl Acad Sci U S A*. 106(31):12571-12572.

26. Hawkes, C.H.; Del Tredici, K.; Braak, H. (**2007**). Parkinson's disease: a dual-hit hypothesis. *Neuropathol Appl Neurobiol*. 33(6):599-614.

27. Michell, A.W.; Luheshi, L.M.; Barker, R.A. (**2005**). Skin and platelet α-synuclein as peripheral biomarkers of Parkinson's disease. *Neurosci Lett*. 381 (3):294-298.

28. Shishido, T.; Ikemura, M.; Obi, T.; , Yamazaki, K.; Terada, T.; Sugiura, A.....& Mizoguchi, K. (**2010**). α-Synuclein accumulation in skin nerve fibers revealed by Skin biopsy in pure autonomic failure. *Neurology*. 74(7):608-610.

29. Marek, K.; Jennings, D.; Tamagnan, G.; Seibyl, J. (**2008**). Biomarkers for Parkinson's disease: Tools to assess Parkinson's disease onset and progression. *Ann Neurol*. 64(Suppl 2):S111-S121.

30. Ibrahim, N.; Kusmirek, J.; Struck, A. F.; Floberg, J. M.; Perlman, S. B.; Gallagher, C.; Hall, L. T. (**2016**). The sensitivity and specificity of F-DOPA PET in a movement disorder clinic. *Am. J. Nucl. Med. Mol. Imaging*. 6 (1): 102–109.

31. Arlicot, N.; Vercouillie, J.; Malherbe, C.; Bidault, R.; Gissot, V.; Maia, S.....& Ribeiro, M.J. (**2017**). PET imaging of dopamine transporter with 18F-LBT999: first human exploration. *J. Nucl. Med*. 58(1):276.

32. Emamzadeh, F.N.; Surguchov, A. (**2018**). Parkinson's disease: Biomarkers, Treatment, and Risk Factors. *Front. Neurosci*. 12:612.

33. Yuncheng, Wu.; Weidong, Le.; Joseph J. (**2011**). Preclinical biomarkers of Parkinson disease. *Archives of neurology*. 68(1): 22-30.

34. Marek, K.; Jennings, D. (**2009**). Can we image premotor Parkinson disease? *Neurology*. 72(7 Suppl): S21-S26.

35. Schwingenschuh, P.; Ruge, D.; Edwards, M.J.; Terranova, C.; Katschnig, P.; Carrillo, F.;

....& Bhatia, K.P. (**2010**). Distinguishing SWEDDs patients with asymmetric resting tremor from Parkinson's disease: a clinical and electrophysiological study. *Mov Disord*. 25(5):560-569.

36. Skoloudík, D.; Jelínková, M.; Blahuta, J.; Cermák P.; Soukup T.; Bártová P. ...& Herzig, R. (**2014**). Transcranial sonography of the substantia nigra: digital image analysis. *Am. J.Neuroradiol*. 35(12):2273–2278.

37. Chung, E.J.; Kim, E.G.; Bae, J.S.; Eun, C.K.; Lee, K.S....& Kim, S.J. (**2009**). Usefulness of diffusion-weighted MRI for differentiation between Parkinson's disease and Parkinson variant of multiple system atrophy. *J. Mov. Disord*. 2(2):64-68.

38. Saeed, U.; Compagnone, J., Aviv, R. I.; Strafella, A.P.; Black, S.E...& Masellis, M. (**2017).** Imaging biomarkers in Parkinson's disease and Parkinsonian syndromes: current and emerging concepts. *Transl Neurodegener*.; 6:*8*.

39. Aarsland, D.; Ravina, B. (**2010**). Biomarkers of PD progression: is CSF the answer *Neurology*. 75(12): 1036-1037.

40. Li, Q.X.; Mok, S.S.; Laughton, K.M.; Lean, C.A.; Cappai, R.; Masters, C.L.; Culvenor, J.G.; Horne, M.K. (**2007**). Plasma α-synuclein is decreased in subjects with Parkinson's disease. *Exp Neurol*. 204(2):583-588.

41. Westermann, B.; Wattendorf, E.; Schwerdtfeger, U.; Husner, A.; Fuhr, P.; Gratzl, O.... .&Welge-Lüssen, A. (**2008).** Functional imaging of the cerebral olfactory system in patients with Parkinson's disease. *J Neurol Neuro-surg Psychiatry*. 79(1):19-24.

42. Ozelius, L.J.; Senthil, G.; Saunders-Pullman R.; Ohmann, E,; Deligtisch, A.; Tagliati, M.... ..&Bressman, S.B. (**2006**). *LRRK2* G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *NEngl J Med*. 354(4):424-425.

43. Lesage, S.; Dürr, A.; Tazir, M.; Lohmann, E.; Leutenegger, A.L.; Janin, S.; Pollak,P.; Brice, A. (**2006**). French Parkinson's Disease Genetics Study Group. *LRRK2* G2019S as a cause of Parkinson's disease in North African Arabs. *N Engl J Med*. 354(4):422-423.

44. Veeriah, S.; Taylor, B.S.;Meng, S.;Fang, F.; Yilmaz, E.; Vivanco, I.; ....& Chan,T.A. (**2010**) Somatic mutations of the Parkinson's disease-associated gene *PARK2* in glioblastoma and other human malignancies. *Nat Genet*. 42(1):77-82.

45. Sutherland, G.; Mellick, G.; Sue, C.;Chan, D.K; Rowe, D.; Halliday, G. (**2007**). A functional polymorphism in the parkin gene promoter affects the age of onset of Parkinson's disease. *Neurosci Lett*. 414(2):170-173.

46. Jankovic, J. (**2005**). Progression of Parkinson disease: Are we making progress in charting the Course? *Arch Neurol*. 62(3):351-352.

47. Le, W.; Pan, T.; Huang M.;Xu, P.; Xie, W.; Zhu, W.; Zhang, X.; Deng, H.; Jankovic, J.(**2008**) Decreased *NURR1* gene expression in patients with Parkinson's disease. *J Neurol Sci*. 273(1-2):29-33.

48. Scherzer, C.R.; Eklund, A.C.; Morse, L.J.; Liao, Z.; Locascio, J.J.; Fefer, D.; ....& Gullans, S.R. (**2007**). Molecular markers of early Parkinson's disease based on gene expression in blood. *Proc Natl Acad Sci U S A*. 104(3):955-960.

49. Delenclos, M.; Jones, D.R.; McLean, P.J; Uitti R.J. (**2016**). Biomarkers in Parkinson's disease: Advances and strategies. *Parkinsonism Relat Disord*. 22 (Suppl 1):S106-110.

50. Hagan, J. J., Leslie, R. A., Patel, S., Evans, M. L., Wattam, T. A., Holmes, S., ...& Upton, N. (**1999**). Orexin A activates locus ceruleus cell firing and increases arousal in the rat. *Proc. Natl. Acad. Sci. U.S.A*. 96(19):10911–10916.

51. Imperatore, R.; Palomba, L.; Cristino, L. (**2017**). Role of Orexin-a in hypertension and obesity. *Curr. Hypertens. Rep*. 19(4):34.

52. Fronczek, R.; Overeem, S.; Lee, S.; Y Hegeman, I.M.; VanPelt, J.; Van Duinen S.G.; Lammers, G.J.; Swaab, D.F. (**2007**). Hypocretin (orexin) loss in Parkinson's disease. *Brain* 130, 1577–1585.

53. Takahashi, Y.; Kanbayashi, T.; Hoshikawa, M.; Imanishi, A.; Sagawa, Y.; Tsutsui, K.; Takeda, Y.; Kusanagi, H.; Nishino, S.; Shimizu, T. (**2015**). Relationship of orexin (hypocretin) system and astrocyte activation in Parkinson's disease with hypersomnolence. *Sleep Biol. Rhythms*. 13, 252–260.

54. Shigenaga, M.; Gimeno, C.J.; Ames, B.N. (**1989**). Urinary8-hydroxy-2'-deoxyguanosine as a biomarker of in vivo oxidative DNA damage. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9697–9701.

55. Kikuchi, A.; Takeda, A.; Onodera, H.; Kimpara, T.; Hisanaga, K.; Sato, N.; Nunomura, A.; Castellani, R.J.; Perry G.; Smith, M.A.; Itoyama, Y. (**2002**). Systemic increase of oxidative nucleic acid damage in Parkinson's disease and multiple system atrophy. *Neurobiol. Dis* 9, 244–248.

56. Nagai, Y.; Ueno, S.; Saeki, Y.; Soga, F.; Hirano, M.; Yanagihara, T. **(1996).** Decrease of the D3 dopamine receptor mRNA expression in lymphocytes from patients with Parkinson's disease. *Neurology*. 46, 791–795.

57. Goldstein, D. S.; Holmes, C.; Lopez, G. J.; Wu, T.; Sharabi, Y. (**2018**). Cerebrospinal fluid biomarkers of central dopamine deficiency predict Parkinson's disease. *Parkinsonism Relat. Disord*. 50, 108–112.

58. Caronti, B.; Antonini, G.; Calderaro, C.; Ruggieri, S.; Palladini, G.; Pontieri, F.E.; Colosimo, C. (**2001).** Dopamine transporter immunoreactivity in peripheral blood lymphocytes in Parkinson's disease. *Neural. Transm*; 108, 803–807.

59. Emamzadeh, F. N. (**2017**). Role of Apolipoproteins and α-Synuclein in Parkinson's disease. *J. Mol. Neurosci.* 62, 344–355.

60. Wang, E.S.; Sun, Y.; Guo, J. G.; GAO, X.; Hu, J.W.; Zhou, L.; Hu, J.; Jiang, C.C. (**2010**). Tetranectin and apolipoprotein A-I in cerebrospinal fluids potential biomarkers for Parkinson's disease. *Acta Neurol.Scand*. 122:350–359.

61. Swanson, C. R.; Berlyand, Y.; Xie, S. X.; Alcalay, R. N.; Chahine, L. M.; Chen- Plotkin, A. S. (**2015**). Plasma apolipoprotein A1 associates with age at onset and motor severity in early Parkinson's disease patients. *Mov. Disor*. 30, 1648–1656.

62. Dos Santos, M. C. T., Barreto-Sanz, M. A., Correia, B. R. S., Bell, R., Widnall, C., Perez, L. T., ... & da Costa, A. N. (2018). miRNA-based signatures in cerebrospinal fluid as potential diagnostic tools for early stage Parkinson's disease. *Oncotarget*, *9*(25), 17455.

63. Shadrina, M. I.; Slominsky, P. A. & Limborska, S. A. (**2012**). Molecular Mechanisms of Pathogenesis of Parkinson's disease. *Int. Rev. Cell Mol. Biol.* 281, 229–266.

64. Martins-Branco, D.; Esteves, A. R.; Santos, D.; Arduino, D.M.; Swerdlow, R.H.; Oliveira, C. R. (**2012**). Ubiquitin proteasome system in Parkinson's disease: a keeper or a witness? *Exp. Neurol.* 238, 89–99.

65. Blandini, F.; Sinforiani, E.; Pacchetti, C.; Samuele, A.; Bazzini, E.; Zangaglia, R. (**2006**). Peripheral proteasome and caspase activity in Parkinson disease and Alzheimer disease. *Neurology*. 66, 529–534.

66. Caudle, W.M.; Bammler, T.K.; Lin, Y.; Pan, S.; Zhang, J. (**2010**). Using 'omics'to defines pathogenesis and biomarkers of Parkinson's disease. *Expert Rev Neurother*. 10:925-942.

67. Goldstein, D.S.; Holmes, C.; Sharabi, Y. (**2012**). Cerebrospinal fluid biomarkers of central catecholamine deficiency in Parkinson's disease and other synucleinopathies. *Brain*. 135:1900–1913.

68. Trupp, M.; Jonsson, P.; Ohrfelt, A.; Zetterberg, H.; Obudulu, O.; Malm, L.....& Forsgren, L. (**2014**). Metabolite and peptide levels in plasma and CSF differentiating healthy controls from patients with newly diagnosed Parkinson's disease. *J Parkinsons Dis*. 4:549–560.

69. Öhman, A.; Forsgren, L. (**2015**). NMR metabolomics of cerebrospinal fluid distinguishes between Parkinson's disease and controls. *Neurosci Lett*. 594:36–39.

70. Chang, K.H.; Cheng, M.L.; Tang, H.Y.; Huang, C.Y.; Wu, Y.R.; Chen, C.M. (**2018**). Alternations of metabolic profile and kynurenine metabolism in the plasma of Parkinson's disease. *Mol Neurobiol*. 55:6319–6328.

71. Oxenkrug, G.; van der Hart, M.; Roeser, J.; Summergrad, P. (**2017**). Peripheral Tryptophan -Kynurenine Metabolism Associated with Metabolic Syndrome is Different in Parkinson's and Alzheimer's Diseases. *Endocrinol Diabetes Metab J*. 1(4).

72. Han, W.; Sapkota, S.; Camicioli, R.; Dixon, R.A.; Li, L. (**2017).** Profiling novel metabolic biomarkers for Parkinson's disease using in-depth metabolomic analysis. *Mov Disord*.; 32:1720–1728.

73. Cipriani, S.; Chen, X.; Schwarzschild, M.A. (**2010**). Urate: a novel biomarker of Parkinson's disease risk, diagnosis and prognosis. *Biomark Med*. 4:701–712.

74. Schwarzschild, M.A.; Schwid, S.R.; Marek, K.; Watts, A.; Lang, A.E.; Oakes, D..... & Ondrasik, J. (**2008).** Serum urate as a predictor of clinical and radiographic progression in Parkinson disease. *Arch Neurol*.; 65:716–723.

75. Van der Brug, M.P.; Singleton, A.; Gasser, T.; Lewis, P.A. (**2015**). Parkinson's disease: From human genetics to clinical trials. *Sci Transl Med*. 7(305): 205.

76. Nagesh Babu, G.; Gupta, M.; Paliwal, V.K.; Singh, S.; Chatterji, T.; Roy, R. (**2018**). Serum metabolomics study in a group of Parkinson's disease patients from northern India. *Clin Chim Acta*. 480:214–219.

77. Roede, J.R.; Uppal, K.; Park, Y.; Lee, K.; Tran, V.; Walker, D....& Jones, D.P. (**2013).** Serum metabolomics of slow vs rapid motor progression Parkinson's disease: a pilot study. *PLoS One*. 22; 8(10):e77629.

78. Luan, H.; Liu, L.F.; Tang, Z.; Mok, V.C.; Li, M.; Cai, Z. (**2015**). Elevated excretion of biopyrrin as a new marker for idiopathic Parkinson's disease. *Parkinsonism Relat Disord*. 21:1371–1372.

79. Luan, H.; Liu, L.F.; Tang, Z.; Zhang, M.; Chua, K.K.; Song, J.X.....& Cai, Z. (**2015**). Comprehensive urinary metabolomic profiling and identification of potential non-invasive marker for idiopathic Parkinson's disease. *Sci Rep*. 5:13888.

80. Unger, M.M.; Spiegel, J.; Dillmann, K.U.; Grundmann, D.; Philippeit, H.; Burmann, J.....& Schafer, K.H. (**2016**). Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat Disord*. 32:66–72.

81. Hamm-Alvarez., S.F.; Janga, S.R.; Edman, M.C.; Feigenbaum. D.; Freire, D.; Mack, W.J.; Okamoto, C.Y.; Lew, M.F. (**2019).** Levels of oligomeric α-Synuclein in reflex tears distinguish Parkinson's disease patients from healthy controls. *Biomark Med.* 13(17):1447-1457.

82. Hamm-Alvarez, S.F.; Okamoto, C.T.; Janga, S.R.; Feigenbaum, D.; Edman, M.C.; Freire, D.....& Lew, M.F. (**2019**). Oligomeric alpha-synuclein is increased in basal tears of Parkinson's patients. *Biomark. Med*. 13(11): 941– 952.

83. Al-Nimer, M.S.; Mshatat, S.F.; Abdulla, H.I. (**2014**). Saliva α-Synuclein and a high extinction coefficient protein. A novel approach in assessment biomarkers of Parkinson's disease. *North Am J Med Sci*. 6:633-637.

84. Chen, H.; O'Reilly, E.J.; Schwarzschild, M.A.; Ascherio, A. (**2008).** Peripheral inflammatory bio-markers and risk of Parkinson's disease. *Am J Epidemiol*. 167(1):90-95.

85. Rentzos, M.; Nikolaou, C.; Andreadou, E.; Paraskevas, G.P.; Rombos, A.; Zoga, M.....& Vassilopoulos, D. (**2009).** Circulating interleukin-10 and inter-leukin-12 in Parkinson's disease. *Acta Neurol Scand*.; 119(5):332-337.

86. Huang, X.; Abbott, R.D.; Petrovitch, H.; Mailman, R.B.; Ross, G.W. (**2008**). Low LDL cholesterol and increased risk of Parkinson's disease: prospective results from Honolulu-Asia Aging Study. *Mov Disord*. 23(7):1013-1018.

87**.** Fang, F.; Zhan, Y.; Hammar, N.; Shen, X.; Wirdefeldt, K.; Walldius, G.; Mariosa, D. (**2019**). Lipids, Apolipoproteins, and the Risk of Parkinson Disease : A Prospective Cohort Study and a Mendelian Randomization Analysis. *Circulation Res*. 125: 643-652.

88. Gao, X.; Simon, K.C.; Han, J.; Schwarzschild, M.A.; Ascherio, A. (**2009).** Genetic determinants of hair color and Parkinson's disease risk. *Ann Neurol*. 65(1):76-82.

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Developing, Optimization and In vitro Evaluating of Three-layers Floating Dipyridamole Film in Hard Gelatine Capsule

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ABSTRACT

Dipyridamole's bioavailability decreases with increasing gastric pH. To overcome this problem it was planned to prepare three-layered floating Dipyridamole films by floating enhancers and release controlling polymers for remain buoyant in the stomach. It focuses on the development and in vitro evaluating of the floating film in hard gelatine capsule through the design of experiment using different hydrophilic polymers, hydroxypropylmethylcellulose (HPMC) K4M and hydroxyethylcellulose (HEC). The amounts of HPMC K4M and HEC were independent variables, affecting film formulations found as 0.242 g and 0.337 g, respectively. The thickness of films, swelling index and percent of drug release in 4th hour were dependent variables found as 2.44±0.09 mm, 137.9%±1.11% and 67.07%±3.28%, respectively. In conclusion, the best fitted kinetic model was the Higuchi model where the drug release was controlled by diffusion, and optimized floating film formulation could be offered as a promising strategy to increase the bioavailability of Dipyridamole.

Keywords: Dipyridamole, Design Expert, Floating film, In vitro release, Swelling index

INTRODUCTION

Extended drug delivery systems are increasingly attracted due to reduction in dose frequency, decreasing of fluctuations in plasma concentration level and

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raising patient compliance. During the development of these dosage forms may get into the some difficulties such as the inability to complete absorption of drug in therapeutic diversity, and it is difficult to remain in the desired and adequate period in the stomach[1]. For this reason, there is an increased interest in the floating dosage forms[2] and gastroretentive dosage forms[3].

Floating systems are the part of the extended-release systems and extremely hopeful approaches if there is an unexpectable gastric emptying time problem and the active ingredient has low gastric residence time. Especially if the drug has low solubility and low bioavailability, floating systems may be a different way of new applications. Therefore, as the absorption of the drug increases the effectiveness of the dosage form increases, and it is possible to provide benefits targeting the specific region[4].

As gastric transition time is increased, fluctuations in plasma drug concentration levels can be controlled more effectively with these systems. As a result, good gastroretentive behavior can be achieved. And also, this makes it easier to obtain prolonged drug release period[5].

Dipyridamole is a BSC Class II weakly basic drug that is absorbed in the upper intestine and it has pH-dependent solubility. Its bioavailability decreases with increasing gastric pH. It is used for the treatment of angina pectoris because of preventing effect from the myocardial infarctions and thrombosis. This effect is provided by phosphodiesterase 5A inhibition so prevents aggregation and blocking the reuptake of adenosine via red blood cells. It also scavenges the free radicals that inactivate cyclooxygenase, leading to the inhibition of platelet activation and thrombin generation[6].

HPMC K4M is a bioadhesive material and used for coating agent, controlled-release agent, extended-release agent, film-forming agent, granulation aid, modified-release agent, mucoadhesive, release-modifying agent, solubilizing agent, stabilizing agent, suspending agent, sustained-release agent, tablet binder, thickening agent, viscosity-increasing agent. It is widely used in oral, ophthalmic, nasal, and topical pharmaceutical formulations[7].

HEC is a nonionic, water-soluble polymer used for coating agent, suspending agent, tablet binder, thickening agent, viscosity-increasing agent in pharmaceutical formulations[7].

Experimental researches need a long process, attention and self-sacrifice. They are carried out by experimental design methods recently. So researchers can minimize raw materials, labor and time consumption. That is why they can achieve correct results quickly with less raw materials and there is no need for

lots of energy or labor. One of the most preferred experimental design program Design Expert is a software for high accuracy data in scientific studies. It explains multifactor data and offers interpretation results. For the optimization, it brings out polynomial equations and investigates the response over the experimental data[8].

In this research paper, a new formulation of floating dipyridamole three-layers film was developed and evaluated. Floating systems or dynamically controlled systems are low-density systems and can float over the extended period of time due to remain on the surface of the stomach fluid without being affected by gastric emptying time. Thus, the fluctuations in plasma active substance concentration can better be controlled as it increases the residence time in the stomach.[5] For this reason, preparing the floating formulation of Dipyridamole will bring many advantages for its absorption. The more amount of drug remains in absorption region, the more amount of drug absorbed. An equal amount of active ingredient was added for each layer to fit the desired amount of active ingredient into the dosage form, that is why three-layered film was prepared. Two different polymers were used during the preparation. HPMC K4M (Hypromellose) was responsible for delaying release and, HEC (Cellosize HEC, Natrosol)[9] was responsible for floating in stomach because of its low density and structural integrity.

METHODOLOGY

Materials

Dipyridamole was kindly donated by Sanovel Pharmaceuticals, HPMC K4M and HEC were obtained from Ashland, glycerine and ethyl alcohol were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade.

Methods

Preparation of floating films

The solvent casting method was used to prepare three-layers of floating film formulations[10]. HPMC K4M and HEC were used as a film-forming agent and glycerine was added to the formulation as a plasticizing agent. The amounts of HPMC K4M and HEC included in the formulation were obtained from the design expert software (Table 1).

For the preparation of the first layer of film formulation, half of the amount of HPMC K4M was12 weighed, and 3.5 mL distilled water was added on the polymer, after this the solution left to swell overnight by solvent casting. After that,

200 mg dipyridamole weighed and dissolved in 4.5 mL ethyl alcohol at 40°C in an ultrasonic bath (Bandelin Sonorex-Germany) and poured on to the solution followed by the addition of 1.2 mL glycerine, then the solution was allowed to dry at 37°C for 12 hours. For the second layer, the amount of HEC was weighed and 7 mL distilled water added to the polymer, and it left overnight. Then 200 mg dipyridamole was weighed and dissolved in 4.5 mL ethyl alcohol at 40°C in an ultrasonic bath and poured to the HEC followed by the addition of 1.2 mL glycerine. Then the solution poured to the first layer. This two-layered film was allowed to dry at 37°C for 12 hours. Then, the third layer has prepared with the same method as the first layer. The flow chart of this method is given in Fig. 1. After the drying of the third layer, one film has formed. This film then divided into four equal parts. Each part of them contains 150 mg dipyridamole.

Table 1. Experimental design matrix

Formula	Factor1 (HPMC K4M) mg	Factor2 (HEC) mg
F1	0.27	0.36
F2	0.14	0.36
F3	0.27	0.23
F4	0.36	0.27
F5	0.40	0.36
F6	0.36	0.45
F7	0.18	0.45
F8	0.27	0.49
F9	0.18	0.27
F10	0.27	0.36
F11	0.27	0.36
F12	0.27	0.36
F13	0.27	0.36

*Glycerine (1.2 mL), ethyl alcohol (4.5 mL) and distilled water (7 mL) were added in all formulations.

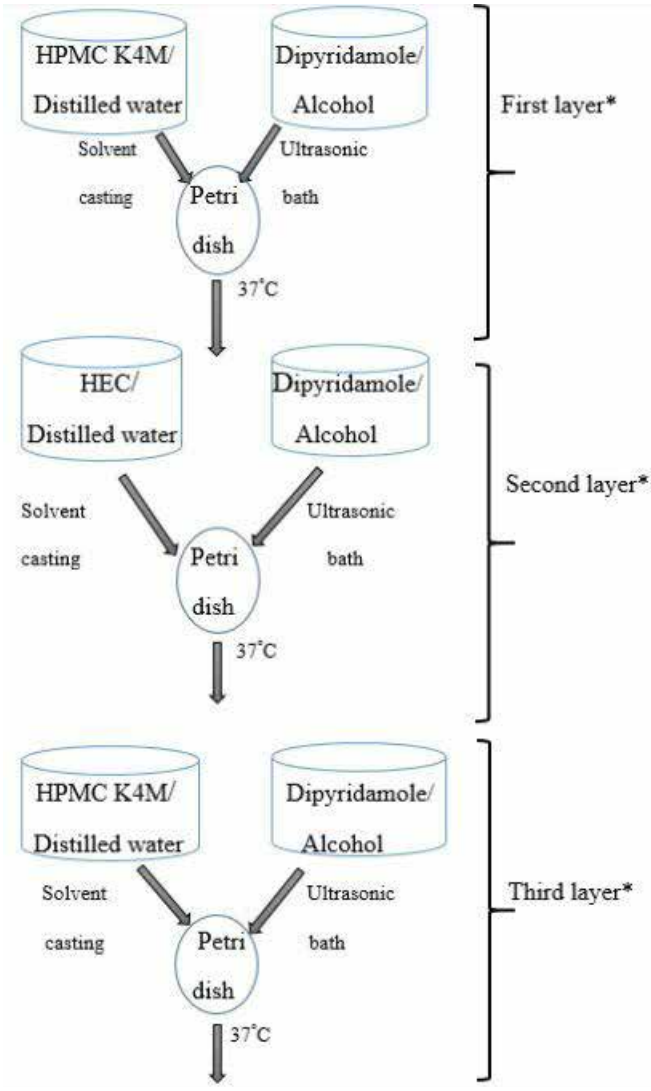

Figure 1. Flow chart of films to be prepared (*Each layer includes 1.2 mL glycerine)

Experimental Design

Film formulations were optimized by the response surface methodology (RSM). According to preliminary studies and literature review, X_1 (HPMC K4M) and X_2 (HEC) were selected as independent variables. X_1 was entered into the design expert software between 0.18 g and 0.36 g, while X_2 was entered between 0.27 g and 0.45 g. Plasticizer concentration was kept constant in all formulations. Independent variables were studied at 5 different levels (-α, -1, 0, +1, +α) using central composite design (CCD). To improve accuracy of the method, the α

value of 1.41 was determined (α value was chosen 1.41 to provide the the rotatability and orthogonality of the design) and total 13 experiments were carried out with 4 factorial points, 4 axial points and 5 replications of the central point (Table 2).

Table 2. Selected variables in central composite design

	Variables	Level of variables				
		-1.41	-1	0	1	1.41
A	HPMC K4M(mg)	0.14	0.18	0.27	0.36	0.40
B	HEC (mg)	0.23	0.27	0.36	0.45	0.49

Y1 (thickness), *Y2* (swelling index) and *Y3* (drug release) were chosen as dependent variables.

Design-Expert software was used for experiment design and statistical analysis. Response was predicted by the quadratic polynomial equation:

$$Y: \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

Where Y is predicted response, X_1 *and* X_2 independent variables, β_0 is the arithmetic mean response of the all runs, β_1 *and* β_2 are the predicted coefficients for the dependent factors X_1 *and* X_2, respectively. X_1X_2 displays how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are used to evaluate nonlinearity.

Analysis of variance (ANOVA) was applied to interpret the effect of independent variables on the dependent variables, and $p<0.05$ was considered to be statistically significant. Optimal responses were determined considering minimum thickness and maximum swelling index and in vitro release values. The optimized formulation was prepared in triplicate, and the obtained experimental results were compared to the predicted values.

Characterizations of floating films

Thickness of films

The thickness of films was measured at different points by using dial caliper (Japan). The average thickness, standard deviation and RSD% were calculated.

Swelling index of films

The swelling behavior of polymers was determined by the water uptake study. 3 cm x 5 cm x 3 cm sponge was soaked with 0.1 N HCl, and placed in a petri dish. Because the sponge is constantly wet, about 1 cm high 0.1 N HCl was added into the petri dish. A filter paper was placed on the upper part of the sponge. It is

made sure that the filter paper was completely wet with buffer. The setup was kept 15 minutes to stabilize before the experiment. Wet paper and the dry film were weighed separately, then it was placed on the setup. A glass fan closed to prevent the system airing. The initial mass of the setup is recorded. For the first six hours, the mass of the setup was recorded, with one-hour interval. The difference between the first weight and "t" time weight was figured out then water absorption capacity was calculated by Equation 1[11]. The experiment was repeated three times for each formulation and standard deviation values were determined.

Swelling index = $(W_t - W_o) / W_o$ x100 Equation 1

W_t = weight of film at t time

W_o = Initial weight

Drug content of films

Three-layer films put in 100 ml alcohol and stirred continuously using a mechanical stirrer (Kika Werke RT15-Germany) at the 40±2°C and the samples were withdrawn at the end of three hours and the drug content was determined spectrophotometrically at 283 nm. The experiment was carried out three times.

Mechanical properties of films

Mechanical properties of optimum film formulation (n=3) were performed using a texture analyzer (TA.XTPlus, Stable Micro Systems-UK) with a load cell of 5 kg. The film (1x4 cm) was placed between clamps at distance of 2 cm. Clamps were removed from each other with a constant crosshead speed of 0.5 mm/s until breakage of the films. The tensile strength and elongation at break (%) were calculated using the following Equations[12].

Tensile strength=Breaking force (N) / Cross-sectional area of film (cm^2) Equation 2

Elongation at break=Increase in length at breaking point (mm)/initial film length (mm) x 100 Equation 3

Dissolution of floating films

Dissolution test was performed in 900 mL 0.1 N HCl at 37°C±0.5. USP apparatus II (Sotax-Switzerland) was used, and the rotation speed was 50 rpm. At the predetermined time intervals samples were withdrawn, and replaced with the same amount of fresh buffer mediums[13]. Schematic illustration of the apparatus used for dissolution studies of films is depicted in Fig. 2. The absorbance of samples was analyzed with required dilutions spectrophotometrically at 283

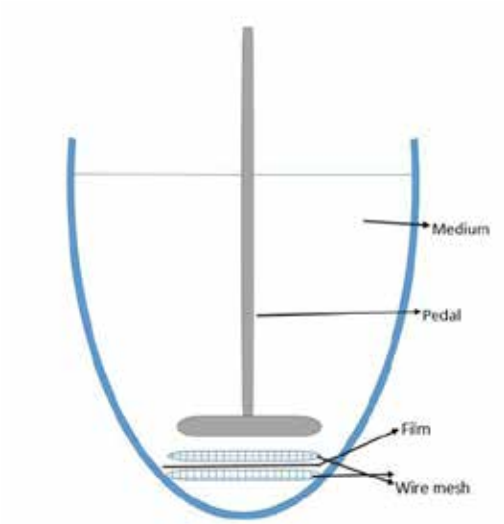

Figure 2. Schematic diagram of film and wire meshes in dissolution wessel

Kinetic models on drug release data

Drug release kinetics have a pivotal role in the field of drug delivery since they get profound vision into the mechanism from the dosage forms. For this reason, zero order, first order, Higuchi, Korsmeyer-Peppas, and Hixson-Crowell release kinetic models were examined to understand drug release kinetics. Kinetic models were evaluated by the coefficient of correlation (r^2). The highest degree of r^2 values determines the best appropriate kinetic model. All calculations were carried out according to the following kinetic equations.

Zero-order model: $Q_t=k_0t$ Equation 2

where Q is the amount of drug released at time t and k_0 is the zero-order release rate constant.

First-order model: $LogQ_t = LogQ_0 - k_1t$ Equation 3

where Q is the amount of drug release at time t and K is the first-order rate constant.

Higuchi-diffusion model

$Q_t = KHt^{1/2}$ Equation 4

where Q is the amount of drug released to the membrane (in mg) at time (t) in minutes. KH is the Higuchi square root of time release constant.

Korsmeyer-Peppas model $Ct/C\infty = Ktn$ Equation 5

where $Ct/C\infty$ is a fraction of drug released at time t, K is the release constant,

and n is the release exponent.

Hixson-Crowell diffusion release model $Q_0^{1/3} - Qt^{1/3} = K_{HC}t$ Equation 6

where Q t/Q is the amount of released drug at time t, K is the constant comprising the structural and geometric characteristics of the formulations, and n is the release of exponent[14].

In vitro buoyancy studies of films

The in vitro buoyancy includes floating lag time and total floating time[15]. In this study, disintegration time of capsules and time of rising to the surface of dissolution medium were floating lag time. The total floating time study was carried out at 37±0.5°C in filled 900 mL 0.1 N HCl dissolution wessel. The turbulence was created in it due to the pedals[10]. The floating behavior was observed. The floating film at different time intervals is depicted in Fig. 3.

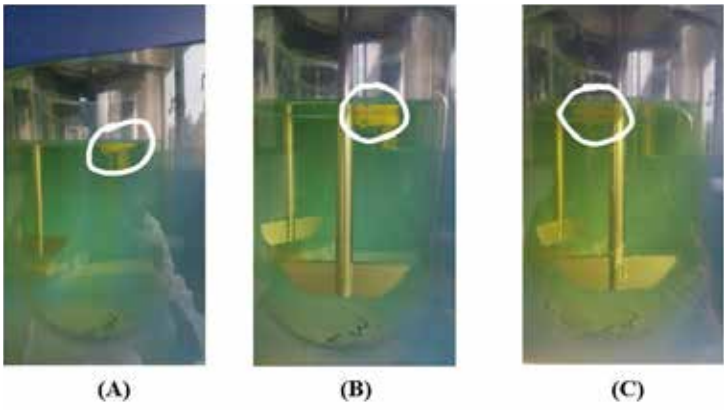

Figure 3. The floating film in 0.1 HCl (a) Initial time (b) 4th hour (c) 8th hour

Floating films into hard gelatine capsule

The prepared films were encapsulated for ease of oral use. For this purpose, the dried 6 cm diameter of film in which area is 28.26 cm² was removed from the petri dish and divided into 4 equal parts. One of them inserted into hard gelatine capsule (size 000-CAPSUGEL) as follows (Fig. 4). Each of them contains 150 mg Dipyridamole.

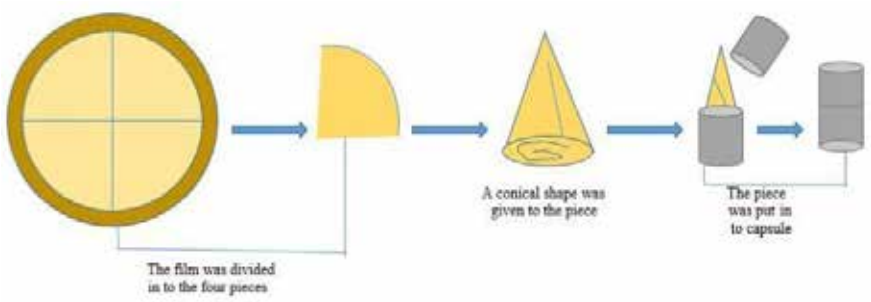

Figure 4. Schematic pattern of three-layers film into hard gelatine capsule

Test on hard gelatine capsules

Disintegration time of hard gelatine capsules

Disintegration time of floating film in hard gelatin capsules (n=6) was determined using disintegration apparatus (Sotax CH-4123, Switzerland). The beaker was filled with 0.1 N HCl. The temperature was maintained at 37° ±0.5°C. The disintegration times noted are the times at which the capsules ruptured, which assists minimize the uncertainty associated with determining the disintegration times based on "complete disintegration". Accordingly, these times are described as the times at which first visible cracks in the capsule shell appear.

Statistical Analysis

All the experiments were carried out in at least triplicate and the resulting data were presented as the mean±standard deviation (SD). Statistical analyses were performed using one-way ANOVA with Tukey's *post hoc* test. A difference with p<0,05 was considered to be statistically significant.

RESULTS and DISCUSSION

Results of test on floating film formulations

Influence of independent variables on thickness

As indicated in Table 3, the thickness of the film was found to be in the range from 1.90 to 3.26 mm. ANOVA results in Table 4 indicated that the amount of HPMC K4M and HEC amounts in the formulation were mainly affected (p value<.001) film thickness. When the 3D response surface graph in Fig. 7A is examined that thickness of film increases with increasing amounts of film forming polymers[16].

The following equation was used to predict the thickness of film:

$$Y1: 2.04 + 0.27 X_1 + 0.23 X_2 + 0.08 X_1 X_2 + 0.17 X_1^2 + 0.3 X_2^2$$

where *Y1* is thickness value, while X_1 and X_2 are the coded values of HPMC K4M and HEC, respectively. The model F value (17.47) and low P-value (0.001) implied the significance of the model equation as displayed in Table 4. The lack of fit of the model (P=0.103) was not significant.

Table 3. The results of in-vitro characterization study of film formulations

Formulation Code	Average thickness of films (mm)	Average drug content (%)	Swelling index (%)	In-vitro release for 4th hour (%)
F1	2.05±0.14	93.42±0.87	149.49±8.70	71.94±5.54
F2	2.16±0.24	97.06±2.35	97.05±1.81	74.40±6.65
F3	2.47±0.44	93.62±0.73	109.35±2.04	74.37±3.60
F4	2.45±0.47	92.58±1.69	89.62±7.63	72.02±5.27
F5	2.66±0.11	92.43±1.21	73.87±1.88	62.60±4.06
F6	3.26±0.54	97.37±0.68	115.21±3.50	65.53±0 .94
F7	2.39±0.12	91.26±1.11	79.15±2.90	73.12±3.08
F8	2.86±0.36	89.32±2.04	104.2±5.81	68.26±3.79
F9	1.90±0.44	89.96±1.15	109.31±1.57	67.93±5.54
F10	2.07±2.07	97.37±1.25	124.15±7.30	66.92±6.37
F11	2.08±2.08	92.37±0.50	134.45±6.70	69.44±0.73
F12	2.04±2.04	92.87±0.11	137.56±8.45	74.37±10.20
F13	1.98±1.98	96.45±2.18	141.71±5.65	74.09±2.90

Table 4. ANOVA Results for response *Y1*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.78	5	0.36	17.47	0.0008	significant
A-X1	0.57	1	0.57	27.79	0.0012	
B-X2	0.43	1	0.43	21.05	0.0025	
AB	0.026	1	0.026	1.26	0.2991	
A2	0.21	1	0.21	10.3	0.0149	
B2	0.63	1	0.63	30.99	0.0008	
Residual	0.14	7	0.02			
Lack of Fit	0.14	3	0.045	29.71	0.1034	not significant
Pure Error	6.12E-03	4	1.53E-03			
Cor Total	1.92	12				

Influence of independent variables on swelling index

The swelling index was calculated by Equation 1 and the results were shown in Fig. 5. When the time increased it was also increased depended on the hydration. Generally, all formulations have good swelling character because of the polymers' natures. HPMC K4M and HEC are good gel-forming, highly swellable and matrix-forming agents. It was observed to have increased swelling index depending on time. It was found to be in the range from 73.87% to 149.49% (Table 3). ANOVA results in Table 5 indicated that the change in the amount of HPMC K4M ($p<0.001$) had more influence than the change in the amount of HEC (P:0.068) on the swelling index.

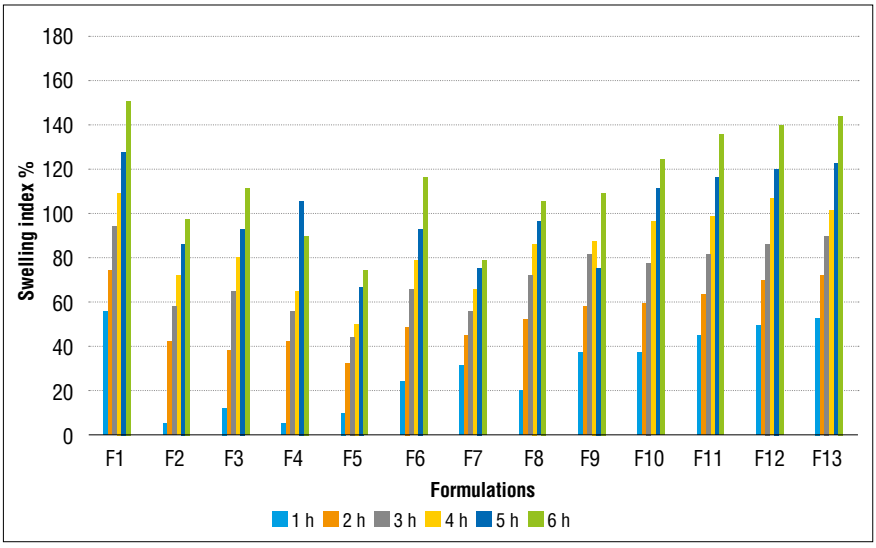

Figure 5. Swelling index cumulative column graph

The following equation was used to predict the swelling index:

$$Y2: 137.47 - 2.05\,X_1 - 1.48\,X_2 + 13.94\,X_1 X_2 - 25.45\,X_1^2 - 14.8\,X_2^2$$

Where $Y2$ is swelling index value, while X_1 and X_2 are the coded values of HPMC K4M and HEC, respectively. The model F value (13.26) and low P-value (0.001) implied the significance of the model equation as displayed in Table 5. Lack of fit of the model (P=0.418) was not significant.

Table 5. ANOVA Results for response *Y2*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6267.88	5	1253.58	13.26	0.0019	significant
A-X1	33.67	1	33.67	0.36	0.0003	
B-X2	17.56	1	17.56	0.19	0.0682	
AB	777.02	1	777.02	8.22	0.0241	
A2	4507.44	1	4507.44	47.69	0.0002	
B2	1523.19	1	1523.19	16.12	0.0051	
Residual	661.64	7	94.52			
Lack of Fit	312.63	3	104.21	1.19	0.4182	not significant
Pure Error	349.01	4	87.25			
Cor Total	6929.51	12				

It has been reported that HPMC matrices were effected more than Carbopol matrices on percentage swelling[17]. HPMC K4M has a positive effect on swelling index due to its hydrophilicity and swellability[18,19]. The hydroxypropyl groups in the HPMC K4M indicate more affinity to water molecules. This behavior can be observed clearly in Fig. 7B as an increase in HPMC K4M amount resulted in increased swelling index until a value near 0.27 g. The increase in the amount of HPMC K4M after this value led to a decrease in the swelling index possibly due to the presence of more physical entanglements between film-forming polymers[20-22].

Influence of independent variables on in vitro release profile

Since the drug release profiles of all formulations reached the plateau value in the first 4 hours (Fig. 6), the cumulative drug release values at 4th hour were chosen to evaluate the effect of the independent variables on the drug release profile.

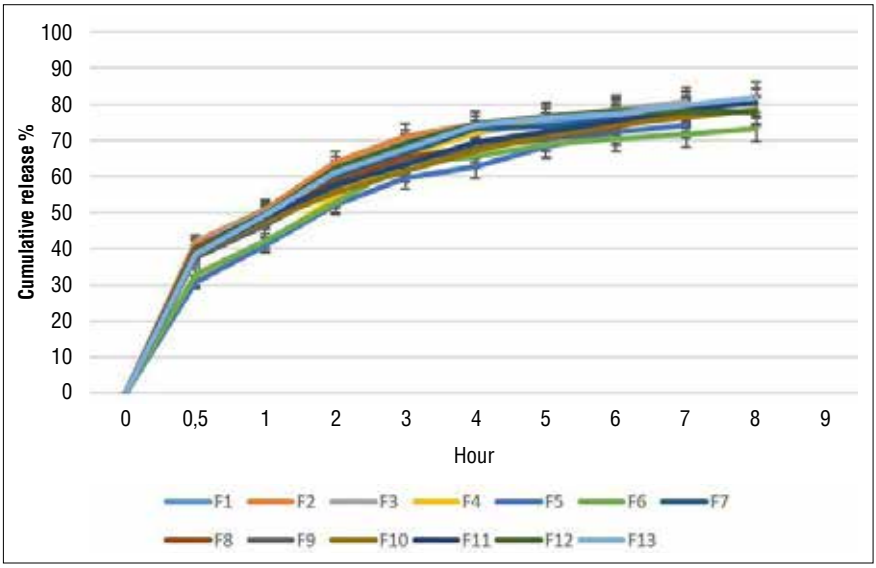

Figure 6. *In vitro* release profiles of formulations (n=6)

As indicated in Table 3, the in vitro drug release was found to be in the range from 62.6% to 74.4%. ANOVA results in Table 6 indicated that the change in the amount of HPMC K4M ($p<0.005$) had more influence than the change in the amount of HEC (P:0.299) on the drug release profile. When the 3D response surface graph in Fig. 7C is examined that the amount of drug released of the formulations decreases with increasing amounts of HPMC K4M[23,24].

The following equation was used to estimate the in vitro drug release:

$$Y3: 71.35 - 2.52\,X_1 - 1.24\,X_2 - 2.92\,X_1 X_2 - 1.49\,X_1^2 - 0.083\,X_2^2$$

where $Y3$ is in vitro release value, while X_1 and X_2 are the coded values of HPMC K4M and HEC, respectively. The model F value (12.3) and low P-value (0.001) implied the significance of the model equation as displayed in Table 6. Lack of fit of the model (P=0.497) was not significant.

Table 6. ANOVA results for response *Y3*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	112.94	5	22.59	12.3	0.0013	significant
A-X1	50.94	1	50.94	5.18	0.0048	
B-X2	12.35	1	12.35	1.26	0.2994	
AB	34.11	1	34.11	3.47	0.1049	
A2	15.45	1	15.45	1.57	0.2503	
B2	0.048	1	0.048	4.86E-03	0.9464	
Residual	68.86	7	9.84			
Lack of Fit	28.61	3	9.54	0.95	0.4974	not significant
Pure Error	40.25	4	10.06			
Cor Total	181.8	12				

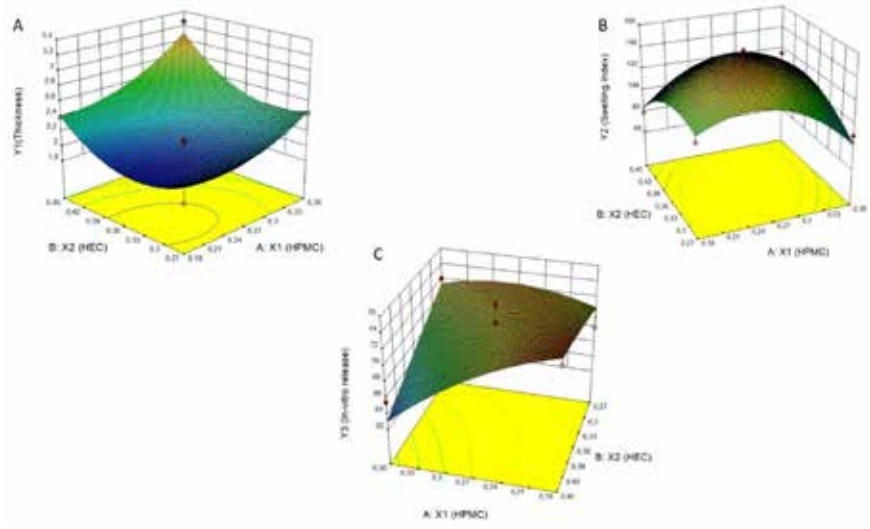

Figure 7. 3D response surface plots for (A) thickness, (B) swelling index, and (C) in vitro release as a function of HPMC and HEC amounts.

HPMC K4M has higher crosslinking degree, this hydrophilic matrix agent was supported the extended release period and floatation[25]. HPMC was used to make Alfuzosine floating beads, delaying release in a region-specific manner[26]. Gastroretentive dosage form was developed for cefuroxim axetil with HPMC and drug release was prolonged to 12 hours[27]. Domperidone floating tablet study showed that HPMC was effected on drug release rate and diffusion coefficient significantly[28]. Floating gastroretentive delivery system was prepared for fexofenadine

hydrochloride and HPMC which showed good floating properties in this study[29]. Also, HEC has good swelling properties and floating behavior[30].

The optimum floating film formulation prolonged drug release to 9 hours and its total floating time is 9 hours also. If the drug remains longer in the absorption region, the more drug is absorbed. Its percent drug release was found 85.78% in 9th hour. In this hour, buffer reaches the depths of matrices of rapidly hydrating polymers. When HPMC K4M contacted with buffer, it absorbs water which causes the polymer to swell. Due to this swelling, a viscous gel was formed around matrices. This matrice is resistant to water penetration. The drug which is inside matrice is released by diffusion due to this gel layer. The drug release is prolonged as the diffusion pathway increases, when the gel layer thickness increases[31].

Results of in vitro release kinetic studies

The results of release kinetic analyses of floating films are shown in Table 7. The release mechanism was calculated by finding the r^2 value for each model. The highest coefficient of correlation (r^2) value shows the most suitable kinetic model of drug release profile[32]. As seen from Table 7, drug release was found to be best fitted by Higuchi model for all formulations. It is pointed that the drug release happens from matrix as a square root of time-dependent process and diffusion controlled.

Polymeric systems commonly releases drug through Fickian diffusion. Atenolol floating sustained release matrix tablet was prepared that drug release kinetic was Higuchi and mechanism was diffusion. Furthermore, Metronidazole floating matrix tablet was developed which uses the same mechanism and has the same kinetic[33,34].

Table 7. Results of different kinetic models' r^2 values

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
Model name							r^2						
Zero order	0.740	0.708	0.727	0.848	0.789	0.773	0.717	0.701	0.724	0.718	0.722	0.737	0.746
First order	0.893	0.873	0.891	0.964	0.908	0.893	0.875	0.858	0.867	0.872	0.877	0.898	0.904
Higuchi	0.940	0.922	0.931	0.987	0.964	0.956	0.926	0.916	0.931	0.923	0.928	0.939	0.943
Hixson–Crowell	0.847	0.823	0.842	0.934	0.872	0.857	0.827	0.809	0.823	0.824	0.830	0.850	0.857
Korsmeyer–Peppas	0.549	0.515	0.523	0.669	0.605	0.589	0.515	0.493	0.526	0.498	0.515	0.547	0.553
							rss*						
Zero order	18734.1	18646.3	18721.2	10432.6	14654.9	16438.8	18138.1	18723.3	17495.5	18123.9	17481.5	16187.4	15983.1
First order	7128.5	7274.1	7122.4	3945.5	4123.6	5809.5	6128.4	6165.4	6105.4	5993.6	5984.3	5913.5	5843.7
Higuchi	440.1	465.3	459.1	286.4	305.1	314.6	476.3	487.1	391.4	406.3	394.9	319.4	295.1
Hixson–Crowell	10165.1	11654.4	10879.4	8756.5	11870.9	12136.4	14781.9	19767.8	14436.9	14315.7	13917.4	13773.7	13175.1
Korsmeyer–Peppas	40583.5	48982.3	46447.3	38415.8	42715.8	46881.5	48912.5	51925.6	47176.4	53751.8	48691.8	41829.8	40198.4

*The values of the sum of squared residuals

Optimization study results

The optimum film formulation was determined by Design-Expert software based on the obtained results from CCD study. Desired limits were set considering the minimum thickness, maximum swelling index and in vitro dissolution values. After the statistical calculations performed by the software, the X1 and X2 quantities which are the critical parameters to be entered into the formulation were determined as 0.242 g and 0.337 g, respectively. The optimized formulation was prepared in triplicate to evaluate the model accuracy for the optimum conditions. As shown in Table 8 the experimental responses were found to be in close agreement with the predicted responses. The concordance between the results showed the importance and validity of the model.

Table 8. Predicted and experimental values of the optimized formulation (n=3)

Response	Predicted Value	Experimental Value	Prediction Error (%)
Thickness (mm)	2.39	2.44±0.09	2.09
Swelling Index (%)	135.91	137.9±1.11	1.46
In-vitro Release (%)	68.02	67.07±3.28	1.40

Drug Content of Floating Film Formulation

In order to determine the homogeneity of the Dipyridamole amount in different parts of the film, drug content determination studies were performed. The results achieved from the pieces taken from different parts of the film indicate that the Dipyridamole is highly recovered from the formulation (92,4%±0,92%) and has a homogeneous distribution in the film (Table 9).

Mechanical Properties of Floating Film Formulation

Tensile strength is one of the important properties for defining the mechanical performance of the material. As indicated in Table 9, the tensile strength value of optimum floating film formulation was found to be 2.65±0.162 N/cm^2. Elongation at break is the ratio between altered length and initial length after breakage of the film formulation. The elongation at break value of optimum floating film formulation was found to be 37.21%±0.875%. According to results obtained from the study of mechanical proporties, optimum three-layer of floating film formulation represents the capability of film formulation to maintain changes of shape without fracture formation.

Table 9. The results of in-vitro characterization study of optimum floating film formulations

Release kinetics and parametres		Optimum value
Korsmeyer–Peppas	r^2	**0.8203**
Higuchi		**0.9679**
First order		**0.9481**
Zero order		**0.8298**
Hixson–Crowell		**0.9174**
Average Thickness (mm)		**2.42±0.31**
Drug content (%)		**92.4±0.92**
Swelling index (%)		**137.9±1.11**
Tensile strength (N/cm^2)		**2.65±0.162**
Elongation at break (%)		**37.21±0.875**

In-vitro buoyancy studies

The in vitro buoyancy analysis was conducted at 37° ±0.5°C. The floating film at different time intervals is depicted in Fig.3. Optimum film formulation in hard gelatin capsules showed sufficient buoyancy time of 9 hours of, as desired.

Results of films in hard gelatine capsules

Disintegration time

Disintegration time is a significant method to determined the quality of the oral dosage forms. It is a process of the oral dosage whereby disintegrates into smaller particles before dissolution happens. During the in vitro disintegration test, the rupture of the capsules was visually evaluated. Their disintegration time was observed less than 4.5 minutes of all formulations. It is easier to disintegrated because gelatin is a globular protein and low molecular weight.

In this research, three-layers of Dipyridamole floating film formulations were optimized with an experimental study using RSM. The floating films were produced using solvent casting method. The effect of the interaction of two independent variables (the amounts of HPMC K4M and HEC) on respective dependent variables (the thickness of films, the swelling character of polymers and the percent of *in-vitro* release in the 4^{th} hour) were investigated. It was observed that experimental results and predicted values showed similar results. Polymers showed good swelling characteristic in the 6^{th} hour. HPMC K4M is more effective on swelling than HEC because of its polymeric features (independence from the medium' pH and its chains disentangled from the matrix through hydration). If the amount of polymer in floating film increases, the thickness of the film increases. The drug release rate slows with increasing amount of polymer in formulations. The release was fitted to Higuchi kinetic model for each formulations. Optimum formulation was obtained in 9 hours of buoyancy time and prolonged release in 0.1 N HCl. The film was cut four quarter-circle then one of them with a conical shaped inserted into a hard gelatine capsule. This provided us with ease of oral use. In conclusion, floating film in a hard gelatine capsule is an innovative approach for remain buoyant in the stomach. With this approach it is possible to overcome the bioavailability problem in high pH values of the Dipyridamole.

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REFERENCES

1. Darandale, S. S., & Vavia, P. R. **(2012).** Design of a gastroretentive mucoadhesive dosage form of furosemide for controlled release. *Acta Pharmaceutica Sinica B*, *2*(5), 509-517.

2. Naveen, N. R., Gopinath, C., & Rao, D. S. (**2017).** Design expert supported mathematical optimization of repaglinide gastroretentive floating tablets: In vitro and in vivo evaluation. *Future Journal of Pharmaceutical Sciences*, *3*(2), 140-147.

3. Verma, S., Nagpal, K., Singh, S. K., & Mishra, D. N. **(2014).** Unfolding type gastroretentive film of Cinnarizine based on ethyl cellulose and hydroxypropylmethyl cellulose. *International journal of biological macromolecules*, *64*, 347-352.

4. Chandel, A., Chauhan, K., Parashar, B., Kumar, H., & Arora, S. **(2012).** Floating drug delivery systems: A better approach. *International Current Pharmaceutical Journal*, *1*(5), 119-127.

5. Tripathi, J., Thapa, P., Maharjan, R., & Jeong, S. H. **(2019).** Current state and future perspectives on gastroretentive drug delivery systems. *Pharmaceutics*, *11*(4), 193.

6. Harker, L. A., & Kadatz, R. A. **(1983).** Mechanism of action of dipyridamole. *Thrombosis research*, *29*, 39-46.

7. Rowe, R. C., Sheskey, P., & Quinn, M. **(2009).** *Handbook of pharmaceutical excipients*. Libros Digitales-Pharmaceutical Press.

8. Chakraborty, P., Dey, S., Parcha, V., Bhattacharya, S. S., & Ghosh, A. **(2013).** Design expert supported mathematical optimization and predictability study of buccoadhesive pharmaceutical wafers of loratadine. *BioMed research international*, *2013*.

9. Chen, Y. C., Ho, H. O., Chiu, C. C., & Sheu, M. T. **(2014).** Development and characterization of a gastroretentive dosage form composed of chitosan and hydroxyethyl cellulose for alendronate. *Drug design, development and therapy*, *8*, 67.

10. Sağıroğlu, A. A., Özsoy, Y., & Özer, Ö. **(2020).** Design, optimization and characterization of novel topical formulations containing Triamcinolone Acetonide. *Journal of Drug Delivery Science and Technology*, *58*, 101594.

11. Bertram, U., & Bodmeier, R. **(2006).** In situ gelling, bioadhesive nasal inserts for extended drug delivery: in vitro characterization of a new nasal dosage form. *European Journal of Pharmaceutical Sciences*, *27*(1), 62-71.

12. Khan, S., Boateng, J. S., Mitchell, J., & Trivedi, V. **(2015).** Formulation, characterisation and stabilisation of buccal films for paediatric drug delivery of omeprazole. *Aaps Pharmscitech*, *16*(4), 800-810.

13. The United States Pharmacopeia: USP 31; The National Formulary: NF 26; by Authority of the United States Pharmacopeial Convention; Washington, D. C., 2005.

14. Gouda, R., Baishya, H., & Qing, Z. **(2017).** Application of mathematical models in drug release kinetics of carbidopa and levodopa ER tablets. *J. Dev. Drugs*, *6*(02), 1-8.

15. Gharti, K. P., Budhathoki, U., Thapa, P., & Bhargava, A. **(2012).** Formulation in vitro evaluation of floating tablets of hydroxypropyl methylcellulose and polyethylene oxide using ranitidine hydrochloride as a model drug. *Journal of Young Pharmacists*, *4*(4), 201-208.

16. Muzib, Y. I., & Kumari, K. S. **(2011).** Mucoadhesive buccal films of glibenclamide: Development and evaluation. *International journal of pharmaceutical investigation*, *1*(1), 42.

17. Jindal, S., Jindal, K., Gupta, G., Garg, R., & Awasthi, R. **(2016).** Gastroretentive floating tablets: An investigation of excipients effect on tablet properties.

18. Agarwal, S., & Murthy, R. S. R. **(2015).** Effect of different polymer concentration on drug release rate and physicochemical properties of mucoadhesive gastroretentive tablets. *Indian journal of phar-*

maceutical sciences, *77*(6), 705.

19. Viridén, A., Wittgren, B., & Larsson, A. **(2009).** Investigation of critical polymer properties for polymer release and swelling of HPMC matrix tablets. *European journal of pharmaceutical sciences*, *36*(2-3), 297-309.

20. Baumgartner, S., Lahajnar, G., Sepe, A., & Kristl, J. **(2002).** Investigation of the state and dynamics of water in hydrogels of cellulose ethers by 1 H NMR spectroscopy. *Aaps Pharmscitech*, *3*(4), 86-93.

21. Nokhodchi, A., Raja, S., Patel, P., & Asare-Addo, K. **(2012).** The role of oral controlled release matrix tablets in drug delivery systems. *BioImpacts: BI*, *2*(4), 175.

22. Colombo, P. **(1993).** Swelling-controlled release in hydrogel matrices for oral route. *Advanced Drug Delivery Reviews*, *11*(1-2), 37-57.

23. Narendra, C., Srinath, M. S., & Babu, G. **(2006).** Optimization of bilayer floating tablet containing metoprolol tartrate as a model drug for gastric retention. *AApS pharmSciTech*, *7*(2), E23-E29.

24. Lefnaoui, S., & Moulai-Mostefa, N. **(2013).** Investigation of the effects of cellulose derivatives on the kinetics of drug release from cellulose-based hydrogel using a response surface method. *Journal of Pharmaceutical Technology, Research and Management*, *1*(1), 119-132.

25. Chen, Y. C., Ho, H. O., Lee, T. Y., & Sheu, M. T. **(2013).** Physical characterizations and sustained release profiling of gastroretentive drug delivery systems with improved floating and swelling capabilities. *International journal of pharmaceutics*, *441*(1-2), 162-169.

26. Fahmy, R. H. **(2012).** Statistical approach for assessing the influence of calcium silicate and HPMC on the formulation of novel alfuzosin hydrochloride mucoadhesive-floating beads as gastroretentive drug delivery systems. *AAPS PharmSciTech*, *13*(3), 990-1004.

27. Bomma, R., & Veerabrahma, K. **(2013).** Development of gastroretentive drug delivery system for cefuroxime axetil: in vitro and in vivo evaluation in human volunteers. *Pharmaceutical development and technology*, *18*(5), 1230-1237.

28. Prajapati, S., Patel, L., & Patel, C. **(2011).** Floating matrix tablets of domperidone formulation and optimization using simplex lattice design. *Iranian journal of pharmaceutical research: IJPR*, *10*(3), 447.

29. Saab, M., Samy, W., Issa, M., & El-Maradny, H. **(2018)**. Design and preparation of controlled floating gastroretentive delivery systems for enhanced fexofenadine hydrochloride oral bioavailability. *Tropical Journal of Pharmaceutical Research*, *17*(4), 569-576.

30. Chen, Y. C., Ho, H. O., Liu, D. Z., Siow, W. S., & Sheu, M. T. **(2015).** Swelling/floating capability and drug release characterizations of gastroretentive drug delivery system based on a combination of hydroxyethyl cellulose and sodium carboxymethyl cellulose. *PloS one*, *10*(1), e0116914.

31. Matharu, A. S., Motto, M. G., Patel, M. R., Simonelli, A. P., & Dave, R. H. **(2011)**. Evaluation of hydroxypropyl methylcellulose matrix systems as swellable gastro-retentive drug delivery systems (GRDDS). *Journal of pharmaceutical sciences*, *100*(1), 150-163.

32. Mhlanga, N., & Ray, S. S. **(2015).** Kinetic models for the release of the anticancer drug doxorubicin from biodegradable polylactide/metal oxide-based hybrids. *International journal of biological macromolecules*, *72*, 1301-1307.

33. Gunjal, P. T., Shinde, M. B., Gharge, V. S., Pimple, S. V., Gurjar, M. K., & Shah, M. N. **(2015).** Design, development and optimization of s (-) Atenolol floating sustained release matrix tablets using surface response methodology. *Indian journal of pharmaceutical sciences*, *77*(5), 563.

34. Ovenseri, A. C., Clifford, O., & Uwumagbe, U. M. **(2018).** Development and in vitro analysis of floating matrix tablets of metronidazole using Brachystegia eurycoma gum. *Pakistan journal of pharmaceutical sciences*, *31*(4).

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Molecular Inclusion -Novel Approach to Enhance Solubility of Olmesartan Medoxomil using Hydroxypropyl β cyclodextrin (HP β-CD)

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ABSTRACT

Olmesartan medoxomil (OLM) is a BCS Class II anti-hypertensive drug, which is having low aqueous solubility and low bioavailability of 26%. In the present study, the objective of the study was to improve the pharmaceutical limitation of OLM by using Molecular Inclusion Technique. Inclusion complex of OLM with Hydroxypropyl β cyclodextrin (HP β-CD) were prepared in drug/carrier molar ratios of 1:1, 1:2, and 1:3 by Solvent evaporation and Kneading method along with the Physical mixtures. The formation of molecular inclusion complex at 1:2 ratio in the solution was confirmed by Job's Plot study. The inclusion complex were characterized by Phase solubility study, Fourier transform infrared spectroscopy, Scanning electron microscopy, Differential scanning calorimetry and X-ray diffraction studies. *In- vivo* pharmacokinetic study reveals enhanced bioavailability of inclusion complexes. Thus, it can be concluded that the formulation of molecular inclusion complex can enhance Solubility and Dissolution making the drug more suitable for treatment.

Keywords: Olmesartan medoxomil, Molecular Inclusion, Stability Constant, Dissolution, HP β-CD.

INTRODUCTION

In recent trends, there are many of newly synthesized active agents that suffer from low aqueous solubility and dissolution and therefore have low bioavailability. There are two approaches that focus on improving the oral bioavailability that

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is enhancement of solubility and dissolution rate and increasing permeability of such drugs. Solubilisation, salt formation, complexation, solid dispersion and size reduction are commonly used approaches to increase dissolution rate. Amongst all these, solid dispersion technology is the promising approach for improvement of solubility and dissolution rate of poorly water soluble drugs[1]. Recently, Solid dispersion technology was explored extensively for the delivery of the drugs having poor solubility. Solid dispersions are mixtures in which drugs exist either in an amorphous form dispersed in the carrier molecule or included into the cavity of the carrier molecule[2]. Molecular inclusion technology has been proved a promising form of drug delivery. It offers dispersion or inclusion of a hydrophobic drug in a hydrophilic matrix of a carrier molecule in order to improve the dissolution rate which eventually improves the bioavailability of the drug.

Cyclodextrin (CD) inclusion complexation has been proved as a promising technique in enhancing solubility of insoluble drugs. There is the formation of host–guest inclusion complexes by weak intermolecular interaction[3]. The structure of cyclodextrin appears as a truncated cone, which has hydrophilic exterior surface and a lipophilic interior cavity into which drug molecules get entrapped[4]. Hydroxypropyl-βCD (HPβCD) is the commonly used excipients in several pharmaceutical formulations[5]. From the literature survey it was revealed that the solubility of βCD in water is relatively low (approximately 18.5 mg/mL at 25 °C), as compared to its derivative HPβCD which has a higher aqueous solubility (approx. 600 mg/mL at 25 °C). The reason behind the higher solubility of HPβCD is the chemical modification in the βCD by the addition of hydroxy propylene oxide group[6]. Also, HP-β cyclodextrin is most useful drug complexing agent because of its ability to readily form complexes and reasonable cost. Therefore, it was thought worthwhile to select HPβCD as a carrier for the formation of molecular inclusion complex.

Olmesartan medoxomil is a specific angiotensin II type I antagonist used alone or with other anti-hypertensive agents to treat hypertension. Olmesartan has poor aqueous solubility and low bioavailability of 26%[7]. In the recent study, it was also explored that OLM has a better renal protective effect than other Angiotensin receptor blockers. Therefore, improvement of solubility of OLM can prove this drug as a better option for the treatment of Hypertension for the patients suffering from renal disorder[3]. HP-βCD forms inclusion complexes in both solid and solution state, which can lead to modification in physical as well as chemical properties of the guest molecule. Solid drugs can be complexed with HP-βCD by using solvent evaporation, freeze drying, kneading, roll mixing etc. techniques.

The objective of the present study is to develop OLM-molecular inclusion complex with improved solubility using Hydroxypropyl-β cyclodextrin (HPβCD) as a carrier with various techniques like physical mixing, solvent evaporation and kneading. The study includes phase solubility study, characterization of inclusion complex by DSC and, SEM, determination of stability constant of the complex, solubilisation and stability studies of the product.

METHODOLOGY

Materials

Olmesartan medoxomil (Mylan Laboratories, Hyderabad), HP-β Cyclodextrin (Kleptose@ Roquette, France) and other reagents used were of AR Grade.

Preparation of Inclusion Complexes

1) Physical mixtures: The physical mixtures of Drug OLM and HP-β Cyclodextrin in different ratios were prepared by mixing the pulverised powders and passing them through #100 mesh sieve

2) Kneading: The Drug and HP-β Cyclodextrin were triturated in different ratios using sufficient amount of water to form a thick paste, which was further kneaded for 15 minutes. Then it was allowed to dry in a hot air oven. The dried mass was then triturated using mortar and pestle, sieved through #100 mesh sieve, stored in a desiccator.

3) Solvent evaporation: To a drug solution in 70% v/v of Ethanol (10 ml) appropriate amount of HP-β Cyclodextrin, in different ratios were added. This solution was allowed to stir continuously using hot plate magnetic stirrer until the solvent evaporated. Solid dispersion thus obtained was then pulverized, sieved through mesh 100, stored in a desiccator.

Characterization of Drug, Excipients and Solid Dispersion

Fourier transform infra-red spectroscopy (IR)

To study and confirm any drug-excipients interaction, Fourier Transform Infrared (FTIR) spectroscopy was performed. Initial spectra of the drug (OLM) and HP- βCD as control along with the solid dispersions were recorded. About 5-10 mg of test samples was used to study FTIR. FTIR spectroscopy of the test samples were performed by using the KBr scanning over wave number range of 4000–400 cm^{-1} with FTIR spectrophotometer (FTIR-8001, Shimadzu, Japan) operated with IR Solution Software.

Drug (OLM), HP- βCD, physical mixture and Solid Dispersion were filled in amber colored vials sealed with bromo butyl rubber stoppers and kept in the

environmental stability chamber (Remi Lab, Mumbai, India) for accelerated stability condition at 40 ± 2°C temperature and 75 ± 5 % relative humidity for a period of 30 days. FTIR spectra of samples were obtained and spectra of solid dispersions were compared with the initial spectra of drug and excipients.

Differential Scanning Calorimetry (DSC)

DSC curves of OLM, HP-β Cyclodextrin, physical mixture and solid dispersion prepared by kneading method and solvent evaporation were obtained by using DSC (SHIMADZU DSC 60 PLUS, Japan) at a heating rate of 5 °C/min from 30 °C to 300 °C in a nitrogen atmosphere. DSC curves of samples were obtained, and spectra of solid dispersions compared with the initial spectra of drug and excipients to confirm the formation of inclusion complex.

X-Ray Diffractometry (XRD)

XRD was carried out at room temperature using a D/max 2500VL/PC powder X-ray diffractometer (Rigaku, Japan) was operated at 40 kV and 35 mA to analyse the physical nature of the drug and inclusion complexes. Test samples were scanned over a 2θ range of 3–40 with a step size of 0.020° and a step time of 59.7 s.

Scanning electron microscopy

The SEM images of OLM, HP-β Cyclodextrin and inclusion complexes prepared was observed by scanning electron microscope (JSM- 5510 (Jeol Ltd. Tokyo, Japan) with the accelerating voltage 10-kV and current of 25 mA. In this technique, the test samples were placed in the metal stub having two way adhesive tape and under reduced pressure of about 2.54 pa; it was coated with the very fine platinum film. The SEM images obtained were observed and analysed at different scale or resolutions.

Determination of stability constant of the complex

Phase solubility studies

Higuchi and Connors method is a classical method for the Solubility studies, thus used here to perform phase solubility studies. In this method, excess drug was added to 30 ml of distilled water containing different molar concentrations of HP-β cyclodextrin (0.002-0.01 M). These mixtures were shaken on the orbital shakers at 250 revolution per minute for 24 hrs at the room temperature. The samples were then removed from shaker and allowed to achieve equilibrium. The aliquots were then filtered through wattman filter paper. The samples were suitably diluted and analysed using UV spectrophotometer by measuring the absorbance at 254 nm wavelength.

Spectrophotometric studies

Complex formation between OLM and HP-β cyclodextrin was studied by keeping the OLM concentration fixed as 2.445 x 10-8 M whereas the concentration of HP-β cyclodextrin was increased from 0.002 to 0.01M. The spectra of the drug were recorded on a UV-Vis spectrophotometer, Shimadzu (UV-1800 240V, Japan). The change in the absorbance of drug on the addition of various concentration of the HP-β cyclodextrin were measured at 254 nm to evaluate the stability constant of the complex. A stability constant is a measure of the strength of the interaction which represent the equilibrium constant for the formation of a complex in solution.

Similarly, the UV spectra of the OLM, physical mixtures and inclusion complexes prepared by solvent evaporation and kneading method in different ratios were recorded and overlayed. The absorbance values of each sample containing fixed concentration of drug were measured at 254 nm to evaluate the enhanced solubility.

Dissolution and Accelerated Stability studies

In vitro dissolution studies

In vitro dissolution studies of pure drug, inclusion complexes and innovator (marketed product) were performed using LABINDIA DS 8000 USP paddle method at 50 rpm in 900 ml of 0.1 N HCl acid (pH 1.2) as dissolution media. The temperature was maintained at 37±0.5°C. 5 ml sample was withdrawn at the time intervals of 5, 10, 15, 30, 40 and 50 minutes, filtered through wattmann filter paper and analyzed spectrophotometrically at 254 nm. After each withdrawal of the sample an equal volume of prewarmed fresh medium at the same condition, was replaced into the dissolution media to maintain the constant volume throughout the test[9].

Accelerated stability testing

Inclusion complexes were kept in sealed amber coloured bottles, protected from light and stored at 40°C/75% RH for 6 months. The products were analyzed for FTIR spectroscopy, drug content and dissolution after 6 month.

RESULTS and DISCUSSION

Physical characterization

FT-IR Spectroscopy:

The FT-IR spectra of pure Olmesartan medoxomil shows characteristic peaks at 2929.87, (C-H, str), 1707, 1739.79, 1830.45 (C-O, str) and 3288.63 cm-1 (N-H,

str) The spectra of molecular inclusion complex of Olmesartan medoxomil with HP-β cyclodextrin showed (Fig. 1) The characteristic peaks of Olmesartan medoxomil at initial conditions was maintained indicating that there is no chemical interaction between drug and excipients. Also, it was observed that there were no changes in the physical appearance of the samples.

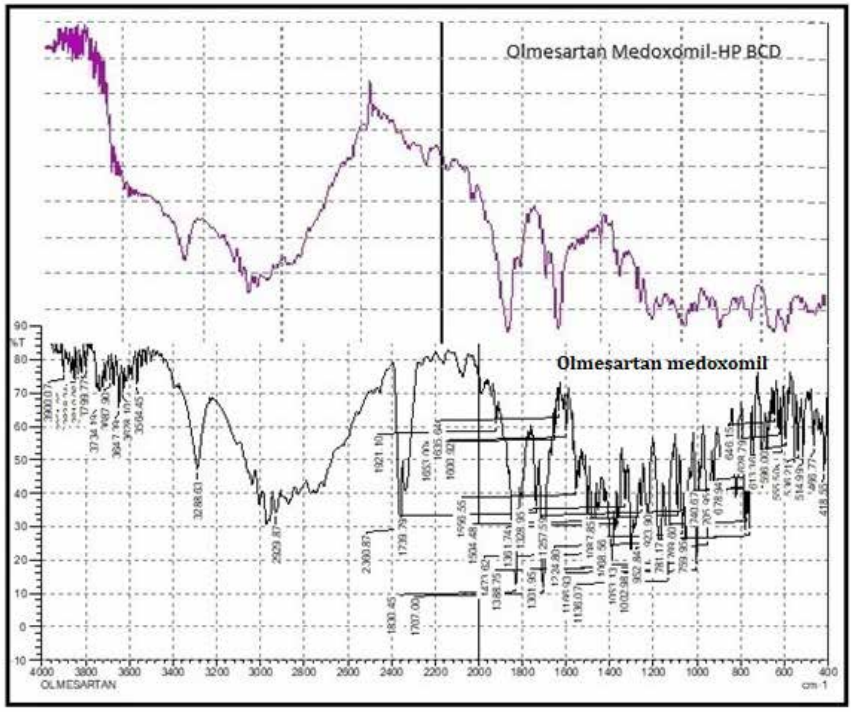

Figure 1. FTIR spectra of Pure Olmesartan Medoxomil (OLM) and complex of Hydroxypropyl β cyclodextrin (HP βCD) and Olmesartan Medoxomil (OLM).

DSC studies

The DSC thermograms of Olmesartan medoxomil, HP-β cyclodextrin and the inclusion complexes are shown in Fig. 2. The DSC curve of HP-β cyclodextrin shows a broad endothermic peak which was due to its amorphous nature. The sharp peak of Olmesartan medoxomil appeared at 184.02 °C, whereas no such peak was observed in inclusion complex prepared with HP-β cyclodextrin suggesting that Olmesartan was molecularly dispersed. It might have been no longer available in a crystalline state, and was converted into the amorphous state.

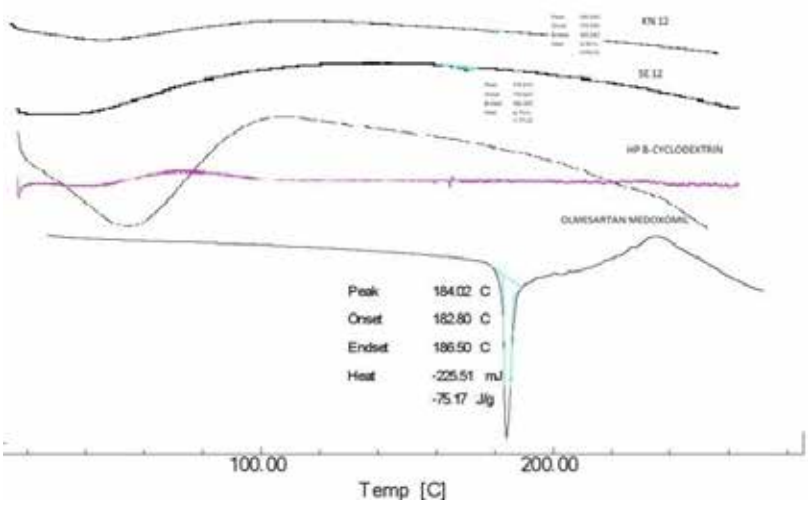

Figure 2. DSC curves of Pure Olmesartan Medoxomil (OLM), Hydroxypropyl β cyclodextrin (HP βCD), Olmesartan Medoxomil (OLM): Hydroxypropyl β cyclodextrin (HP βCD) kneaded complex (1:2), and Olmesartan Medoxomil (OLM): Hydroxypropyl β cyclodextrin (HP βCD) solvent evaporated complex (1:2).

SEM ANALYSIS

The surface morphological features of OLM, HP- βCD, and inclusion complexes are shown in figure 3. The complexes appeared as agglomerates rather than individual crystalline structures. This change of the particle shape was indicative of a conversion of solid crystalline state to amorphous state of the complexes.

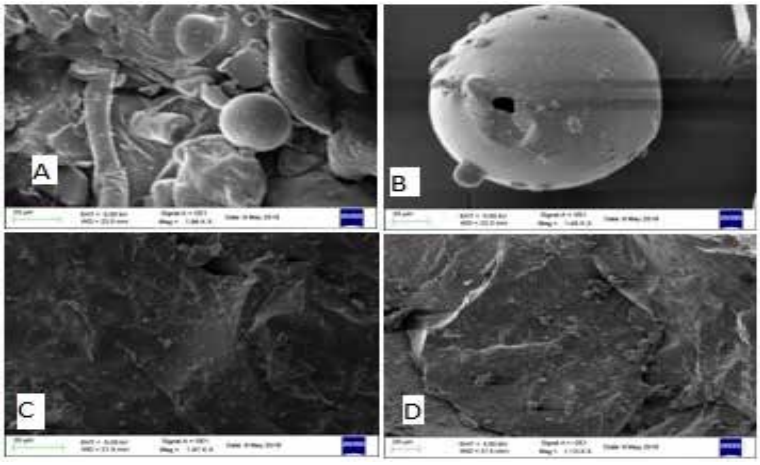

Figure 3. Scanning electron microscopic images of A- OLM and HP- β CD, B- HP- β CD, C -Inclusion complex of HP β-CD (SE 12) and D- Inclusion complex of HP β-CD (KN 12)

XRD analysis

The crystallinity of the pure drug and inclusion complexes could be studied through XRD patterns as shown in the fig 4. It was observed that pure OLM exhibited a characteristic peaks in the 2ø angle located at 7º, 9 º, 11 º, 12 º, 14º, 16 º, 19 º, and 21º which indicates the crystalline nature of OLM. Whereas, X-ray diffractometer graph of HP-βCD shows no characteristic peak, which indicates the amorphous nature of the carrier. When the inclusion complex prepared by solvent evaporation and kneading technique were analysed, they also showed completely diffused diffraction patterns indicating conversion of crystalline form into amorphous. This suggests that OLM was entrapped within the hydrophobic cavity of HPβCD. Absence of crystallinity and developed amorphous nature of the evaporated and kneaded complexes of OLM: HPβCD suggests the improved solubility and dissolution profile of OLM in gastrointestinal media.

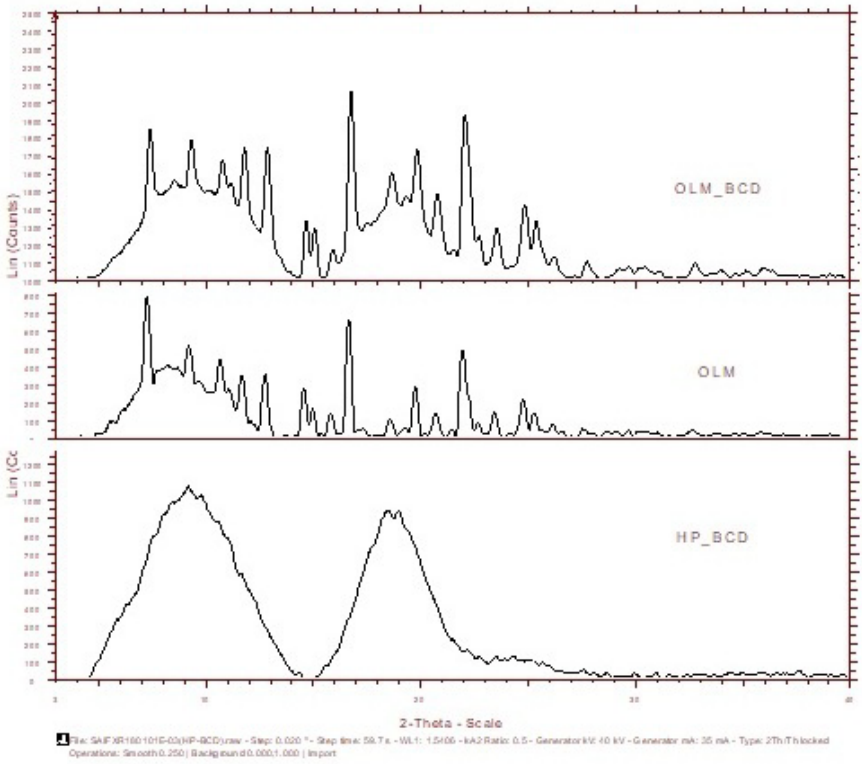

Figure 4. X-ray diffraction patterns of Pure Olmesartan Medoxomil (OLM), Hydroxypropyl β cyclodextrin (HP βCD) and kneading complex of Hydroxypropyl β cyclodextrin (HP βCD) and Olmesartan Medoxomil (OLM)

UV Spectroscopy

The Olmesartan medoxomil solution in the presence of increasing molar concentration of HP-βCD was scanned and analysed on UV spectrophotometer. The spectra of each solution recorded are shown in Fig. 5. There are changes observed in the peak intensity in-spite of addition of fixed amount of drug in solution. This may be the result of transfer of the drug molecule from water to the HP-βCD cavity, resulting in enhanced solubility of drug as indicated by the increased peak intensity[10].

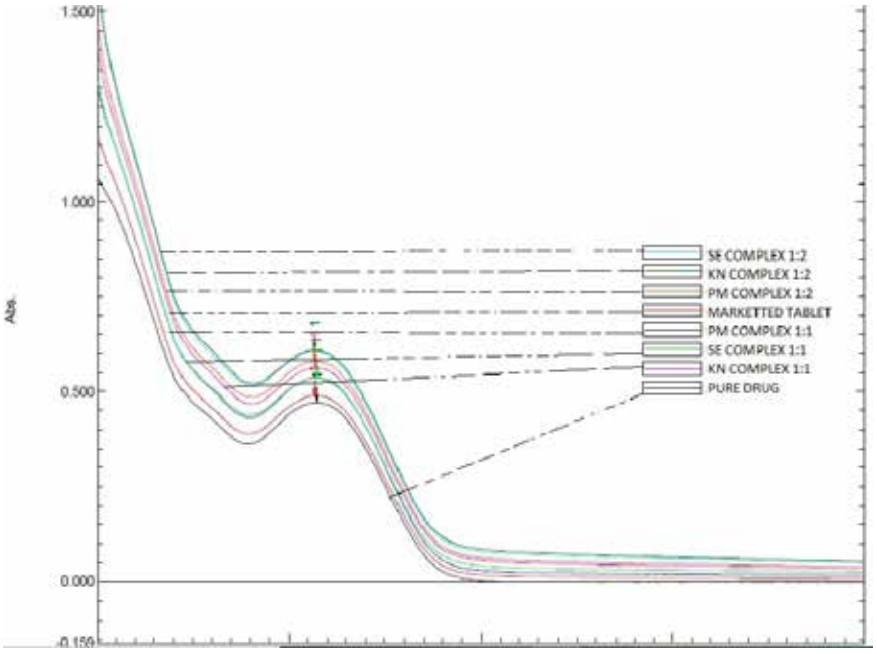

Figure 5. Overlay Spectra of Olmesartan Medoxomil (OLM) and various complexes prepared with Hydroxypropyl- β cyclodextrin (HP βCD).

Determination of Stability Constants

The complexing behaviour of Olmesartan Medoxomil with the HP-β cyclodextrin was studied by the phase-solubility method. The phase solubility diagram for the inclusion complex formation between Olmesartan medoxomil and HP-β cyclodextrin is shown in Fig. 6. It was observed from the phase solubility diagram that the aqueous solubility of Olmesartan medoxomil have increased linearly, as a function of HP-β cyclodextrin concentration. The graph shows the value with a slope 0.1957 (r= 0.9643). The stability constant (Kc) of Olmesartan medoxomil was calculated as 11858.15 M^{-1} from the plot of the phase solubility diagram and using the equation

$$Kc = \frac{Slope}{So(1\text{-}Slope)} \quad (1)$$

where, So is the solubility of drug. It can be concluded from the results obtained by using method of continuous variation (Job's Plot) as shown in fig.7 that the increase in solubility observed was due to the formation of a 1:2 M inclusion complex.

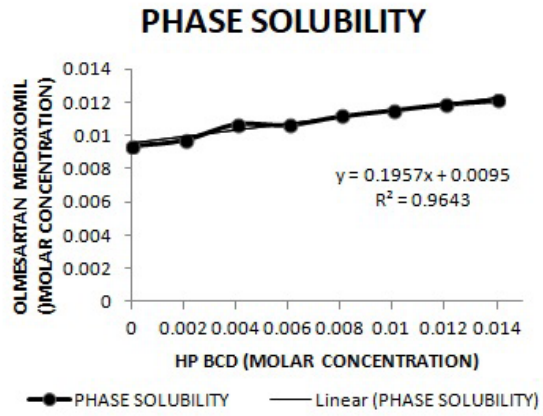

Figure 6. Phase solubility diagram of Olmesartan Medoxomil (OLM) in Hydroxypropyl β cyclodextrin (HP βCD) solution.

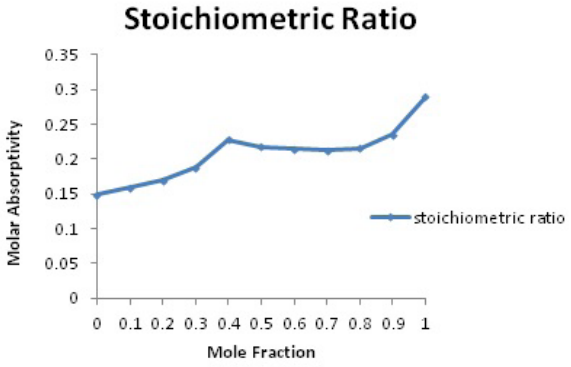

Figure 7. Job's Plot showing stoichiometric ratio of Olmesartan Medoxomil (OLM) in Hydroxypropyl β cyclodextrin (HP βCD) molecular inclusion complex.

Absorption spectra used to confirm the formation of inclusion complex and to compare the degree of enhancement of solubility. In this study, absorption spectra of HP-β cyclodextrin, Olmesartan medoxomil, physical mixture and inclusion complex were taken into consideration. In the absorption spectra for Olmesartan medoxomil and the physical mixture there is only a slight increase in the absorbance peak along the wavelength was recorded which may be due to the higher wetting effect of the available HP-β cyclodextrin with drug. Whereas, inclusion complex showed much more rise in intensity at all points of wavelength, this may be due to the inclusion or molecular dispersion of drug in HP-β cyclodextrin cavity.

Stability constant was determined using the Benesi–Hildebrand equation

$$\frac{1}{\Delta A} = \frac{1}{(D)} \; \boldsymbol{Kc}\Delta\epsilon \, \boldsymbol{X} \; \frac{1}{(CD)} + \frac{1}{(D)} \; \Delta\epsilon \qquad 2$$

where ΔA is the difference of absorbance at 254 nm, (CD) the HP- β cyclodextrin concentration, (D) the drug concentration (constant), and Δϵ is the difference in the molar absorptivity values between the complex and the free drug. The Kc value was calculated to be 5531.12 M^{-1}. It was found that the stability constant which was calculated by a spectral shift technique was relatively small compared to that obtained by the phase solubility studies. However, from the literature chaudhary et.al it was noted that the use of the spectral shift technique is not the method of choice for a reliable determination the drug with low aqueous solubility, because the difference in the absorptivity values will be too small[11]. In contrast, the other phase solubility method provides a much better and reliable results in these cases[12].

In vitro dissolution studies

The dissolution curves of Pure drug, Physical mixtures, Solvent evaporation and Kneading solid dispersions in 0.1 N HCl (pH =1.2) at 37 ± 0.5°C are shown in Fig. 8 and the corresponding values are given in Table 2. From the results obtained, it was observed that all the binary systems of Olmesartan Medoxomil demonstrated higher dissolution rates than pure drug and its corresponding physical mixtures. The physical mixtures also showed improved dissolution rate as compared with OLM but the highest drug release is observed for molecular inclusion complexes prepared by Kneading method. It is evident that the inclusion complex improved the dissolution rate of OLM to the greatest extent. Amongst all preparations, OLM: HP-βCD 1:2 inclusion complex produced by kneading method showed highest dissolution rate of about 96.73 % of drug in pH 1.2 at the end of 30 min.

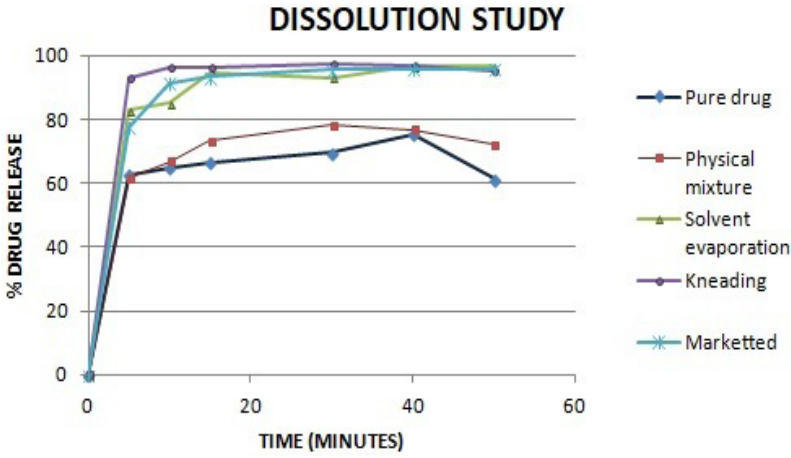

Figure 8. Dissolution profile of Olmesartan Medoxomil, prepared Olmesartan Medoxomil (OLM) : Hydroxypropyl β cyclodextrin (HP βCD) molecular inclusion complexes and Marketed preparation in 0.1 N hydrochloric acid.

Table 1. Olmesartan medoxomil content in HPβCD inclusion complex systems prepared.

Method	Formulation code	Complex system	Olmesartan medoxomil content (%)
Physical mixing	PM 11	Olmesartan medoxomil: HP-βCD (1:1)	98.52
	PM 12	Olmesartan medoxomil: HP-βCD (1:2)	98.64
	PM 13	Olmesartan medoxomil: HP-βCD (1:3)	98.45
Kneading	KN 11	Olmesartan medoxomil: HP-βCD (1:1)	99.21
	KN 12	Olmesartan medoxomil: HP-βCD (1:2)	99.10
	KN 13	Olmesartan medoxomil: HP-βCD (1:3)	98.78
Solvent evaporation	SE 11	Olmesartan medoxomil: HP-βCD (1:1)	98.11
	SE 12	Olmesartan medoxomil: HP-βCD (1:2)	98.24
	SE 13	Olmesartan medoxomil: HP-βCD (1:3)	98.37

PM 11; Physical Mixture (1:1), PM 12; Physical Mixture (1:2), PM 13; Physical Mixture (1:3), KN 12; Kneading Complex (1:1),KN 12; Kneading Complex (1:2), KN 13; Kneading Complex (1:3), SE 11; Solvent Evaporation Complex (1:1) ,SE 12; Solvent Evaporation Complex (1:2), SE 13; Solvent Evaporation Complex (1:3).

Table 2. Dissolution profile of Olmesartan medoxomil in 0.1 N hydrochloric acid

TIME (min)	Mean percentage of drug dissolved (±SD)* n=3				
	PD	PM 12	SE12	KN 12	INN
5	62.52(1.56)	61.69(1.01)	82.86(1.19)	92.88(0.59)	77.56(1.14)
10	64.66(1.75)	66.88(1.14)	85.25(1.63)	96.46(1.53)	91.47(1.01)
15	66.27(1.68)	73.32(1.11)	94.73(0.73)	96.29(1.01)	93.32(1.01)
30	69.52(0.83)	78.08(1.20)	93.08(1.40)	97.40(1.17)	95.54(1.5)
40	75.09(1.63)	76.86(1.23)	96.61(1.15)	96.73(0.97)	95.79(0.74)
50	61.18(1.00)	71.92(2.14)	96.51(1.00)	95.09(1.57)	95.84(1.37)
DE_{15}%	59.66	61.75	77.23	83.52	77.79
Similarity factor	36.94	42.01	78.37	65.59	Reference

PD, Pure Drug; PM 12; Physical Mixture (1:2), SE 12; Solvent Evaporation Complex (1:2), KN 12; Kneading Complex (1:2), INN, Marketed Tablet (Powdered)

Dissolution efficiency (% DE) is the area under the dissolution curve between time point's t_1 and t_2 expressed as a percentage of the curve at maximum dissolution, y100, over the same time period and is expressed by the following expression:

$$\textit{Dissolution efficiency } (DE) = \frac{\int_{t1}^{t2} y dt}{y100(t2-t1)} \quad (3)$$

DE_{15}% values of PD, PM12, SE12, KN12 and INN are found to be 59.66, 61.75, 77.23, 83.52 and 77.79 respectively.

Accelerated stability testing:

Accelerated stability testing results showed no significant variations neither in the content of drug nor in dissolution profiles after storage at 40 °C/75% RH for 6 months. Therefore, all these results imply that the prepared molecular inclusions are stable over the storage period and no effect on its stability on storage.

In the present study it was clearly observed that OLM immediate release inclusion complex can be effectively produced by processing via HP-βCD with enhanced solubility and Dissolution Rate. The results of the studies also indicated the formation of HP βCD- Olmesartan medoxomil molecular inclusion complex prepared by kneading method at different ratios of drug and HP-βCD. The inclusion complex had enhanced dissolution rate of OLM to a greater extent compared to the corresponding solvent evaporation, physical mixture and pure OLM. Further, it was noted that OLM-incorporated molecular inclusions in 1:2 ratio aqueous solution provides the highest dissolution and solubility

values compared to the marketed product and OLM pure drug, which indicate that the oral bioavailability of OLM might be improved. Kneading method was the most effective method in terms of OLM solubility and dissolution. This enhanced dissolution from the inclusion complexes may be the result of increased hydrophilicity and wetting effect that results from the interaction between the drug and the carrier [13,14]. The another reason for the rapid dissolution of OLM from inclusion complexes may be attributed to the entrapment of drug in the hydrophilic carrier matrix of carrier HP-βCD and a decrease in the crystallinity of drug as observed in XRD and DSC studies. Further, in the mechanism of quick dissolution of drug from the inclusion complexes could be due to the soluble carrier dissolving into the dissolution medium and allowing the entrapped drug release to the medium in the form of very fine particles.

Thus, we can conclude that HP-βCD is useful as a primary carrier in the formulation of molecular inclusion complex of the drug (OLM) prepared by kneading method so as to enhance its solubility and dissolution rate.

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REFERENCES

1. Aggarwal, S.; Gupta G.D.; Chaudhary S. Solubility and dissolution enhancement of poorly aqueous soluble drug. Int. J. of Drug Deliv. 2012, 4, 341-365.

2. Kalepua, S.; Nekkant, V. Insoluble drug delivery strategies: review. Acta Pharmaceut.Sin. B. 2015, 5.5,442–453.

3. Takashi, O.; Oru, S.;Yoshito, M.; Ritsuya N. Olmesartan is More effective than other Angiotensin receptor antagonist in reducing proteinuria in patients with chronic kidney disease other than Diabetic Nephropathy. Cur.Therap.Res. 2013, 74,63-67.

4. Loftsson, T.; Brewster, M.E. Pharmaceutical applications of cyclodextrins 1. Drug solubilisation and stabilization. J Pharm Sci 1996, 85, 1017–1025.

5. Singh, R.; Bharti, N.; Madan, J. et al. Characterization of cyclodextrin inclusion complexes – a review. J Pharm Sci Technol. 2010, 2, 171–183.

6. Gabriel, O.K.L.; Yvonne, T.F.T.; Kok-Khiang, P.Enhancement of norfloxacin solubility via inclusion complexation with β-cyclodextrin and its derivative hydroxypropyl-β-cyclodextrin. Asian J. of Pharma. Scien. II. 2016, 536-546.

7. Li, N.Inclusion complex of trimethoprim with β-cyclodextrin. J. Pharmaceut. Biomed. 2005 39, 824–829.

8. Sean, C.S. Martindale, The Complete Drug Reference. 36th Edition. Pharmaceutical Press, 2009.

9. Dehghan, M.H.G.; Jafar M. Improving dissolution of meloxicam using solid dispersions. Iranian J Pharm Res., 2006, 4, 231-238.

10. Ismail, S. Interaction of anticonvulsant drugs with alpha and beta-cyclodextrins. I. Methsumide. STP Pharm. Sci. 1991, 321-325.

11. 10. Shah, T.J.; Amin, A.F.; Parikh, J.R. Process optimizationand characterization of poloxamer solid dispersions of a poorly water soluble drugs. AAPS Pharm Sci Tech. 2007, 8.2, E1-E7.

12b Chaudhary, K.P.R.; and Nalluri, B.N.; Studies on nimesulide and bcyclodextrin inclusion complexes. physicochemical characterization and dissolution rate studies. Drug Dev Ind Pharm. 2000;26:1217-1220

13. Yang, L.J.; Chen, W.; Ma, S.X. et al. Host-guest system of taxifolin and native cyclodextrin or its derivative: preparation characterization, inclusion mode, and solubilization. Carbohydr Polym. 2011, 85, 629–637.

14. Yuvaraja, K.; Khanam, J. Enhancement of carvedilol solubility by solid dispersion technique using cyclodextrins, water soluble polymers and hydroxyl acid. J. of Pharmac. and Biomed. Anal. 2014, 96, 10–20.

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Awareness and Knowledge of Autism Spectrum Disorders Among Community Pharmacists in Khartoum state(Sudan), 2018

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ABSTRACT

This study was conducted to assess awareness and knowledge of autism spectrum disorders (ASDs) among community pharmacists in Khartoum state. A cross-sectional study was conducted among 345 community pharmacists. All the data were collected from randomly selected by questionnaire. The results indicated gaps in participant awareness and knowledge regarding ASDs. The participant reported low familiarity with ASDs. The median score on 12 item knowledge section regarding ASDs was about 60%. Having continuing education(CE) or training program on ASDs was significantly associated with familiarity and confidence scores. About 64% of the participants reported as not feeling confident enough in their ability to counsel parents about medications used for their children with ASDs and their side effects. Pharmacists' awareness and knowledge regarding ASDs needs to be improved. Providing continuing education programs should be considered to increase pharmacist's awareness and knowledge regarding ASDs.

Keywords: Autism spectrum disorders, Community pharmacist, Behavioral disorders.

INTRODUCTION

Autism Spectrum Disorders (ASDs) are complex, lifelong neurodevelopmental and behavioral disorders manifests as difficulty communicating, abnormal social interaction, and delays in developmental skills [(1)]. The first reported on ASDs was Kanner in 1943 and since then the global prevalence has been increasing consistently[(2)], The Centers for Disease Control (CDC) estimated the prevalence of ASDs nationally as 1 in every 59 children[(3)], it's 3-4 more prevalent

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among males than females[4], and more prevalent than juvenile diabetes, pediatric cancer, and pediatric AIDS combined[3]. Because of increasing prevalence, ASDs is widely considered to be an emerging public health concern [5], ASDs were not shown to be associated with a certain racial, ethnic and socioeconomic group[2], it occurs because of an interaction of genetic and environmental factors[6]. Currently, there is no cure for autism[7] but there are pharmacological and non-pharmacological treatment options to alleviate the symptoms associated with ASDs. [8].

Pharmacological treatment has become increasingly popular in managing behavioral symptoms of ASDs like aggressiveness, anxiety, hyperactivity, and tantrums. [8-10], Risperidone and aripiprazole are drugs approved by The Food and Drug Administration (FDA) for the management of behavioral symptoms associated with ASDs. [11,12] Other pharmacological classes used to manage symptoms of ASDs include Central Nervous System (CNS) stimulants, antidepressants, and anxiolytics[12]. In today's healthcare system, the role of pharmacists goes beyond merely dispensing medications, they are considered as trusted and easily accessible health care providers[13]. Therefore, the community pharmacist could play an important role in the care of people with ASDs and help them achieve the best results from their medications. The pharmacological treatment of children with ASDs can be challenging. A previous study showed that children with ASDs are particularly susceptible to adverse effects and may exhibit paradoxical reactions to medications. [14,15]. Therefore, improperly trained pharmacists or those with less familiarity and knowledge would fail to do so. This might have consequences on their role in providing healthcare and might result in deteriorating the health and quality of life of their patients.

As the community pharmacist is the first line of interaction with families and/or caregivers in our community, they should be more familiar and knowledgeable about ASDs resources allocated for ASDs in their communities and should guide families of children with ASDs to relevant professional resources. Unfortunately, previous studies reported cases of pharmacists lacking knowledge and awareness of the ASDs area. [16-18] Community pharmacists practicing in Khartoum(Sudan) were not previously assessed for their awareness and knowledge of ASDs. Therefore, this study aims to assess awareness and knowledge of autism spectrum disorders (ASDs) among community pharmacists.

METHODOLOGY

Study setting and population: This study was conducted in Khartoum state, the national capital of Sudan from April to August 2018. The study population was registered community pharmacists practicing in the Khartoum state.

According to the Sudanese General Directorate of Pharmacy, the number of registered community pharmacists when this study started is 2265. [19]

Study design and sampling: A descriptive cross-sectional survey design was used for this study. The sample size needed for this study was calculated according to this equation:

$$(np= \frac{N}{1+N(e)2}).$$

Where N= target population, n= sample size, e= margin of error (0.05). The sample size was estimated at 95% confidence interval with a default margin of error of 5%. The sample size was 345 pharmacists.

Sampling conducted by selecting individual units (Khartoum state pharmacies) by stratified sampling to ensure representativeness of the study. Khartoum state pharmacies were broken down into seven strata by the area (locality). Samples were randomly selected from each stratum according to their percent in the population as follow; Khartoum (23%), Khartoum North (14%), Om-durman (13%), Sharq an-Nīl(13%), Jabal Awliya(14%), Om Badda, (11%), and Karari (12%). One of the pharmacists who are working in the selected pharmacy was randomly selected and included in the study.

Data collection: Face to face paper-based questionnaires were administered after obtaining participants' informed consent by the researcher itself and trained data collectors who visited the community pharmacists in their work-places and explained the purpose of the study. The questionnaire sections were adopted from similar previous studies[16,19-22]. The questionnaire contained four sections, the first section collected demographic and practice details of the study participants, also pharmacist was asked if they had a course(s) or lecture(s) on ASDs during their pharmacy degree program and if they had continuing education (CE) or training program on ASDs. The second section contained six items to measure pharmacists' degree of familiarity with symptoms, treatment, and community resources of ASDs. The participant pharmacist had to indicate the degree of familiarity on each item on a Likert scale of five options. The third section contained 12 items to measure pharmacists' knowledge of the etiology, prevalence, and treatment of ASDs. The fourth section contained four items to measure pharmacists' training and confidence in medication management of ASDs. Pharmacists had to indicate the degree of their agreement with the 4 items on a Likert scale of five options. The questionnaire was tested in twenty community pharmacies as a pilot study, and these pharmacies were excluded when the study was conducted.

Statistical analysis: Statistical Package for Social Sciences software, version 21.0 (IBM SPSSInc., Chicago, IL) was used for the analysis of data. Initially, all information gathered via directly administered questionnaire are coded into variables. The normality of data was tested using the Kolmogorov-Smirnov test. Data that were not normally distributed expressed as medians then categorized into poor (less than the median) and good (median and above) and compared using Mann-Whitney U-Test. The reliability test of the questionnaire was tested using Cronbach's Alpha= 0.618 (62%), Spearman's rho correlation was used to investigate the correlation between familiarity, knowledge, and confidence scores. and binary logistic regression was used to present results. A *p*-value of less than 0.05 was considered statistically significant.

Ethical approval: This study received ethical approval from the ethical committee of the faculty of pharmacy, University of Khartoum. Pharmacists who participated in this study also gave informed consent before they took part.

RESULTS and DISCUSSION

Demographic and practice characteristics of the study participants

A total of 345 community pharmacist participants in the study giving a response rate of 100%. The study participants practicing in the 7 localities of Khartoum state was found between 1996- 2016. The demographic and practice-related characteristics of the participant pharmacists are listed in Table 1.

Table 1. Demographic and practice characteristics of the participant community pharmacists surveyed (n= 345)

Demographic variables	Number	Percentage
Age (Years)		
(≤35)	328	95.1%
(>35)	17	4.9%
Gender		
Male	93	27%
Female	252	73%
Number of years in practice		
(<5)	248	71.9%
(≥5)	97	28.1%
Job Title		
Staff pharmacist	271	78.6%
Others(manager,owner)	74	21.4%
Highest degree		
Higher degree (MSc and Ph.D.)*	70	20.3%
Basic degree (BSc)	275	79.7%
Had attend ASDs course(s) or lecture(s) during pharmacy degree		
Yes	34	9.9%
No	311	90.1%
Had attended ASDs CE program*		
Yes	23	6.7%
No	322	93.3%

*ASDs autism spectrum disorders, BSc bachelor degree in pharmacy, MSc master of science degree, Ph.D. doctor of philosophy degree, CE continuing education

The demographic and practice-related characteristics of the pharmacists from different localities were similar. About 73% of community pharmacists were female and Nearly 78% of community pharmacists identified themselves as staff pharmacists. About 77% of the community pharmacists had basic pharmacy degrees and about 80% had less than 5 years practicing pharmacy in Khartoum. Only about 10% had either course(s) or lecturer(s) on ASDs during their pharmacy school program, and about 6% had Continuing Education Programme (CEP) on ASDs as shown in Table 1.

The familiarity of pharmacists with Symptoms, Treatment, and Community Resources of ASDs

Table (2) describes the participant pharmacists' degree of familiarity with ASDs. Majority of the participants showed a low degree of familiarity (67.9%) with symptoms, treatment, and community resources of ASDs, the median score on the Likert scale was 13. Furthermore, about 74% of participants showed low familiarity with classes of medications used to manage symptoms of ASDs, and nearly 59% low familiar with their side effects. About 74% were not sufficiently familiar with the specific behavior that medication seeks to alleviate.

Table 2. Degree of familiarity with ASDs symptoms, treatment, and community resources of the participant community pharmacists surveyed (n= 345)

Familiarity variables (n=345)	Not at all familiar N(%)	Not familiar N(%)	Somewhat familiar N(%)	Familiar N(%)	Extremely familiar N (%)
Are you familiar with the different symptoms of ASDs*?	24	94	165	54	8
	7%	27.2%	47.8%	15.7%	2.3%
Are you familiar with different classes of medications? (e.g.antipsychotics, antidepressants, stimulants) that are used in treating the symptoms of ASDs?	127	128	76	12	2
	36.8%	37.1%	22%	3.5%	0.6%
Are you familiar with specific behaviors associated with ASDs that medications seek to alleviate? (e.g. hyperactivity, OCD, and self-injury)	124	131	72	16	2
	35.9%	38%	20.9%	4.6%	0.6%
Are you familiar with various side effects produced by medications used in the treatment of ASDs symptoms (e.g. sedation, irritation, and extrapyramidal symptoms)?	78	125	118	21	3
	22.6%	36.2%	34.2%	6.1%	0.9%
Are you familiar with how to help families sort through informed decisions about their children with ASDs?	61	172	76	34	2
	17.7%	49.9%	22%	9.9%	0.6%
Are you familiar with community resources in your region that can be used for the referral of a child who is exhibiting symptoms commonly associated with ASDs?	73	161	77	33	1
	21.2%	46.7%	22.3%	9.6%	0.3%

*ASDs autism spectrum disorders, OCD obsessive-compulsive disorder

Knowledge of Pharmacists of Etiology, Prevalence, and Treatment of ASDs

Table (3) describes a participant's knowledge of ASDs based on their response to a series of true/false statements. The median score was nearly 58%. About 95% of participants knew that the children with ASDs have impairment in so-

cial interaction, communication or language, and behavioral development (variable 1), and about 87% knew that ASDs are developmental disorders(variable 2). Only about 30% of participants knew that ASDs are more prevalent than down syndrome and juvenile diabetes (variables 5 and 4). About 51% knew that ASDs are not curable (variable 6), and 55% knew that risperidone and aripiprazole have been approved by the FDA for the treatment of irritability associated with ASDs (variable 7). Only 14% and 25% don't know that vaccine cannot cause ASDs and ASDs not exists only in childhood (variable 8,9 respectively). Nearly half of the participants knew ASDs are rare disorders (variable 12), and about81% knew that genetic factors play a major role in the etiology of ASDs(variable 11). Only about 29% knew that ASDs are not caused because of emotionally distant, rejecting parents(variable 10) and about 43% knew ASDs occur more commonly among males than females(variable 3).

Table 3. Knowledge of etiology, prevalence, and treatment of ASDs of the participant community pharmacists surveyed (n= 345).

Knowledge variables (n=345)	True N (%)	False N (%)
1- ASDs are developmental disorders	**302**	43
	87.5%	12.5%
2- Children with ASDs have impairments in social interaction, communication or language, and behavioral development	**330**	15
	95.7%	4.3%
3- ASDs occur more commonly among males than females	**151**	194
	43.8%	56.2%
4- ASDs is more prevalent than juvenile diabetes	**97**	248
	28.1%	71.9%
5- ASDs is more prevalent than down syndrome	**115**	230
	33.3%	66.7%
6- ASDs is curable	168	**177**
	48.7%	**51.3%**
7- Risperidone and aripiprazole have been approved by the FDA for the treatment of irritability associated with ASDs	**191**	154
	55.4%	44.6%
8- Vaccines can cause ASDs	50	**295**
	14.5%	**85.5%**
9- ASDs exists only in childhood	89	**256**
	25.8%	**74.2%**
10- ASDs are caused because of emotionally distant, rejecting parents	243	**102**
	70.4%	**29.6%**
11- Genetic factors play a major role in the etiology of ASDs	**281**	64
	81.4%	18.6%
12- ASDs are rare disorders	171	**174**
	49.6%	**50.4%**

ASDs autism spectrum disorders, FDA food, and drug administration
Correct answers are in boldface

Training and Confidence of Pharmacists in Medication Management of ASDs

Table (4) described the training and confidence of pharmacists in medication management of ASDs. About 64% of the participants reported as not feeling confident enough in their ability to counsel parents about medications used for their children with ASDs and their side effects, and about 54% reported not feeling comfortable enough dispensing medications for individuals with ASDs. But 89% agreed that they would benefit from taking CE or training programs in the ASDs area, and nearly 91% agreed that pharmacy curricula should include courses or lectures on ASDs.

Table 4. Training and confidence of the participant community pharmacists surveyed in medication management of ASDs (n= 345).

Confidence Variables (n=345)	Strongly disagree N (%)	Disagree N (%)	Neutral N (%)	Agree N (%)	Strongly agree N (%)
I feel confident in my ability to counsel parents about the medication profile and side effects of prescriptions being used for the treatment of their child with ASDs	47	176	68	43	11
	13.6%	51%	19.7%	12.5%	3.2%
I feel comfortable dispensing medications used in the treatment of ASDs	32	152	74	75	12
	9.3%	44.1%	21.4%	21.7%	3.5%
I feel that I could benefit from taking a continuing education or training program in the area of ASDs	14	10	13	179	129
	4.1%	2.9%	3.8%	51.9%	37.4%
I feel that the pharmacy school curriculum should include a course or lecture in the area of ASDs	11	6	12	179	137
	3.2%	1.7%	3.5%	51.9%	39.7%

ASDs autism spectrum disorders

Association of characteristics of the participants and familiarity and knowledge

Comparing the characteristic of the participant using the Mann-Whitney *U* test, a certain variable like having a CE program on ASDs was significantly associated (p value<0.05) with familiarity score and confidence score, as shown in Table 5. On the other hand, age, gender, practice setting, number of years in practice, job title, and a degree in pharmacy were not significantly associated with familiarity score.

Table 5. Association of characteristics of the study pharmacists with familiarity, knowledge and confidence scores

Mann-Whitney U-Test							
Variables		**Familiarity**		**Knowledge**		**Confidence**	
Mean Rank		**P-Value**	**Mean Rank**	**P-Value**	**Mean Rank**	**P-Value**	
Age (Years)	(≤35)	170.62	0.051*	174.24	0.301*	173.17	0.889*
	(>35)	218.85		149.03		169.76	
Gender	Male	180.47	0.396*	184.01	0.205*	184.11	0.201*
	Female	170.24		168.94		168.9	
Number of years in practice	(<5)	170.96	0.541*	169.9	0.347*	170.04	0.370*
	(≥5)	178.23		180.94		180.57	
Job Title	Staff pharmacist	167.2	0.038**	168.88	0.135*	174.45	0.599*
	Others	194.25		188.1		167.68	
Highest degree	MSc and PhD	190.61	0.096*	188.39	0.141*	161.33	0.265*
	BSc	168.52		169.08		175.97	
Had attend ASDs course(s) or lecture(s) during pharmacy degree	Yes	201.25	0.081*	187.85	0.352*	187.26	0.371*
	No	169.91		171.38		171.44	
Had attended ASDs CEP	Yes	215.52	0.033**	194.63	0.273*	213.22	0.042**
	No	169.96		171.45		170.13	

**P-value ≤ 0.05 that's considered as statistically significant.

*P-value >0.05 that's considered as statistically insignificant.

Confidence scores significantly correlated with familiarity scores (spearman's rho=0.312, *p*-value < 0.01) and knowledge scores (spearman's rho=0.169 *p*-value <0.01), also there were significant correlation between familiarity and knowledge scores (spearman's rho=0.227 *p*-value<0.01). Binary logistic regression analysis was conducted to know factors that could be associated with the low familiarity, knowledge, and confidence scores. The analysis revealed that older age is significantly associated with poor knowledge (*p-value* 0.037). Moreover, participant's poor knowledge and poor familiarity was significantly contributed to poor confidence with *p-value* 0.001 and 0.047 respectively.

To the best knowledge of the investigators, this is the first study to assess community pharmacist's awareness and knowledge regarding Autism Spectrum Disorders(ASDs) in Sudan. The present study used variables from previous studies that were used to measure similar constructs among healthcare provid-

ers. [16,18, 20-22]. As shown in Table 3, about 87% of participants knew that ASDs are developmental disorders. This finding was higher than the result observed in studies from Palestine[18]and Mississippi[20], in which the percentage was 40% and 76% respectively.

In agreement with the studies from Mississippi surveyed pharmacists and pharmacy students [16,20], in the current study, about 96% of the participants knew that children with ASDs have impairments in social interaction, communication or language, and behavioral development. However, only about 44% of the participant knew that ASDs are more common in males than in females. This finding is close to the study conducted in Palestine [18], but lower than the findings obtained from the pharmacists and pharmacy students from Mississippi were about (85%) of participants correctly answered this statement [16,20]. On the other hand, only about 30% of the participants knew about the prevalence of ASDs compared to juvenile diabetes and Down syndrome, respectively. This finding is consistent with a study carried out in Palestine, However, it is contrary to the study from Mississippi [20].

Although the primary duty of the community pharmacist is not diagnosis, participant pharmacists were somewhat familiar with the different symptoms of ASDs. About 34% of the participant were minimally familiar with signs and symptoms of ASDs, our results were consistent with those reported among nurses in the state of Virginia and pharmacists in Palestine [22, 18]. However, pharmacists and pharmacy students in Mississippi were relatively more familiar with the signs and symptoms of ASDs as only 25% of them reported as either not familiar at all or not familiar with the different symptoms of ASDs.[16,20].

Pharmacists assuming educative roles might need to educate families of people with ASDs on these symptoms. Pharmacists who are not familiar with symptoms of ASDs cannot assume this role. This can be even worse when pharmacists are not familiar with how to help families sort through information to make informed decisions about their child with ASDs. This study revealed that about 67% of the pharmacists reported themselves as either not familiar at all or not familiar with how to help families sort through information to make informed decisions about their child with ASDs (Table 2). This percentage was comparable to those reported by Palestinian pharmacists and Mississippi pharmacists and pharmacy students. [16,18, 20]. Moreover, about 68% of the pharmacists in this study were not familiar with community resources in their region that can be used for the referral of a child who was exhibiting symptoms commonly associated with ASDs. These results were comparable to those reported by Mississippi pharmacists and pharmacy students [16, 20] and similar to the studies done on Palestinian pharmacists [18].

Although, some studies concluded that early diagnosis improves the prognosis of ASDs in children [23]. Unfortunately, in many cases there were significant delays in diagnosing children with ASDs[24]. In modern healthcare delivery systems, the role of pharmacists in caring for patients with chronic diseases is expanding. Subsequently, patients often report a good relationship with their pharmacists[13] previous research showed that both patients and primary healthcare physicians wanted pharmacists to play a larger role in providing information to patients. [13][25]. When asked to indicate the degree of familiarity of participants with the different classes of medications (e.g. antipsychotics, antidepressants, stimulants) that are used in treating the symptoms of ASDs, nearly 74% of the participants in this study reported themselves as either not familiar at all or not familiar. This value was quite higher than that reported in study conducted in Palestine, where 62% of the pharmacists reported as not feeling confident enough in their ability to counsel parents about ASDs medications[18]. This finding highlight the need to increase knowledge of pharmacists on the classes of medications used to treat symptoms of ASDs.

Furthermore, about 58% of the pharmacists were either not familiar at all or not familiar with various side effects produced by medications used in the treatment of symptoms of ASD. These results are worrisome, as pharmacists are expected to be familiar with medications used in the management of different diseases and the side effects associated with the use of these medications. Therefore, it was not surprising that nearly 64% of the pharmacists in this study were not confident in their ability to counsel parents about the medications being used for the treatment of their child with ASDs.Previous studies reported that children with ASDs are prescribed psychotropic medications[11,12] These medications are associated with weight gain, sedation, and/or tardive dyskinesia which might further complicate the management of ASDs in children[26].

Approximately 49% of the participant in this study thought ASDs were rare disorders, 70% thought ASDs are caused because of emotionally distant, rejecting parents, 25% thought that ASDs exist only in childhood, and about 18% did not know that genetics play a major role in the etiology of ASDs. About 14% believed that the vaccine causes ASDs. Pharmacists and other healthcare professionals are in a position to influence parent's decisions on vaccinations[27]. Previous studies showed that believing in this myth had negatively affected vaccination rates in the US and UK.[28,29].

Having CEP in the area of ASDs was significantly associated with familiarity and confidence scores. In this study, confidence scores correlated with both familiarity and knowledge scores. Despite the obvious low Spearman's rho, there

was a significant positive correlation between the scores. This indicates that the pharmacists who were relatively familiar and knowledgeable of ASDs were more confident in their ability to counsel parents about the medication profile and side effects of prescriptions being used for the treatment of their child with ASDs as well as dispensing medications used to treat symptoms of ASDs. A previous study showed that on many occasions, education increases familiarity and knowledge in certain domains [30]. In this study, about 89% of the participants agreed that they could benefit from taking a CE or training program in the area of ASDs. Similarly, about 95% agreed that the pharmacy curriculum should include a course or lecture in the area of ASDs.

These results highlight the need to integrate educational interventions on ASDs in pharmacy curricula. Policymakers and health authorities should consider educational interventions to increase awareness and knowledge of pharmacists on ASDs. Similarly, pharmacy schools should revise, widen, and deepen educational materials on ASDs in the current curricula.

Community pharmacists are in a key position to provide crucial information to individuals with chronic disease and disabilities, including ASDs. The current study revealed gaps in awareness and knowledge of ASDs among community pharmacists in, Khartoum state, Sudan. Participants in this study reported low confidence in their ability to dispense and counsel individuals with ASDs and their families on medications used to alleviate symptoms of ASDs. Community pharmacists reported low familiarity with resources devoted to ASDs in their community, these gaps could be filled by specifically designed educational interventions.

CONFLICTS OF INTEREST

Nadine Hassan Ali and Ahmed H. Arbab report no conflicts of interest associated with this article.

REFERENCES

1. Mormann, M.; Gilbertson, C.; Milavetz, G.; Vos, S. Dispelling vaccine myths: MMR and considerations for practicing pharmacists. *J. Am. Pharm. Assoc.* **2012**, 52, 282-286.

2. Christensen, D.L.; Braun, K.V.; Bao, J.; Bilder, D.; Charles, J.; Constantino, J.N.; Daniels, J.' Durkin, M.S.; Fitzgerald, R.T.; Kurzius-Spencer, M..; Lee, L.C. Prevalence and characteristics of autism spectrum disorder among children aged 8 years autism and developmental disabilities monitoring network, 11 sites, United States, 2012, MMWR Surveill Summ. **2018**, 65,1–23.

3. Autism Speaks. What is autism? Available at:http://www.autismspeaks.org/what-autism. [Accessed on April 5.2019]

4. Leung, A.K.; Kao, C.P. Evaluation and management of the child with speech delay. *Am. Fam. Physician*. **1999**, 59, 3121-3128.

5. Rice, C, Nicholas, J.; Baio, J, Pettygrove, S.; Lee, L.C.; Braun, K.V; Doernberg, N.; Cunniff, C.; Newschaffer, C.; Meaney, F.J; Charles, J. Changes in autism spectrum disorder prevalence in 4 areas of the United States. *Disabil. Health J.* **2010**, 3, 186-201.

6. Hertz-Picciotto, I.; Croen, L.A.; Hansen, R.; Jones, C.R.; Van de Water, J.; Pessah, I.N. The charge study: an epidemiologic investigation of genetic and environmental factors contributing to autism. *Environ. Health Perspect.* **2006**; 114, 1119-1125.

7. Tsai, L. Y. Psychopharmacology in autism. *Psychosom. Med.* **1999**, 61, 651-665.

8. Kaplan, G.; and McCracken, J.T. Psychopharmacology of autism spectrum disorders. *Pediatr. Clin. North Am.***2012**, 59, 175-187

9. Mohiuddin, S.; Ghaziuddin, M. Psychopharmacologyof autism spectrum disorders: A selective review. *Autism* .**2013** ,17, 645–654.

10. Steckler, T.; Spooren, W.; Murphy, D. Autism spectrum disorders–an emerging area in psychopharmacology. *Psychopharmacology*, **2014**, 231, 977–978.

11. Esbensen, A. J.; Greenberg, J. S.; Seltzer, M. M.; Aman, M. G. A. longitudinal investigation of psychotropic and non-psychotropic medication use among adolescents and adults with autism spectrum disorders. *J. Autism Dev. Disord.,* **2009***,* 39, 1339-1349.

12. Mandell, D. S.; Morales, K. H.; Marcus, S. C.; Stahmer, A. C.; Doshi, J.; Polsky, D. E. Psychotropic medication use among Medicaid-enrolled children with autism spectrum disorders. *Pediatrics***. 2008**; 121: e441-e448.

13. McAuley, J. W.; Miller, M. A.; Klatte, E.; Shneker, B. F. Patients with epilepsy's perception on community pharmacist's current and potential role in their care. *Epilepsy Behav*. **2009**, 14, 141-145.

14. Handen, B. L.; Johnson, C. R.; Lubetsky, M. Efficacy of methylphenidate among children with autism and symptoms of attention-deficit hyperactivity disorder. *J. Autism Dev. Disord*. **2000**; 30, 245–255.

15. Marrosu, F.; Marrosu, G.; Rachel, M. G.; Biggio, G. Paradoxicalreactions elicited by diazepam in children with classic autism. *Funct. Neurol.* **1987**, 2, 355-361.

16. Khanna, R.; Jariwala, K. Awareness and knowledge of autism among pharmacists. *Res Social Adm Pharm*, **2012**, 8, 464–471.

17. Luleci, N. E.; Hidiroglu, S.; Karavus, M., Karavus, A; Sanver, F.F.; Ozgur, F.; Celik ,M.; Celik S.C. The pharmacists' awareness, knowledge and attitude about childhood autism in Istanbul. *Int. J. Clin. Pharm.* **2016**, 38, 1477-1482.

18. Shawahna, R.; Fahed, B., Qadri, D.; Sharawi, L.; Soroghli, M.; Dweik, M. Awareness and Knowledge of Autism Spectrum Disorders Among Pharmacists: A Cross-Sectional Study in Palestinian Pharmacy Practice *J. Autism Dev. Disord.* **2017**, 47, 1618-1627.

19. Federal Ministry of Health, Sudan, Annual Pharmaceutical Statistical Report, **2006**. Available,from:http://apps.who.int/medicinedocs/documents/s17176ar/s17176ar.pdf, FMOH Khartoum. [accessed on April7, 2018]

20. Khanna, R.; Jariwala, K.; Holmes, E. R.; Ramachandran, S. Autism familiarity and knowledge among pharmacy students. *Curr Pharm*. Teac.*h Learn.* **2014**, 6, 150–157.

21. Schwartz, H.; Drager, K. D. Training and knowledge in autism among speech-language pathologists: A survey. *Lang. Speech Hear. Ser.* **2008**, 39, 66-77.

22. Strunk, J. A. School nurses' knowledge of autism spectrum disorders. *J. Sch. Nurs., 2009;25*, 445-452.

23. Freeman, B. J. Guidelines for evaluating intervention program for children with autism. *J. Autism Dev. Disord.* **1997**, 27, 641-651.

24. Wiggins, L. D.; Baio, J.; Rice, C. Examination of the time between first evaluation and first autism spectrum diagnosis in a population-based sample. *J Dev Behav Pediatr.* **2006**, 27, S79-S87.

25. McAuley, J.; Mott, D.; Schommer, J.; Moore, J.; AL, R. Assessing the needs of community pharmacists and primary care physicians in providing care to patients with epilepsy. *J. Am. Pharm. Assoc.***1999**, 39: 499-504.

26. Hattier, M. A.; Matson, J. L.; Tureck, K., Horovitz, M. The effects of gender and age on repetitive and/or restricted behaviors and interests in adults with autism spectrum disorders and intellectual disability. *Res Dev Disabil.* **2011**, 32: 2346-2351.

27. Grabenstein, J. D.; Hartzema, A. G.; Guess, H. A.; Johnston, W.P. Community pharmacists as immunization advocates: A pharmacoepidemiologic experiment. *Int.J. Pharm. Pract.* **1993**, 2:5-10.

28. Novella, S. The anti-vaccination movement. *SkepticalInquirer*.**2016**, 31, 26–31.

29. Smith, M. J.; Ellenberg, S. S.; Bell, L. M.; Rubin, D. M. Media coverage of the measles-mumps-rubella vaccine and autism controversy and its relationship to MMR immunization rates in the United States. *Pediatrics.* **2008**, 121, e836–e843.

30. Gillespie-Lynch, K.; Brooks, P.J.; Someki, F.; Obeid, R.; Shane-Simpson, C, Kapp S.K; Daou, N, Smith D. S. Changing college students' conceptions of autism: An online training to increase knowledge and decrease stigma. *J. Autism Dev. Disord.***2015**, 45: 2553-2566.

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Nanosponges (NSs): Using as a Nanocarrier for Anti Cancer Drug Delivery Applications

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ABSTRACT

Nanosponges (NSs) are non-toxic, porous, solid polymers, having three dimensional structures which stable at high temperature and can form inclusion complexes with many molecules due to their cavity size. NSs are prepared by reacting β-cyclodextrin (β-CD) with cross-linkers such as pyromellitic anhydride, hexamethylene diisocyanate, carbonyl-diimidazole or diphenyl carbonate.

Many anti-cancer drugs with both hydrophilic and lipophilic properties can be loaded or integrated into NS and ultimately increase the characteristics of the drugs. The most important properties and advantages of NSs are easily synthesized and modified, provide high drug loading capacity along with controlled release, increase the solubility and bioavailability of water-soluble molecules. In addition, NSs protect the degradable actives from environmental influences, biologically safe and biodegradable compared to other nanocarriers. This review has highlighted the structure and properties of CD-NS as well as demonstrating a new generation of functionalized NS studies as an innovative drug delivery agent for pharmaceutical applications.

Keywords: Nanosponge, Cyclodextrin, Nanocarriers, Controlled Drug Delivery, Guided Drug Delivery, Cancer

INTRODUCTION

According to the data of the world health organization, cancer is defined as the second most important disease which has a fatal impact on human health of the 21st century [1]. Strategies developed for cancer treatment include chemotherapy, surgery, hormone therapy, radiation therapy and recently nanoparticular-based anti-cancer drugs and targeted therapy. However, traditional cancer treatment

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methods have some disadvantages compared to innovative methods. Although it has a positive effect on the destruction of cancer cells in chemotherapy applications where cancer drugs are used, it has many negative features such as damaging normal healthy cells in the tumor environment, showing toxic properties depending on the doses used and having side effects. Surgical treatment, however, involves risks such as tumor cells invading or spreading other tissues, reoccurrence, although local cancerous tissue is removed.

In the radiation therapy that uses high energy radiation, tumor cells can be eliminated and prevent them from dividing and multiplying. During radiotherapy, the tumor area is determined and the rays are sent to the cancerous cell at a higher dose. In the treatments, a large area is used to give adequate amount of irradiation to the tumor tissue, and accordingly, healthy tissue damage and side effects can be seen much more.

Most drugs currently in use have poor biopharmaceutical properties [2], low permeability, solubility and intestinal absorption, and short blood circulation times that cause various toxic side effects, therefore limiting their use in clinical applications. These adverse conditions cause rapid excretion, dose-related side effects, and deterioration in gastrointestinal fluids, in vitro instability and lack of selectivity [3].In order to overcome disadvantages, drug targeting and delivery systems (DTDS) have been broadly developed, investigated and researched, especially with the aim of nano-carrier based DTDS. Treatment using nanoparticles (NPs) is known as a new type of cancer treatment method that improves the pharmacological properties of drugs and removes tumors in normal tissues with minimal effect. In this method, traditional cancer drugs are directly conjugated to NPs or integrated with a suitable ligand, and have positive results such as increased stability of the drugs, biocompatibility, and less impact on healthy cells and much less side effects in traditional methods. The accumulation of drug loaded nanostructure in the tumor cell can vary depending on the circulation time, shape, size of the structure and the targeted tumor type [3].

The main function of NPs develop is the delivery and controlled release of therapeutic drugs to the targeted area. It is prominent that the nanomaterials used for this aim should be biocompatible, soluble and secure [4-7]. It is also important that NPs should be non-toxic, non-mutagenic, have high stability, and have the capacity to deliver therapeutic agent to specific site.

For this objectives, many nano-based drug delivery system was developed, designed and manufactured such as nanosponges, polymeric micelles, solid lipid nanoparticles, metallic nanoparticles (silver, gold), carbon nanotubes, nanogels, dendrimers, liposomes, nanocrystals, magnetic nanoparticles and micro-

capsules. For instance, Nanosponge is a three-dimensional, solid, insoluble structure that is obtained by a variety of synthesis techniques using cyclodextrins (CD) (which may be α-CD, β-CD, γ-CD) with polymeric crosslinkers. Solid lipid nanoparticles (SLNs) are structures that are used as drug delivery material that researchers work on, consisting of emulsifiers, water / solvents and lipids, and have many advantages such as low toxicity, easy production, controlled drug release, and delivering drugs of particular lipofolic nature [8].

Dendrimers are polymers containing a large number of branched monomers consisting of a central core. These structures, which have many functional groups thanks to the branched monomers composed of dendrons, are used as drug delivery agents due to their compatibility and flexible structures. Denimers synthesized from many polymers such as polyamidoamine PAMAM), polypropyleneimine (PPI) have nanoparticle drug delivery potential due to their biological compatibility. In addition, these structures are used for photothermal therapy gene therapy applications [9,10].

The liposomes, consisting of small spherical vesicles, are two-layer structures made of phospholipids containing a hydrophobic hydrocarbon chain and a hydrophilic head, and are used as drug delivery agents. Due to their high compatibility, liposomes, which can be formed in nanometer or micrometer size due to their single or multi-layer structure, can carry structures such as medicine, protein, antigen, DNA and can be used in other therapies as well as cancer therapy [11].

However, due to the disadvantages of NPs, it is difficult to develop an optimal drug delivery system that achieves the desired result, some nanomaterials are difficult to apply as a drug delivery agents due to their degradation or toxicity effects in the body parts, and this requires sufficient time and research. For instance, the disadvantage of solid lipid nanoparticles is that SLNp have a crystalline structure that causes drug release due to unexpected polymeric degradation [8]. Dendrimers, on the other hand, may show toxic properties due to the presence of peripheral amine carboxylate, phosphonate, sulfonate groups, as well as the fact that they can show non-specific drug delivery and sudden and uncontrolled drug release due to its open network [12].

Liposomes also have disadvantages such as low content of drug loading, high cost, poor stability and undesired hydrophobic drug release.

Due to their characteristic structure and properties, NPs can be passively, actively or physically delivered to the target site, e.g. tumor cell [13-16]. Targeting the NPs to the tumor region depends on the structural feature of the tumor tissue, that is, the leaky vascular feature and the weak excretory system, which is called

enhanced permeability and retention (EPR) effect. The nanostructure caused by the EPR effect accumulates in the tumor region and this is defined as passive targeting. The NPs whose surface is modified with ligands such as peptides or antibodies binds by recognizing reproducers on the cell surface and internalized into the cell through endocytosis, leaving the loaded drugs. This event is defined as active targeting. On the other hand, accumulation of nanostructure can be performed in tumor tissues through external effects such as ultrasound, magnetic field, heat, light waves (UV, infrared) [17-18].

Again according to the designed NPs structure, internal stimulatory mechanisms (pH-guided [19], enzymes[20], temperature [21]) or as external stimuli (electric field [22], the magnetic field [23], Light [24], voltage excitations [25] ultrasound [26], and other chemicals [27-29]) may release the drug in the cell.

In researches on some nanoparticles used for drug delivery strategies, the solubility and effectiveness of drugs have been increased and it has been proven that these structures can be used as effective delivery agents. For instance, dendrimers can be used as delivery agents for non-steroidal anti-inflammatory lyophilic drugs. For example, in a study, the drug containing the amino group was encapsulated into PPI or PAMAM type dendrimers and its solubility was increased [12,30].

In a study with solid lipid nanoparticles, tamoxifen loaded SLNp was prepared and its effectiveness on MCF7 cancer cells was investigated. In in vitro cytotoxide research, they emphasized that the efficiency of tamoxifen loaded SLNp increased when compared to free tamoxifen[31,32].

METHODOLOGY

Cyclodextrin Based Nanosponges (CD-NSs)

Nanosponges are effective nano-carriers that researchers focus on as a drug delivery agent, which has recently attracted the attention of many researchers due to their characteristic structures. NSs have been developed to eliminate the disadvantages of the traditional medicines described in detail above and increase the effectiveness of drugs. Briefly, Nanosponges are obtained by using cyclodextrins (CD) (α-CD, β-CD, γ-CD) with polymeric crosslinkers (such as diphenyl carbonate). Since the main component is cyclodextrins, it is useful to mention the definition and structure of CDs.

Chemical Structure and Properties of CDs

Cyclodextrin (CD) is defined as a water-soluble, biocompatible cyclic oligisaride, which is formed by the enzymatic degradation of starch, elementary polysaccharides in nature, by the *Bacillus amylobacter* [33] bacterium, and con-

tain a hydrophilic outer surface and a lipophilic cavity that is formed by the α-1,4 glycosidic binding of D-glycosupranoses (Figure 1.). β-CD, γ-CD, α-CD are named depending on the number of glucosupranose units in their structure. For instance, the structure formed by connecting 7 glucosupranose units with glycosidic bond is called β-CD (Figure 1.).

Despite the fact that CDs in higher structures are found in nature, the difficulty of obtaining and being able to form an inclusion complex with very few substances were not preferred in terms of research. Among them, β -CD is the most preferred and profound studied structure because its convenient cavity, which can interact with many inorganic and organic substances, in addition, ensures high drug loading capacity, easy availability and affordability.

Since 1970, CDs have been used in medicine, food, cosmetics, textiles, catalysts, biotechnology, and more recently as a drug targeting and delivery system in pharmacology, nanotechnology and the pharmaceutical industry.

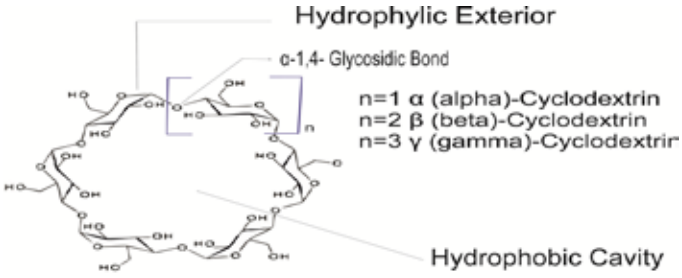

Figure 1. Dimensions and chemical structure for α-, β- and γ-CD (n = 6, 7 and 8, respectively).

Cyclodextrin-Based Nanosponge (CD-NS) Concept

Natural CDs do not form inclusion complexes with particular drugs which has high molecular weight or hydrophilic property, in addition some CDs limit their use since they show toxic properties when injected intravenously [34-36]. Therefore, many chemical modification studies have been carried out in order to use them as a nano drug carrier system, eliminate the disadvantages of CDs, and improve their physical, chemical properties. One of these alterations is the formation of NSs by reacting natural CDs with the crosslinkers described below. In 1998, DeQuan Li and Min Ma were the first researchers to use the term cyclodextrin-based Nanosponges [37] and develop the NS obtained by bonding β-CD with a cross-linker (such as diisocyanate) for the treatment and purify of wastewater [38]. In their research, they showed that CD- NSs completely removed wastes such as p-chlorophenol from wastewater at even a billionth level.

However, the development of CD-based NSs by reacting of natural CDs with crosslinkers and the application of NSs as a nano drug delivery system was first demonstrated by Trotta and his team [39-41].

Nanosponge's are defined as nanoscale hyper-crosslinked polymers which is non-toxic, stable at high temperature, spongy pores and a three-dimensional network structure. It is synthesized by using main component of β, α, or γ-cyclodextrins and crosslinked by proper amount of crosslinkers (such as pyromellitic anhydride, hexamethylene diisocyanate, carbonyl-diimidazole, diphenyl carbonate, (Figure 2,3.) NSs can preserve the hydrophilic and hydrophobic drugs in their three dimensional cavity. It is usually prepared from β-cyclodextrins compared with other native CDs (α, and γ) because β-CD has the highest drug encapsulation sites, higher complexes and stability due to the appropriate cavity size [39,40].

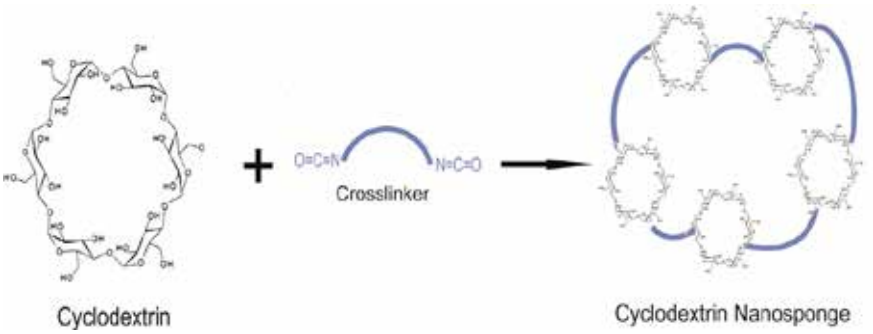

Figure 2. Cyclodextrins bind to the crosslinking polymer to form NanospongeThe four most basic cross-linking polymers are used in the synthesis of NSs with appropriate synthesis methods, such as anhydride crosslinkers, carbonyl crosslinkers, diisocyanate linkers, and epichlorohydrin (Figure 3.).

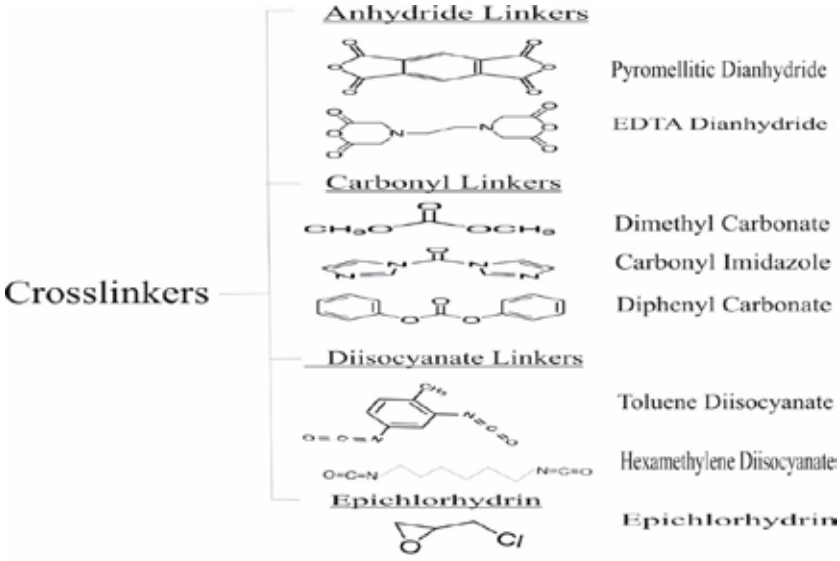

Figure 3. Common crosslinkers used in Nanosponge synthesis.

RESULTS and DISCUSSION

Methods Used in Nanosponge Synthesis

Melting method [42], solvent method [43], ultrasound assisted synthesis [42] microwave-assisted synthesis [44,45] methods are used to obtain desired characteristics and different sizes of NS. In the melting method, briefly, the crosslinker such as diphenyl carbonate and beta cyclodextrin in certain molar ratios are melted and gradually heated to a temperature of 90-100 °C under magnetic stirring, and the by-product formed is removed and cooled at room temperature. The solid product **(Figure 4.)** formed is washed with distilled water and acetone, and the unreacted product is removed [42].

In solvent method, cyclodextrin is dissolved in a polar solvent such as dimethylsulfoxide and reacted with a crosslinker such as carbonyldiimidazole at a certain molar ratio for about 4-5 hours at 90 °C. When the reaction is over, an excess of water is added to remove the unreacted crosslinker and the solid is obtained by filtration. Then Sohxlet- extracted with acetone to remove from by-products **(Figure 4.)** [43, 44,46].

In ultrasound assistand synthesis, cyclodextrin and cross-linker are put in a bottle in a solvent-free environment and subjected to sonication in the ultrasound bath for approximately 5 hours at 90 °C. After cooling at room temperature, processes are performed as specified in the solvent method [39,42].

The product obtained by the solvent method has a spherical morphology and a high dissolution power for water-soluble molecules. In addition, submicron spherical nanosponges can be obtained by ultrasound assisted synthesis using a suitable crosslinker.

Microwave assisted synthesis is known as the easiest method in obtaining cyclodextrin based nanosponge compared to other methods. The method is used to obtain homogeneous crystallinity of NSs as well as a synthesis method which reduces reaction time four times compared to melting method.

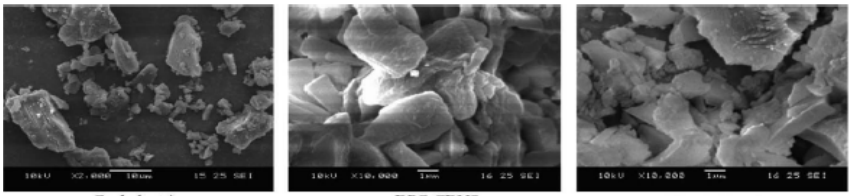

Figure 4. SEM image of a) Cyclodextrin b) Diphenyl Carbonate crosslinked NS c) Pyromellitic Anhydride crosslinked NS [46]

Factors Affecting Formation of NSs

There are also important factors affecting the formation of NS. These are:

Drug Type: Besides the fact that the drugs used are hydrophilic or hydrophobic, the properties of the drugs known as 'Five rules of Lipinski' affect the formation of NSs.

Polymer Type and Crosslinkers: The type of CD (being α -, β- and γ-CD) and the crosslinkers used play an important role in NSs formation. Specifically, the determination of CD and cross-linker molar ratios causes the particle size of NS to form in the desired size; however, in the cross-linker types play an important role in transforming NSs into a three-dimensional structure suitable for hydrophilic or hydrophilic drugs.

Temperature: Increasing the temperature, for example, can especially lead to a decrease or weakening of intermolecular hydrophobic forces (such as Vander waals force) between NSs and the drug [47].

Nanosponge Preparation Method: The effectiveness of the method used is important in terms of both the formation of NS and drug coplex and integration of the drug into NS, depending on the nature of the drug and polymer used in the formulation. One of the most useful techniques for the drug / nanosponge complex is the freeze-drying technique [48].

Degree of Substitution: The ability to form nanosponge complexes may vary depending on the number, position and structure of the substituent on the parent polymer. For example, the higher the degree of substitution on the parent polymer, the more cross-linking can take place between CD and crosslinker [49].

Nanosponge Toxicity

Toxicity studies are necessary studies that are extremely important in terms of whether or not the dose of the drug used and the nanocarrier designed for drug delivery is toxic to humans and animals, hence to evaluate the usability of the structure.

Toxicity studies with NS have shown no toxic effects or adverse effects. For example, by injecting NS onto Swiss albino mice, the acute systemic toxicity of NS has been shown to be safe from 500 to 5000 mg / kg and does not show any signs of toxicity or adverse reactions [50]. In addition, the study of oral administration of nanosponge was tested in mice [51] without significant side effects. In vitro toxicity studies using cell cultures such as MCF7, COS, HELA using the MTT test also showed that NSs had no cytotoxic effect [39].

The Most Common General Applications of Nanosponge

Due to their sophisticated structure and biocompatibility, they can deliver hydrophobic and a hydrophilic drug, provide controlled release, enhance the satiability of drugs as well as increases the dissolution rate of drugs in aqueous environments. NSs can be prepared and used in the pharmaceutical field as capsules or tablets, parenterally, topically in hydrogel forms and as adjuvants as well as in applications such as delivery and targeting of drugs [41,52-54].

To summarize a few of the general drug delivery application areas of NSs,

Increasing solubility of drugs: Shende et al.[54] researched the effect of the low-soluble meloxicam drug on the stability, solubility, slow and sustained release of the inclusion complex formed with β-CD alone and NS.

For this goal, 1: 1 molar ratio of β-CD with meloxicam drug and 1: 8 molar ratio of NS obtained by using β-CD and pyromellitic dianhydride cross-linker (PMDA). Meloxicam, which has a solubility of 9.45 / g / ml in aqueous medium alone has increased to 19.07 g / ml after combining with β-CD, and this value has increased to 36.61 g / ml in integration with β-CD based NS. Therefore, they have successfully proved that NS increases the solubility of the drug.

Controlled and sustained drug release: Cavalli et al.[55] has obtained NS by using beta cyclodextrin and DFC crosslinker and integrates 3 different type of drugs with both hydrophilic (eg Doxorubicin) and lipophilic (eg flurbiprofen or dexamethasone) property to NS, and conducted a research to examine drug

solubility and its effect on drug release profile.

Effect of NS study has indicated that the solubility increases about 4% by weight for doxorubicin and 15% by weight for lipophilic drugs. Compare to release profile, doxorubicin is released very slowly in the physiological medium at pH 1.1, but it is faster when the pH is increased to 7.4. In addition, they demonstrated the interaction between the drug and nanosponge and sustaine drug release over time, emphasizing that drug release from NSs was less than 20% for dexamethasone and for flurbiprofen less than 10% after 2 hours.

Protecting drugs from light or distortion: S. Anandam et al.[56] conducted discrete irradiation tests for complex quercetin and pure quercetin at the equal concentration so the protective effect of NS on photodegradation of quercetin investigated. As a result, they have proved that pure quercetin disorder is much faster than NS encapsulated quercetin. In addition to this study, they proved that concentration of pure quercetin decreased by more than 50% in simulated intestinal fluid (SIF) within 6 hours, but NS encapsulated formulation did not show significant reduction, therefore NS proved that it effectively protects drugs from photodegradation. In addition, they emphasized that the antioxidant activity of quercetin and the radical scavenging activity of DPPH increased 569 times with the NS complex. [56].

Protein and enzyme transport: Bovine has been used as a model protein and has been shown to increase protein stability by incorporating serum albumin into poly amidoamine cross-linked NS [57].

As oral and topical drug delivery systems: Gangadharappa, H. V. et al.[58] conducted a research on pharmacokinetics and skin irritation on mice for the topical application of Nanasponges by integrating the water-soluble lipophilic celecoxib drug with NS formed in hydrogel. They emphasized that the optimized formulation NS-4 does not irritate the skin on mice, nanosponge hydrogels obtained can be used as topical drug release for celecoxib, as well as meeting the requirements in human use in this dosage form [58].

As a gas delivery agent: Nanosponge formulations have demonstrated the ability to function and store as a reservoir for large quantities of various gas types such as carbon dioxide and oxygen [59]. In addition, NSs are used as cosmetics [60], agricultural [61] and water treatment [62].

Recently, researchers have been focusing on designing functionalized NS by surface modification of NSs and enhancing intracellular cell interaction by using ligands. In a study conducted for this purpose, NS was synthesized with β-CD and diphenyl carbonate (DPC) crosslinker and functionalized the surface of NS with hydrogen succinate (CHS) to investigate cellular interaction and

protein binding by adding cholesterol [63]. In the study used doxorubicin DOX as a model drug, the cellular binding activity revealed an increase in cellular uptake and Dox adsorption of Dox-loaded β-CD-NS using the surface functionalization approach. They emphasized that they show preferable internalization in cells due to the interaction among cell membrane and CHS. In the same study, in vitro cytotoxicity of surface-modified β-CD-NS was investigated in the HeLa cell line and the data obtained showed that surface-modified β-CD-NS samples were biologically friendly and did not show significant toxicity in HeLa cells [63].

Drug targeting provides significant advantages in terms of drug delivery and drug release compared to traditional treatment methods, as well as significant potential advantages such as reducing the side effects of drugs, increasing their bioavailability and biocompatibility (thus encapsulating lipophilic and / or hydrophilic materials and keeping them in the blood for a long time and in a controlled manner). In addition, it is a promising nanocarrier that can increase the bioavailability and aqueous solubility by protecting the drugs from degradation in the physiological environment, which may result in increased specificity with yield when used with ligands for targeting.

There are many more things to discover, research and develop for NS. For example, obtaining functionalized NS, integrating ligands into NS, delivering the nanostructure to the targeted region through active or passive mechanisms and binding to the cell receptors. In addition, many studies are needed, such as intracellular uptake and drug release using internal and external stimulants, to integrate NS with metallic or inorganic nanostructures to increase drug loading capacity. However, functionalized NS surface engineering studies are limited to several publications.

In addition to increasing the efficiency and specificity of the targeted regions and reducing the toxicity of the drug, many researches are needed to increase the effect level of the drug by taking the drug release slowly and gradually.

However, mathematical modeling of NS integration into the cell and conducting positively charged NS studies to overcome the problems encountered for gene or nucleic acid delivery may be considered as future approaches.

CONFLICT OF INTEREST

The authors have not declared any personal or financial conflicts of interest with any other parties or organizations.

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REFERENCES

1. Health topics https://www.who.int/health-topics/ (accessed Mar 20, 2020).

2. Siegel, R.; Ma, J.; Zou, Z.; Jemal, A. Cancer statistics. CA: *A Can. J. For Clinic.* **2014**, 64, 9–29.

3. Valente, F.; Astolfi, L.; Simoni, E.; Danti, S.; Franceschini, V.; Chicca, M.; Martini, A. *J. Drug Del. Sci. and Tech.* **2017**, 39, 28.

4. Torchilin, V. P. Drug targeting. *Eu. J. Pharmaceut. Sci.* **2000**, *11*.

5. Jemal, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA: A Can. J. For Clinic.* **2011**, *61*, 69–90.

6. Chen, Z. (G. Small-molecule delivery by nanoparticles for anticancer therapy. *Trends in Mole. Med.* **2010**, *16*, 594–602.

7. Jin, S.-E.; Jin, H.-E.; Hong, S.-S. Targeted Delivery System of Nanobiomaterials in Anticancer Therapy: From Cells to Clinics. *BioMed Res. Int.* **2014**, *2014*, 1–23.

8. Amoabediny, G.; Haghiralsadat, F.; Naderinezhad, S.; Helder, M. N.; Kharanaghi, E. A.; Arough, J. M.; Zandieh-Doulabi, B. *Int. J. Poly. Mater. and Poly. Biomater.* **2017**, 67 (6), 383.

9. Wang, H.; Huang, Q.; Chang, H.; Xiao, J.; Cheng, Y. Biomater. Sci. **2016**, 4 (3), 375.

10. Cai, X.; Zhu, H.; Zhang, Y.; Gu, Z. ACS App. *Mater. & Interfaces* **2017**, 9 (11), 9402.

11. Yingchoncharoen, P.; Kalinowski, D. S.; Richardson, D. R. *Pharmaco. Rev.* **2016**, 68 (3), 701.

12. Patel, V.; Rajani, C.; Paul, D.; Borisa, P.; Rajpoot, K.; Youngren-Ortiz, S. R.; Tekade, R. K. *Drug Del. Sys.***2020**, 333.

13. Colombo, P. Opinion Paper: Modular Drug Delivery Systems for Personalized Oral Dosage Forms. *R. Pat. on Drug Del. & Form.* **2016**, *10*, 3–3.

14. Maeda, H.; Nakamura, H.; Fang, J. The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo. *Ad. Drug Del. Rev.* **2013**, *65*, 71–79.

15. Decuzzi, P.; Godin, B.; Tanaka, T.; Lee, S.-Y.; Chiappini, C.; Liu, X.; Ferrari, M. Size and shape effects in the biodistribution of intravascularly injected particles. *J. Cont. Re.* **2010**, *141*, 320–327.

16. Jeong, S.-Y.; Woo; Chung; Jung; Ju; Kang; Lee; Seo; Lee; Lee; et al. Preclinical evaluation of injectable sirolimus formulated with polymeric nanoparticle for cancer therapy. *Int. J. Nanomed.* **2012**, 2197.

17. Bidram, E.; Esmaeili, Y.; Ranji-Burachaloo, H.; Al-Zaubai, N.; Zarrabi, A.; Stewart, A.; Dunstan, D. E. *J. Drug Del. Sci. and Tech.* **2019**, 54, 101350.

18. Chertok, B.; Langer, R. *Theranostics* **2018**, 8 (2), 341.

19. Chen, Y.-C.; Liao, L.-C.; Lu, P.-L.; Lo, C.-L.; Tsai, H.-C.; Huang, C.-Y.; Wei, K.-C.; Yen, T.-C.; Hsiue, G.-H. The accumulation of dual pH and temperature responsive micelles in tumors. *Biomaterials* **2012**, *33*, 4576–4588.

20. Lee,Y.;Miyata, K.; Oba, M.; Ishii, T.; Fukushima, S.; Han, M.; Koyama, H.; Nishiyama,N.; Kataoka,K. Charge-Conversion Ternary Polyplex with EndosomeDisruption Moiety:ATechnique for Efficient and Safe Gene Delivery. *Angewandte Chemie Int. Ed.* **2008**, 47, 5163–5166.

21. Zhang, J.; Feng, K.; Cuddihy, M.; Kotov, N. A.; Ma, P. X. Spontaneous formation of temperature-responsive assemblies by molecular recognition of a β-cyclodextrin-containing block copolymer and poly(N-isopropylacrylamide). *Soft Matter* **2010**, *6*, 610–617.

22. Ge, J.; Neofytou, E.; Cahill, T. J.; Beygui, R. E.; Zare, R. N. Drug Release from Electric-Field-Responsive Nanoparticles. *ACS Nano* **2011**, *6*, 227–233.

23. Namiki, Y.; Namiki, T.; Yoshida, H.; Ishii, Y.; Tsubota, A.; Koido, S.; Nariai, K.; Mitsunaga, M.; Yanagisawa, S.; Kashiwagi, H.; et al. A novel magnetic crystal–lipid nanostructure for magnetically guided in vivo gene delivery. *Nature Nanotech.* **2009**, *4*, 598–606.

24. Chen, J.; Guo, Z.; Wang, H.-B.; Gong, M.; Kong, X.-K.; Xia, P.; Chen, Q.-W. Multifunctional Fe3O4@C@Ag hybrid nanoparticles as dual modal imaging probes and near-infrared light-responsive drug delivery platform. *Biomaterials* **2013**, *34*, 571–581.

25. Chakravarty, P.; Qian, W.; El-Sayed, M. A.; Prausnitz, M. R. Delivery of molecules into cells using carbon nanoparticles activated by femtosecond laser pulses. *Nature Nanotech.* **2010**, *5*, 607–611.

26. Stanley, S. A.; Gagner, J. E.; Damanpour, S.; Yoshida, M.; Dordick, J. S.; Friedman, J. M. Radio-Wave Heating of Iron Oxide Nanoparticles Can Regulate Plasma Glucose in Mice. *Science* **2012**, *336*, 604–608.

27. Korin, N.; Kanapathipillai, M.; Matthews, B. D.; Crescente, M.; Brill, A.; Mammoto, T.; Ghosh, K.; Jurek, S.; Bencherif, S. A.; Bhatta, D.; et al. Shear-Activated Nanotherapeutics for Drug Targeting to Obstructed Blood Vessels. *Science* **2012**, *337*, 738–742.

28. Takae, S.; Miyata, K.; Oba, M.; Ishii, T.; Nishiyama, N.; Itaka, K.; Yamasaki, Y.; Koyama, H.; Kataoka, K. PEG-Detachable Polyplex Micelles Based on Disulfide-Linked Block Catiomers as Bioresponsive Nonviral Gene Vectors. *J. Am. Chem.Soc.* **2008**, *130*, 6001–6009.

29. Broaders, K. E.; Grandhe, S.; Fréchet, J. M. J. A Biocompatible Oxidation-Triggered Carrier Polymer with Potential in Therapeutics. *J. Am. Chem.Soc.* **2011**, *133*, 756–758.

30. Markowicz-Piasecka, M.; Mikiciuk-Olasik, E. *Nanobiomater. in Drug Del.* **2016**, 39.

31. Eskiler, G. G.; Cecener, G.; Dikmen, G.; Egeli, U.; Tunca, B. *Eu. J. Pharmaceut. Sci.* **2018**, 120, 73.

32. Daima, H. K.; PN, N.; Ranjan, S.; Dasgupta, N.; Lichtfouse, E. Nanoscience in medicine; Springer: Cham, Switzerland, **2020**.

33. Jansook, P.; Ogawa, N.; Loftsson, T. Cyclodextrins: structure, physicochemical properties and pharmaceutical applications. *Int. J. Pharmaceut.* **2018**, *535*, 272–284.

34. Davis, M. E.; Brewster, M. E. Cyclodextrin-based pharmaceutics: past, present and future. *Nature Rev. Drug Del.* **2004**, *3*, 1023–1035.

35. Loftsson, T.; Jarho, P.; Másson, M.; Järvinen, T. Cyclodextrins in drug delivery. *Ex. Op.on Drug Del.* **2005**, *2*, 335–351.

36. Gray, J. E.; Weaver, R. N.; Purmalis, A. Ultrastructural Observations of Chronic Progressive Nephrosis in the Sprague-Dawley Rat. *Vet. Pathology* **1974**, *11*, 153–164.

37. Li, D.; Ma, M. Cyclodextrin polymer separation materials, **1998**, *WO 9822197.*

38. Li, D.; Ma, M. Nanosponges For Water Purification. *Clean Prod. and Process.* **2000**, 2, 0112-0116.

39. Trotta, F.; Zanetti, M.; Cavalli, R. Cyclodextrin-based nanosponges as drug carriers. *Beilstein J. Org. Chem.* **2012**, *8*, 2091–2099.

40. Trotta, F. Cyclodextrin Nanosponges and their Applications. *Cyclodextrins in Pharma., Cos., and Biomed.* **2011**, 323–342.

41. Swaminathan, S.; Cavalli, R.; Trotta, F. Cyclodextrin-based nanosponges: a versatile platform for cancer nanotherapeutics development. *Wiley Interdisciplinary Reviews: Nano. and Nanobiotech.* **2016**, *8*, 579–601.

42. Inamuddin; Asiri, A. M.; Mohammad, A. Applications of nanocomposite materials in drug delivery; *Woodhead Pub., an imprint of Elsevier: Oxford*, **2018**.

43. Osmani, R. A. M.; Bhosale, R. R.; Hani, U.; Vaghela, R.; Kulkarni, P. K. Cyclodextrin Based Nanosponges: Impending Carters in Drug Delivery and Nanotherapeutics. *C. Drug Therap.* **2015**, *10*, 3–19.

44. Singireddy, A.; Pedireddi, S. R.; Nimmagadda, S.; Subramanian, S. Beneficial effects of microwave assisted heating versus conventional heating in synthesis of cyclodextrin based nanosponges. *Mater. Today: Proceedings* **2016**, *3*, 3951–3959.

45. Anandam, S.; Selvamuthukumar, S. Optimization of microwave-assisted synthesis of cyclodextrin nanosponges using response surface methodology. *J. Porous Mater.* **2014**, *21*, 1015–1023.

46. Pushpalatha, R.; Selvamuthukumar, S.; Kilimozhi, D. Cross-Linked, Cyclodextrin-Based Nanosponges For Curcumin Delivery - Physicochemical Characterization, Drug Release, Stability And Cytotoxicity. *J. Drug Del. Sci. and Tech.* **2018**, 45, 45-53.

47. Sehgal, N.; N, V. G.; Kanna, S. A Review On Nanosponges A Review On Nanosponges: A Boon To Targeted Drug Delivery For Anticancer Drug. *Asian J. Pharmaceut. and Clinic. Re.* **2019**, 1–7.

48. Bhowmik, H.; Venkatesh, D. N.; Kuila, A.; Kumar, K. H. Nanosponges: A Review. *Int. J. Appl. Pharmaceut.* **2018**, *10*, 1.

49. Pandey, P.; Purohit, D.; Dureja, H. Nanosponges –A Promising Novel Drug Delivery System. *Rec. Pat. on Nanotech.* **2018**, *12*, 180–191.

50. Manakker, F. V. D.; Vermonden, T.; Nostrum, C. F. V.; Hennink, W. E. Cyclodextrin-Based Polymeric Materials: Synthesis, Properties, and Pharmaceutical/Biomedical Applications. *Biomacromole.* **2009**, *10*, 3157–3175.

51. Shende, P.; Kulkarni, Y. A.; Gaud, R.; Deshmukh, K.; Cavalli, R.; Trotta, F.; Caldera, F. Acute and Repeated Dose Toxicity Studies of Different β-Cyclodextrin-Based Nanosponge Formulations. *J. Pharmaceut. Sci.* **2015**, *104*, 1856–1863.

52. Minelli, R.; Cavalli, R.; Ellis, L.; Pettazzoni, P.; Trotta, F.; Ciamporcero, E.; Barrera, G.; Fantozzi, R.; Dianzani, C.; Pili, R. Nanosponge-encapsulated camptothecin exerts anti-tumor activity in human prostate cancer cells. *Eu. J. Pharmaceut. Sci.* **2012**, *47*, 686–694.

53. Daga, M.; Ullio, C.; Argenziano, M.; Dianzani, C.; Cavalli, R.; Trotta, F.; Ferretti, C.; Zara, G. P.; Gigliotti, C. L.; Ciamporcero, E. S.; et al. GSH-targeted nanosponges increase doxorubicin-induced toxicity "in vitro" and "in vivo" in cancer cells with high antioxidant defenses. *Free. Rad. Bio. and Med.* **2016**, *97*, 24–37.

54. Shende, P. K.; Gaud, R.; Bakal, R.; Patil, D. Effect of inclusion complexation of meloxicam with β-cyclodextrin- and β-cyclodextrin-based nanosponges on solubility, in vitro release and stability studies. *Colloids and Surfaces B: Biointerfaces* **2015**, *136*, 105–110.

55. Cavalli, R.; Trotta, F.; Tumiatti, W. Cyclodextrin-based Nanosponges for Drug Delivery. *J. Inclu. Pheno. and Macrocyc. Chem.* **2006**, *56*, 209–213.

56. Anandam, S.; Selvamuthukumar, S. Fabrication of cyclodextrin nanosponges for quercetin delivery: physicochemical characterization, photostability, and antioxidant effects. *J. Mater. Sci.* **2014**, *49*, 8140–8153.

57. Swaminathan, S.; Cavalli, R.; Trotta, F.; Ferruti, P.; Ranucci, E.; Gerges, I.; Manfredi, A.; Marinotto, D.; Vavia, P. R. In vitro release modulation and conformational stabilization of a model protein using swellable polyamidoamine nanosponges of β-cyclodextrin. *J. Inc. Phenom. and Macrocyc. Chem.* **2010**, *68*, 183–191.

58. Gangadharappa, H.; Prasad, S. M. C.; Singh, R. P. Formulation, in vitro and in vivo evaluation of celecoxib nanosponge hydrogels for topical application. *J. Drug Del. Sci. and Tech.***2017**, *41*, 488–501.

59. Cavalli, R.; Akhter, A. K.; Bisazza, A.; Giustetto, P.; Trotta, F.; Vavia, P. Nanosponge formulations as oxygen delivery systems. *Int. J. Pharmaceut.* **2010**, *402*, 254–257.

60. Sapino, S.; Carlotti, M. E.; Cavalli, R.; Ugazio, E.; Berlier, G.; Gastaldi, L.; Morel, S. Photochemical and antioxidant properties of gamma-oryzanol in beta-cyclodextrin-based nanosponges. *J. Inc. Pheno. and Macrocyc. Chem.* **2012**, *75*, 69–76.

61. Lien, N.; Telford, J. An investigation of the inclusion complex of cyclomaltoheptaose (β-cyclodextrin) with N-methylanthranilic acid in the solid state. *Carbohyd. Re.* **2009**, *344*, 2606–2608.

62. Euvrard, É.; Morin-Crini, N.; Druart, C.; Bugnet, J.; Martel, B.; Cosentino, C.; Moutarlier, V.; Crini, G. Cross-linked cyclodextrin-based material for treatment of metals and organic substances present in industrial discharge waters. *Beilstein J. Org. Chem.* **2016**, *12*, 1826–1838.

63. Singh, P.; Ren, X.; Guo, T.; Wu, L.; Shakya, S.; He, Y.; Wang, C.; Maharjan, A.; Singh, V.; Zhang, J. Biofunctionalization of β-cyclodextrin nanosponges using cholesterol. *Carbohydr. Polym.* **2018**, *190*, 23–30.

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Formulation Design and Optimization of Sustained Released Matrix Tablets of Propranolol HCl Using Natural and Synthetic Polymers

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ABSTRACT

The proposed research work aimed to design, formulate and finally to evaluate sustained released matrix tablets of propranolol hydrochloride using the combination of hydrophilic and hydrophobic polymers. Formulation and optimization of Propranolol HCl was done by direct compression technique using 3^2 factorial design. The amount of polymer Mastic gum (X_1) and HPMC (X_2) were chosen as independent variables and their effect on amount of drug release at 2 hours (Y_1) , 4 hours (Y_2) and 8 hours (Y_3) at three levels low (-1), medium(0) and high(+1) was taken as dependent variable. Drug-excipient compatibility studies were performed by FTIR and DSC analysis. A total of 9 combinations of sustained released tablets were formulated and evaluated for both pre and post compression parameters. Design expert software version 10 was used to evaluate the effect of independent variable over dependent variable and to generate polynomial equation to represent experimental results. The B7 formulation containing 5 % of mastic gum and 25% of HPMC K-15 combination showed 60.13% drug release in 8 hours and was chosen as optimized formulation. Release kinetic mechanism indicated that the optimized formulation fitted well into Kosmeyer Peppas model (R^2= 0.9974). Stability studies indicated that selected formulation was stable for 90 days. Formulation containing 5% of mastic gum and 25% HPMC was found to be effective and can be explored further to develop sustained released formulations.

Keywords: Sustained release, mastic gum, factorial design, propranolol HCl.

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INTRODUCTION

Sustained drug delivery is specialized form of modified drug delivery that offer the advantage of reducing the dose frequency as compared to conventional dosage forms for those drug candidate that have rapid clearance rate due to short elimination half life. Oral sustained release formulations are capable of achieving a steady therapeutic drug blood level by continuously releasing the drug for long duration after a single dose administration thus offering better patient compliance and control over therapy.[1, 2] Depending upon release kinetics these systems are designated as continuous release, delayed transit and continuous release and delayed released systems. The mechanism involved in drug release may be dissolution, diffusion, and dissolution along with diffusion. Among all these systems, dissolution controlled system are the most acceptable one. The dissolution controlled release system is further classified as matrix dissolution control/ encapsulating/ or reservoir device type. Matrix systems are mostly preferred for sustained release dosage forms because they are simple to design, economical. Formulation of matrix systems involves the dispersion or dissolving the drug into polymeric matrix that retard the drug release and then blending with other additives to formulate a tablet dosage form.[3, 4] Releases retardant mainly used are hydrophilic and hydrophobic polymers. Different natural and synthetic polymers are matix forming agents in order to control the drug release. Mastic gum is a natural oleoresin exudate isolated from the stems and leaves of *Pistacia lentiscus*. It is known to posse's anti-oxidant, antimicrobial, anti-inflammatory and hepatoprotective activity. Recent studies demonstrated its use as tablet binder, microencapsulating agent and matrix former in the formulation of sustained released dosage forms. Propranolol hydrochloride is a non selective beta adrenergic blocking agent prescribed in high blood pressure, angina pectoris and many other cardiovascular disorders.[5] The oral bioavailability of Propranolol hydrochloride is low. It is a highly water soluble drug with relatively short biological half life of 3-6 hours and a dose of 40 mg thrice daily. This high dosing frequency results in fluctuation of plasma drug level; therefore it is needed to have in sustained release dosage form to reduce dose frequency and improve patient compliance. The purpose of the present study is to formulate and optimize sustained released matrix tablets of Propranolol HCl using hydrophilic polymer HPMC K-15 and hydrophobic polymer mastic gum as a material for matrix formation with improved patient compliance.[6]

METHODOLOGY

Propranolol HCl and mastic gum were procured from Yarrow chem, Mumbai, India. HPMC K-15 was taken from Loba chemie Pvt. Ltd, Mumbai, India. All other chemical used were of laboratory and analytical grade

Standard calibration of Propranolol hydrochloride

A standard calibration curve of Propranolol HCl was plotted in Phosphate buffer pH 6.8 and 0.1N HCl having pH 1.2. A stock solution of drug was prepared by dissolving 100 mg of drug in phosphate buffer pH 6.8 and 0.1N HCl. The volume was made up to 100ml to prepare stock solution (A) to get a concentration of 1000 µg/ml.10 ml of stock solution (A) was further diluted to 100 ml to obtain stock solution (B) with concentration 10 µg /ml. Aliquots of stock solution (B) was diluted to obtain working solutions of concentration 2 to 20 µg/ml.[7] The absorbance of the final solutions were taken at 290nm using double beam UV spectrophotometer, Systronics (AU – 2701).

Compatibility studies of drug and excipients

Compatibility studies were carried by FTIR and DSC analysis. FTIR spectrum of pure drug Propranolol HCl, Polymer (mastic gum) and mixture of drug with polymer (1:1) was taken using Bruker Alpha T instrument by KBr pellet method. The spectra were scanned over a frequency range 4000-400 cm^{-1}. The possibility of drug-excipient interaction was also investigated by DSC. The samples set in a DSC instrument Mettler Toledo (model number: Star 1). The DSC thermograms of pure drug, a mixture of drug with mastic gum, HPMC, Magnesium stearate, aerosil, microcrystalline cellulose were taken. The thermal analysis was performed at a heating rate of 10.00° C/min over a temperature range 98-80°C.

Design of Experiment

A 3^2 full factorial design given in **Table 1** was used to assess the combined effect of independent variables on the dependent variable. Two factors were assessed at three levels, high, medium and low. The experimental trials were taken on all 9 possible combinations. Statistical model including mathematical polynomial equation was generated to study the response.[8]

Table 1. Design format of 3^2 factorial designs

Formulation batches	Variable X_1	Variable X_2
B1	-1	-1
B2	0	-1
B3	+1	-1
B4	-1	0
B5	0	0
B6	+1	0
B7	-1	+1
B8	0	+1
B9	+1	+1

Where +1 is higher level,-1is lower level and 0 is mid level for the independent variables.

Polynomial equation generated by this design is as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1X_1 + b_{22}X_2X_2 + b_{12}X_1X_2 \qquad \text{Equation: 1}$$

Where Y is the response variable b_0 is the arithmetic mean response of the 9 trials, and b_1 and b_2 are the regression coefficients. The main effects (X_1 and X_2) are represents the average results of changing 1 factor at time from its low to high value. The interaction terms (X_1 X_2) show how the response varies when two factors are simultaneously varied. The polynomial terms (X_1 X_1 and X_2X_2) are included to investigate nonlinearity. The level of independent variables and their coding is given in **Table 2**.

Table 2. Levels for independent variables and coding of variable

Levels	Coded value	Concentration of HPMC (%)X_1	Concentration of Mastic gum (%)X_2
Low	-1	5	5
Medium	0	15	15
High	+1	25	25

Preparation of sustained release tablets

The propanolol HCl matrix tablets having a net weight of 200 mg were compressed by direct compression method. All the excipients as per the composition were previously passed through sieve no.60 to get uniform particle size were weighed accurately and mixed thoroughly for 15 min. After mixing powder blend was transferred to double punch tablet punching machine (A.K industries) for compression. The detailed composition of the prepared tablets using 3^2factorial design is given in **Table 3**.

Table 3. Composition of factorial design batches

Ingredients (mg)	B1	B2	B3	B4	B5	B6	B7	B8	B9
Propanolol HCl	40	40	40	40	40	40	40	40	40
HPMC K 15	10	10	10	30	30	30	50	50	50
Magnesium stearate	2	2	2	2	2	2	2	2	2
Mastic gum	10	30	50	10	30	50	10	30	50
Microcrystalline cellulose	136	116	96	76	96	76	96	76	56
Aerosil	2	2	2	2	2	2	2	2	2

Net weight of each tablet = 200 mg

Evaluation of sustained released matrix tablets

Pre compression parameters

The micromeritics of all the compositions (B1to B9) were evaluated by calculating their bulk density, tapped density, angle of repose, carr's index and hausner's ratio.[9]

Post compression parameters:

Weight variation

To ensure uniformity in tablets weight, twenty tablets were selected at random from each batch and average weight was determined. Then the individual tablet weight was compared with the average weight. The percentage deviation was calculated and checked for weight variation as per the official specifications.[10]

% deviation= Average weight - Individual weight /Average weight X 100

Thickness

The thickness of tablets was measured by Vernier calipers to ensure uniformity in thickness. This is done by taking three tablets at random from each batch. It is expressed in mm.

Hardness

To find tablet hardness Monsanto hardness tester was used. Three tablets from each batch were taken and the average value with standard deviation was taken as tablet hardness. It is expressed in kg/cm^2.

Percent Friability

Tablets friability was assessed using Roche friabilator. Ten tablets from each formulation batch were initially weighed and placed in Roche friabilator rotated at 25 rpm for 4 minutes with 100 revolutions.[11] Then final weight was taken. The percentage friable loss was calculated using the formula:

% Friability = initial weight –final weight / initial weight X 100

Content uniformity

Uniformity in drug distribution was carried out by triturating ten tablets to fine powder. Powder equivalent to the 40 mg of drug was weighed and dissolved in 100 ml of phosphate buffer pH 6.8 and after suitable dilution absorbance was measured using UV-visible spectrophotometer at λ_{max} 290nm.[12]

Swelling index

Swelling index of formulated batches was estimated by placing the initially weighed tablet into a petriplate containing 5ml of phosphate buffer pH 6.8. After a regular interval of time the tablets were removed and swollen tablets were weighed. This was done for the period of 6hours.[13]

In vitro dissolution studies

The *in vitro* drug release studies were done using basket type dissolution test apparatus in 900 ml of 0.1 N HCl pH 1.2 as dissolution medium for 2 hours followed by phosphate buffer pH 6.8. The basket was adjusted at 50rpm and the temperature of 37±1°C was maintained throughout the experiment. A sample of 5ml was withdrawn at different time intervals for 8 hours. Each sample was filtered using membrane filter with a pore size of 0.45 mm and was analyzed after appropriate dilution by UV spectrophotometer at λ_{max} 290nm.[14]

Drug release kinetic study

To assess release kinetics, various mathematical equations have been used, namely zero order, first order, Higuchi model and Kosmeyer Peppas equation.[15] The most suitable model was selected on the basis of the value of regression coefficient near to unity.

Selection of optimized formulations

For the selection of optimized formulation, a simple exhaustive grid search was done. The regression equations were calculated for different combinations of independent variables and the response values were compared for the selection of optimized formulation. The areas that give the optimum value for each studied response was found using overlay plot.

Comparison of optimized formulation with commercial brand

The optimized formulation selected by design expert software was then compared with one of commercial brand of propranolol HCl tablet, Inderal 40 (Abott India Ltd) for its *in vitro* drug release.

Stability studies

The optimized formulation was subjected to stability studies after packing in aluminum pack as per ICH guidelines for 90 days at 40°C ± 2°/ 75% RH ± using stability test chamber (Remi elektrotechnik Ltd; Vasai, India). Test sampling was done at different time points for analysis.[16]

RESULTS and DISCUSSION

Preformulation studies

Standard calibration curve of propranolol Hydrochloride

Standard calibration curve of drug plotted in 0.1N HCl and pH 6.8 phosphate buffers is shown in **Figure 1 and 2,** the R^2 value was found to be 0.9995 and 0.9857 respectively which indicated the linearity of the graph.

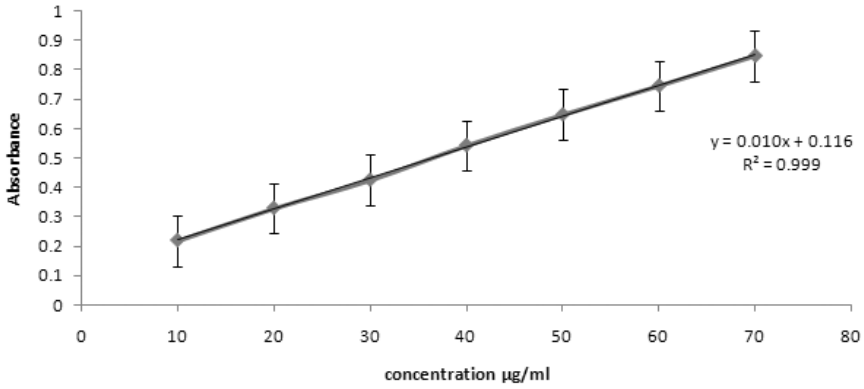

Figure 1. Standard plot of drug in 0.1N HCL

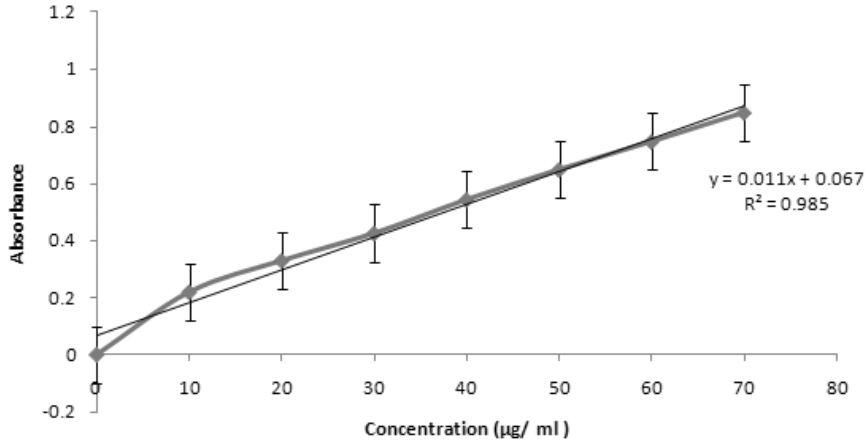

Figure 2. Standard plot of drug in Phosphate buffer pH 6.8

Compatibility studies of drug and excipients

FTIR Analysis

FTIR spectra of propanolol hydrochloride showed a characteristics peak of OH stretch at 3435.84 cm^{-1}. -NH stretch at 3330.11 cm^{-1}, -CH stretch at 2928.33. A Peak of acryl C=C symmetric aromatic ring stretching at 1632.65 cm- ¹and aryl coupling C-O-Stretching at 1268.17 cm^{-1} which peak was obtained from 1500 cm^{-1}. An aryl O-CH_2 asymmetric stretching at 1240.96 and symmetric stretching at 1074.95 cm^{-1}. A peak at 796.90 $cm^{-1}$is due to naphthalene ring. Spectra of mastic gum showed a characteristics peak of OH group at 3440.25 cm^{-1}. FTIR spectra of mastic gum and drug showed a characteristics peak of -NH stretching at 3330.34, -CH aromatic at 2925.40, C=C aryl group attached at 1691.09 and 1637.70, –CH_3 bending at 1456.71and 1401.01, C-O-C Stretching at 1267.46. All characteristic peaks of drug are shown in FTIR spectra of drug and mastic gum which indicates the compatibility of drug and mastic gum. FTIR spectra of Propranolol hydrochloride, Mastic gum and physical mixture of mastic gum HPMC K-15 and drug are given in **Figure 3, 4 and 5** respectively.

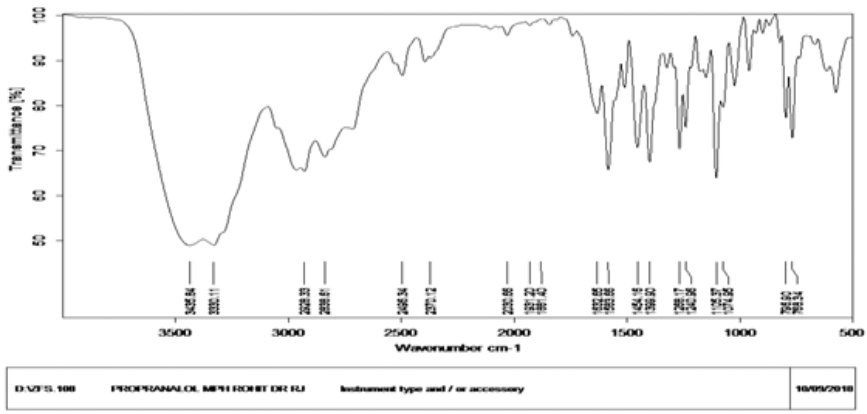

Figure 3. FTIR of drug Propanolol HCl

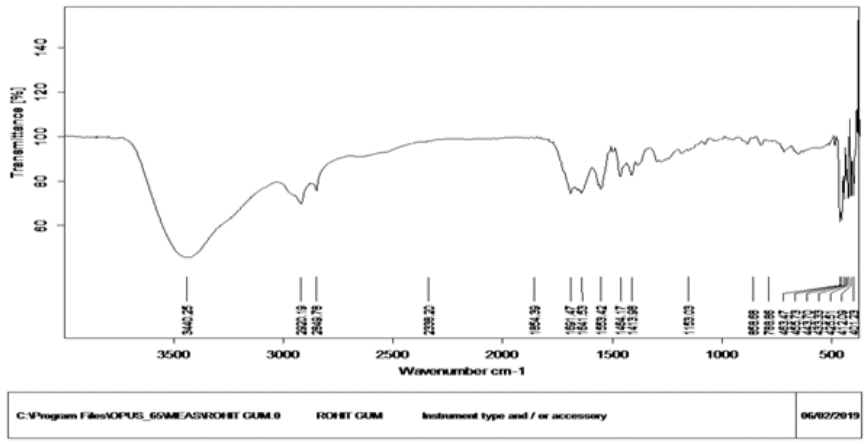

Figure 4. FTIR of Mastic gum

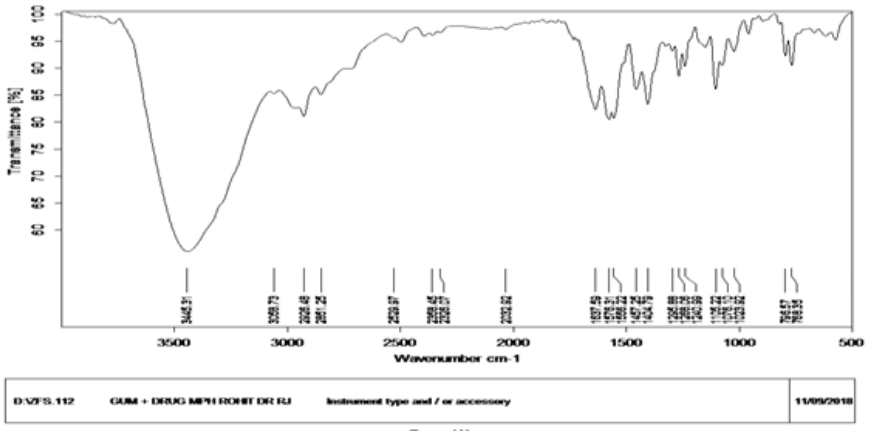

Figure 5. FTIR of HPMC-K 15

DSC Analysis

The possibility of drug excipient interaction was further investigated by DSC. DSC curve of pure drug as shown in **Figure 6** give endothermic peak at 168.74°C. DSC curve of mastic gum as shown **Figure 7** give endothermic peak at 98.80°C. DSC curve of drug and mastic gum as depicted in **Figure 8** give endothermic peak at 155.43°C. From the DSC results it was concluded that there is no incompatibility between the drug and excipient selected.

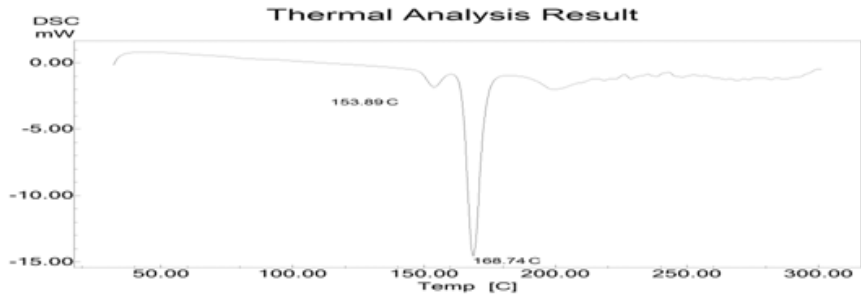

Figure 6. DSC analysis of Propanolol Hydrochloride

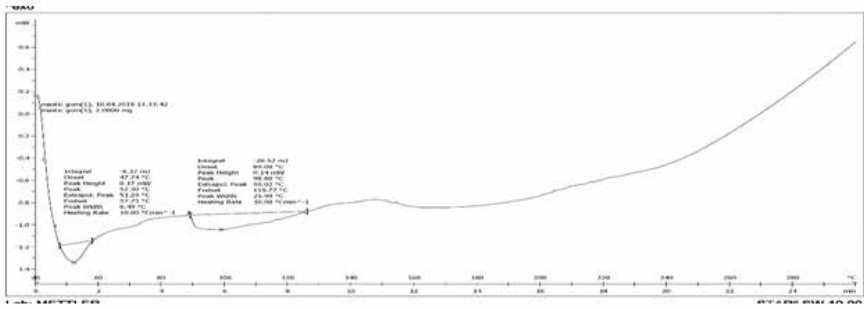

Figure 7. DSC analysis of Mastic Gum

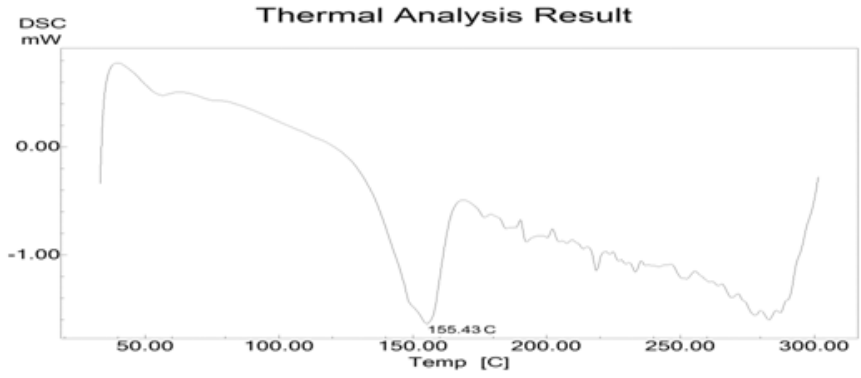

Figure 8. DSC analysis of HPMC-K15

Evaluation of 3^2 full factorial design batches B1 to B9:

Pre compression Evaluations

Bulk density was found in range of 0.24 to 0.645 gm/cm^3, tapped density ranged from 0.535 to 0.6, Angle of repose 14.0 to 26.26, Carr's index range from 22.3 to 4.2, Hausner's ratio ranged from 1.04to 1.181. Results of evaluation of precompression parameters as shown in **Table 4** indicated good micromeritic properties of all formulation batches.

Table 4. Characterization of pre compression parameters of design batches B1 to B9

Parameters	B1	B2	B3	B4	B5	B6	B7	B8	B9
Bulk density (g/cm^3)	0.475± 0.01	0.5128±0.03	0.4736±0.2	0.24±0.01	0.52±0.1	0.66±0.05	0.62±0.1	0.645±0.3	0.495±0.1
Tapped density (g/ml)	0.61±0.02	0.60±0.03	0.52±0.2	0.27±0.01	0.58±0.4	0.68±.01	0.6±0.01	0.71±0.03	0.535±0.01
Angle of repose	14.0±0.03	16.69±0.12	16.17±0.04	22.7±0.05	17.7±0.12	18.2±0.11	26.26±0.1	23.26±0.2	25.17±0.21
Carr's index (%)	22.3±0.1	15.37±0.5	10.47±0.11	11.1±0.12	11.5±0.1	4.2±0.3	5.30±0.1	5.90±0.01	7.47±0.13
Hausenr's ratio	1.28±0.03	1.181±0.11	1.116±0.01	1.121±0.02	1.13±0.02	1.04±0.03	1.05±0.01	1.10±0.02	1.080±0.21

Values are expressed in mean ±SD, n=3

Post compression Evaluations

The Weight of the formulated batches passed the weight variation test as the % weight variation was within the Pharmacopoeial limits of ±7.5 % of the weight. The thickness of all tablets was in the range between 3.23 to 3.98 mm which indicates uniformity in size and shape of the tablets. The hardness of tablets was in the range between 4.66 to 7.5 kg/ cm^2 which indicated the good mechanical strength of the prepared formulations that could maintain physical integrity

during the normal course of handling, also increase in content of mastic gum increases hardness due to the strong binding character of gum mastic.[17] Friability was in the range between 0.5% to 0.94%. Friability values were in agreement with official limit of less than 1% in all cases which indicated good mechanical strength required for handling and transportation. Content of drug distributed in all tablets was found in the range between 95.3 to 99.68% this ensured the uniformity and homogeneity of the drug distribution in the tablets. Results of post compression parameters are given in **Table 5.**

Table 5. Characterization of Post compression parameters of batches B1 to B9

Parameters	B1	B2	B3	B4	B5	B6	B7	B8	B9
Weight variation	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Thickness (mm)	3.79±0.095	3.98±0.27	3.8±0.15	3.4±0.047	3.93±0.31	3.23±0.02	3.7±0.021	3.92±0.03	3.82±0.02
Hardness (kg/cm^2)	5.0±0.22	6.16±0.015	7.5±0.23	5.6±0.25	6.33±0.058	7±0.026	4.66±0.036	5.27±0.07	7±0.06
(%) Friability	0.5%±0.03	0.94%±0.08	0.5%±0.45	0.09%±0.05	0.6%±0.045	0.53%±0.015	0.21%±0.33	0.28%±0.06	0.82%±0.02
(%)Drug content	95.3±0.25	98.2±0.05	98±2.23	99.4±0.36	99.68±0.21	97.6±0.11	99.1±0.71	99.51±1.18	99.1±0.01
Swelling index (%)	135.29±0.027	22.2±0.03	31.25±0.05	66.6±0.023	35.29±0.06	26.31±0.12	56.25±0.23	16.66±0.25	25±0.045

All values are expressed as mean ±SD, n=3

In vitro drug release studies

From *in vitro* drug release it was observed that as the concentration of the mastic gum increases, the amount of drug release decreases. This may be explained due hydrophobic nature of gum which delay the hydration and swelling of polymer matrix to release drug. Decreasing content of mastic gum and increasing amount of HPMC stimulates the drug release, due to the hydrophilic nature of HPMC which dissolve the coating of mastic gum around the drug particles and increase release in early stage. From the results of *in vitro* drug study it was found that B7 tablet batch containing 5% (10mg) of mastic gum and 25% (50mg) of HPMC-K15 could sustain the drug release for 8 hours and was selected as optimized formulation, showing 19.43 at 2hours, 31.55 at 4 hours and 60.13 % drug release at 8 hours which was found to uniform and consistent, this may be attributed due to more free drug: polymer ratio and constant release of the drug embedded in the mastic gum and HPMC-K15 matrix which has a hydrophilic gel forming nature. On coming in contact with liquid medium

this polymer hydrate and swell, forming a hydro gel layer which regulate the further penetration of the liquid into the tablet matrix and control the dissolution and diffusion of drug out of the polymer matrix and there by sustain the drug release. [18]As the concentration of mastic gum increases the hydrophobic nature of the gum retarded the drug release which was due to the gummy nature and therefore poor solvent penetration into the tablet matrix that reduces the wetting and drug dissolution. Results of in vitro drug release study are shown in **Figure 9 (a), (b) and (c)**

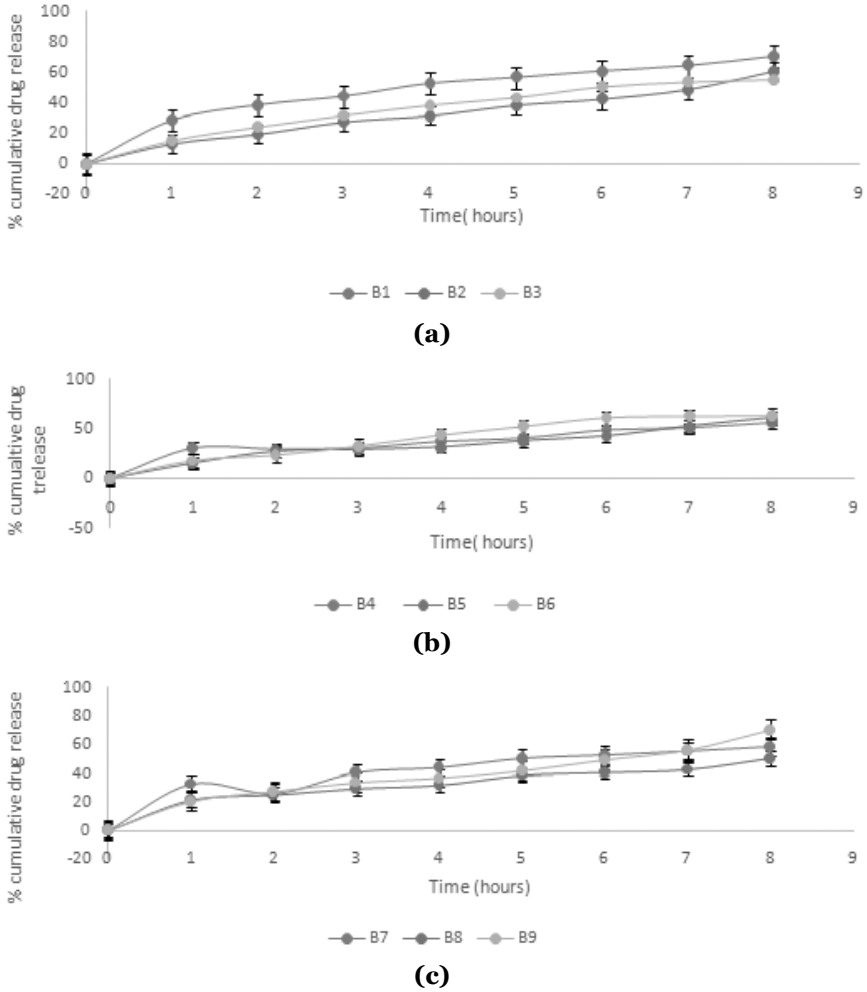

Figure 9. *In vitro* drug release of formulations. (a), B1, B2 and B3; (b), B4, B5 and B6; and (c), B7, B8 and B9.

Regression Analysis

Mathematical relationship generated in the form of polynomial equations for the studied responses are expressed as follows:

$Y1 = +26.75444 - 0.58667X_1 - 3.22500X_2 + 3.89250X_1X_2$

$Y2 = +30.75889 - 1.62833X_1 - 2.88500X_2 + 1.66500X_1X_2 + 1.42167X^2_1 \cdot 58167X^2_2$

$Y3 = +60.28444 - 0.9600X_1 + 0.61833X_2 + 6.85500X_1X_2$

The above equation demonstrates the effect of various process variables over the studied responses. The analysis of variance (ANOVA) was performed to estimate the level of significance of model at 5%. A model is said to be significant if the p value is less than 0.05. Summary of regression analysis for studied response is given in **Table 6**; all measured responses were found to be statistically significant as indicated by P value. F value measures the equality of two variances. The results are shown in **Table 7**.

Table 6. Summary of regression analysis of measured Responses

Coefficient	X_0	X_1	X_2	X_{12}	X_{11}	X_{22}	R^2
% CDR at 2 hours	+26.75444	0.58667	3.22500	+3.89250	-	-	0.998
% CDR at 4 hours	+30.75889	-1.62833	-2.885000	+1.66500	-	-	0.9782
% CDR at 8 hours	+60.28444	-0.96000	+0.61833	+6.85500	-	-	0.9360

Table 7. Results of analysis of variance of all three responses

Source	%CDR at 2 hours		%CDR at 4 hours		%CDR at 8 hours	
Model	F	P Value	F	P Value	F	P value
X_1	1.21	0.03627	0.60	0.057	5.76	0.061
X_2	3.04	0.07903	0.17	0.06936	0.70	0.05635
X_1X_2	0,82	0.0173	0.17	-0.1507	0.84	0.06103
X_1^2	0.71	-	3.80	0.01092	-	-
X_2^2	-	-	-	-	-	-

The relationship between dependent and independent variables was further elucidated using surface response, contour plots and diagnostic graph as given in **Figure. 10, 11and 12**. Correlation between predicted and actual values as indicated by diagnostic graph was found linear with R^2 value 0.998, 0.9782 and 0.9360 indicating the excellent fit.

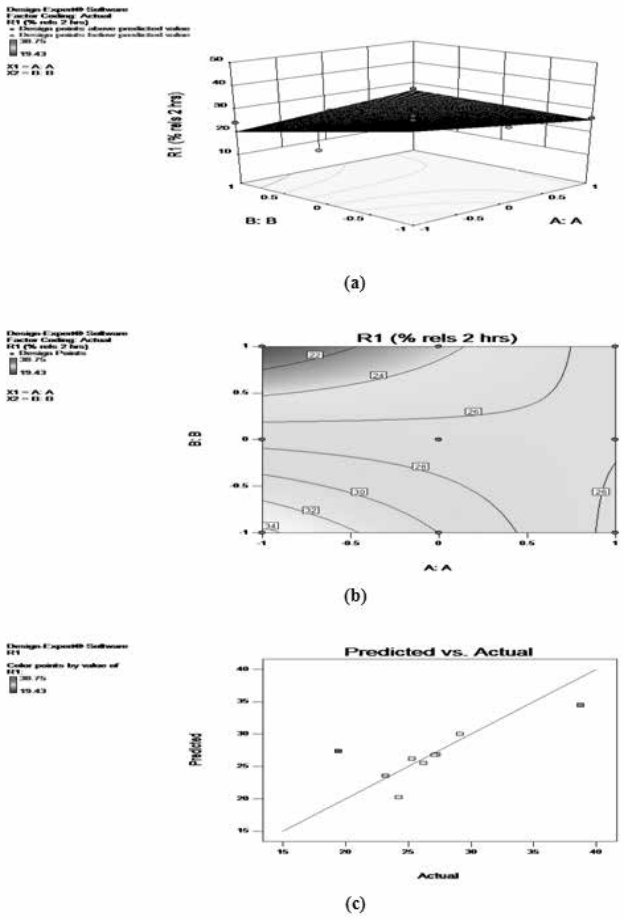

Figure 10. (a) Response surface plot for 2 hours drug release; (b) Contour plot for drug release at 2 hours and (c) Diagnostic plot for drug release at 2 hours.

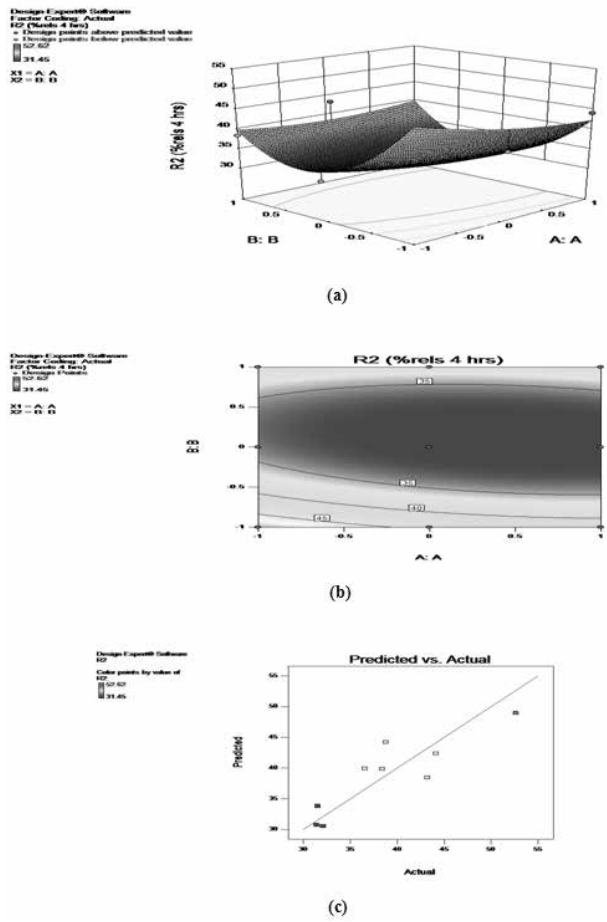

Figure 11. (a) Response surface plot for drug release at 4 hours; (b) Contour plot for drug release for 4 hours; and (c) Diagnostic plot for drug release at 4 hours.

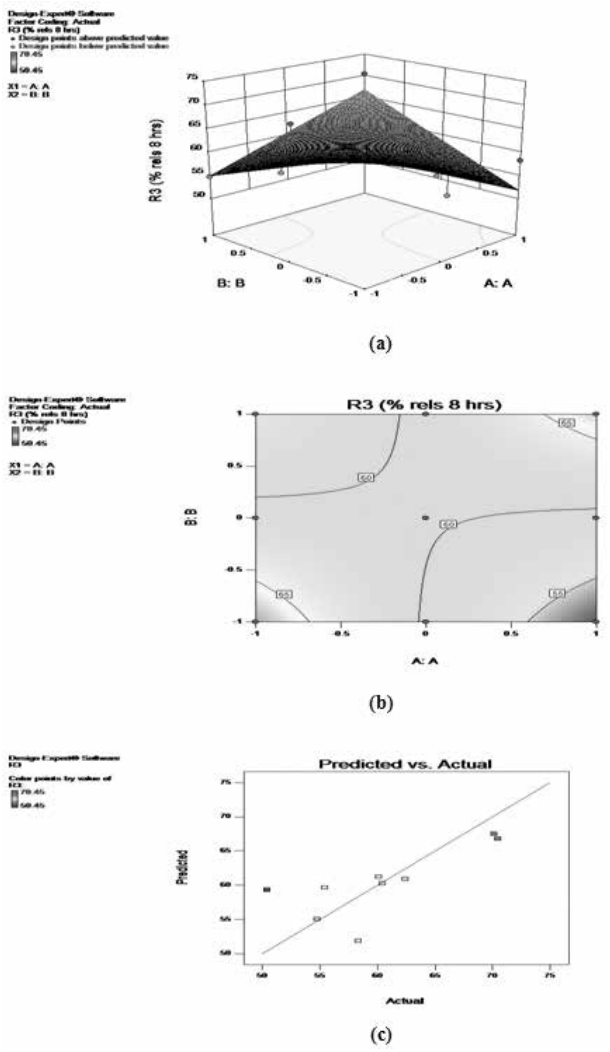

Figure 12. (a) Response surface plot for drug release at 8 hours; (b) Contour plot for drug release for 8 hours; and (c) Diagnostic plot for drug release for 8 hours

Formulation optimization

A numerical optimization using desirability approach was used to develop a new formulation with desired response. Upon evaluation a formulation having desirability closer to 1 was observed. The percentage prediction error between the predicted and experimental values for each response was calculated which was found to be within 5%. The formulation B7 was selected as optimized batch as error was minimum for studied responses.[19, 20] ,

Table 8. Predicted and Experimented values of three Responses with % error

Response	Predicted value	Experimental value	% Error
R1(2hours)	19.82	19.43	1.96
R2(4 hours)	32.05	31.45	1.87
R3(8hours)	62.12	60.43	2.27

Comparison between experimented (E) and predicted value (P) of B2 formulation are given in **Table 8**. Desirability and predicted values of the response R1, R2 and R3 are shown in **Figure 13**.

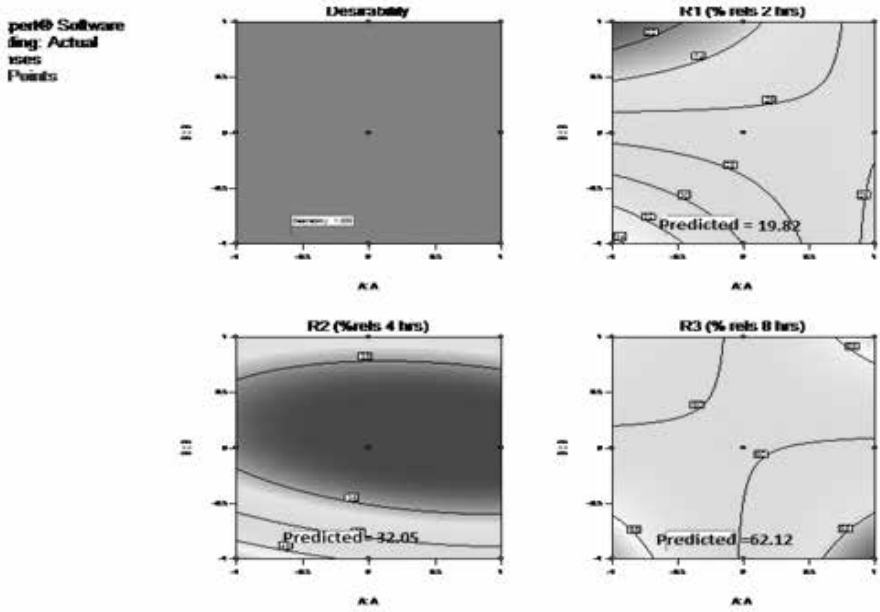

Figure 13. Desirability and Predicted values Responses R1, R2 and R3

Drug Release Kinetic Study

From the results of drug release kinetics it was found that the release mechanism follows swelling and diffusion best demonstrated by Korsmeyer Peppa model giving R^2 value of 0.9942. Results of drug release kinetic study are shown in **Table 9.**

Table 9. *In vitro* release kinetics study of sustained released matrix tablets (B1-B9)

Formulations	Zero order R^2	First order R^2	Higuchi R^2	Korsmeyer-Peppas R^2
B1	0.9747	0.9498	0.9927	0.9922
B2	0.9856	0.9928	0.9586	0.9942
B3	0.9666	0.9908	0.9891	0.9974
B4	0.9428	0.8735	0.9636	0.9358
B5	0.9616	0.9511	0.8917	0.8793
B6	0.9498	0.9867	0.9606	0.9877
B7	0.8957	0.8879	0.9384	0.9233
B8	0.9815	0.9053	0.9607	0.9768
B9	0.9683	0.9709	0.9175	0.9694

Comparison of marketed formulation of Inderal 40 with optimized B7formulation

In vitro drug release of optimized formulation was compared with marketed tablet of Propanolol HCl, Inderal 40 for 8 hours. Results of % cumulative drug release are shown in **Figure 14**. The *in vitro* drug release of B7 formulation showed more sustained release behavior as comparison to Inderal 40

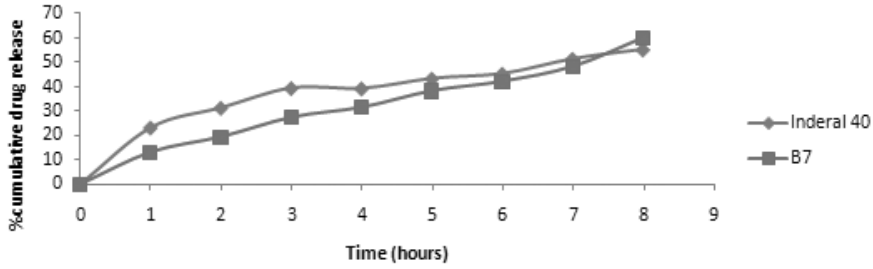

Figure 14. Comparison of marketed formulation with Optimized B7 formulation

Table 10. Stability study of optimized B7 formulation (40^{0C} with 75% RH)

Test Parameters	Time Points (days)					
	0	15	30	45	60	90
% CDR at 2 hours	19.43	18.70	19.40	19.23	19.42	19.41
% CDR at 4 hours	31.45	31.23	31.29	31.43	31.45	31.40
% CDR at 8 hours	60.43	60.45	60.43	61.0	60.89	60.42

Stability Studies

Results of stability studies of optimized batch are give in **Table10** which indicated that all the physical parameters of formulation remains with the prescribed limit during the test periods. The percentage drug release estimated at different time points does not showed any variation, which means that the selected formulation is stable.

The current research work was done with the objective of formulation of sustained released tablets of propanolol HCl using combination of gum mastic and HPMC-K15 utilizing 3^2 factorial design approaches. It was concluded that mastic gum (5%) and HPMC- K15 (25%) exhibited desired sustained drug release and followed Kosmeyer Peppas kinetic; the drug release mechanism may be diffusion or swelling of matrix. Therefore, mastic gum and HPMC- K15 can be a suitable combination for formulation of sustained released tablets.

CONFLICTS OF INTEREST

Authors has no conflicts of interest

AUTHORS' CONTRIBUTIONS

All authors contributed equally

REFERENCES

1. Prakash, P.; Porwal, M.; Saxena, A. Role of natural polymers is sustained release drug delivery systems: applications and recent approaches. *Int. Res. J. Pharm. Techno.,* **2011**, 2, 6-11.

2. Yadav, I.K.; Singh, H.P.; Singh, R.P.; Tiwari, P.K.; Chandra, D.; Jaiswal, D.; Jain, D.A. Formulation, evaluation and optimization of aceclofenac sustained release matrix tablets. *Int. J. Phar. Sci. Dr. Res.,* **2010**, *2*, 107-111.

3. Chowdary, K.; Kalyani. Recent Research on Matrix tablets for controlled release – A Review. *J. Int. Res. Pharm. App. Sci.,* **2013**, 3, 142-48.

4. Singh, K.; Kumar, A.; Langyan, N.; Ahuja, M. Evaluation of Mimosa pudica seed mucilage as sustained-release excipient. *AAPS Pharm. Sci. Tech.*, **2009**, *10*, 1121-1127.

5. Chugh, I.; Seth, N.; Rana, A.C.; and Gupta, S. Oral sustained release drug delivery system.*Int. Res. J. Pharm. Sci.,* **2012**, 3, 57-62.

6. Bhargava, A.; Arthur, R.P.S.; Tanwar, Y, S.; Gupta,S.; Bhaduka, G. Oral sustained release dosage form an opportunity to prolong the release of drug. *Int. J. Res. Pharm. Biomed. Sci.,* **2013**, 3, 7-14.

7. Yan, G.; Li, H.; Zhang, R.; Ding, D. Preparation and evaluation of sustained released formulation of nifedipine HPME tablets. *Drug Dev. Ind. Pharm.,* **2000**, 26,681-86.

8. Ghosh, S.; Ghosh, N. S.; Devnath, S.; Ganesh, J.I.; Chakraborty, R.S. Formulation and evaluation of sustained release dosage form of nifedipine hydrochloride using multi-unit chitosan treated alginate. *Int. J. Res. Pharm. Biomed. Sci.,* **2010**, 1, 124-31.

9. Afsar, C.; Sayyed, N.; Shaikh, S.; Tarique, K.; Siddik, M.; Mohammad.; Shaikh, A. Formulation and evaluation of sustained release tablets of aceclofenac using hydrophilic matrix system. *Int. J. Pharm. Pharm. Sci.*, **2011**, 2, 145-48.

10. Satyaraj, A.; Abhinav, K. Formulation evaluation of metoprolol succinate controlled release tablets using natural and synthetic polymer. *Int. J. Pharm. Sci. Res.*,**2012**, 2, 47-56.

11. Somnath, L.; Manoj, P.K.; Gorakhnath, H.; Development and evaluation of sustained released matrix tablets of naproxen. *Der. Pharmacia. Lettre.,* **2015**, 7, 270-79.

12. Kaleemullah, M.; Jiyauddin, K.; Thiban, E.; Rasha, S.; Al-Dhalli, S.; Budiasih, S.; Gamal, O.E.; Fadli, A.; Eddy, Y. Development and evaluation of Ketoprofen sustained release matrix tablet using Hibiscus rosa-sinensis leaves mucilage. *Saudi. pharm. J.*, **2017**, *25*, 770-779

13. Vlachou, M.; Geraniou, E.; Siamidi, A. Modified release of furosemide from Eudragits® and poly (ethylene oxide)-based matrices and dry-coated tablets. *Acta. Pharmaceutica.* **2020**, *70*, 49-61.

14. Upendra, N.; Charu, B. Formulation and *in vitro* characterization of diclofenac sodium loaded sustained release matrix using natural and synthetic polymers. *Ind. J. Pharm. Edu. Res.*, **2014**, 48, 12-24.

15. Fentie, M.; Belete, A.; Mariam, T.G. Formulation of Sustained Release Floating microspheres of Furosemide from Ethylcellulose and Hydroxypropyl Methylcellulose polymer Blends. *J. Nano. med. Nanotechnol.*, **2015**, 6, 2-5

16. Higuchi, T. mechanism of sustained action medication: theoretical analysis of rate of release of solid drugs dispersed in solid matrices. *Int. J. Pharm. Sci.,* **1963**, 52, 1145-49.

17. Yeole, P.G.; Galgatte, U.C.; Babla, I.B.; Nakhat, P.D. Design and Evaluation of Xanthan gum based Sustained release matrix tablet of Diclofenac sodium. *Ind. J. Pharm. Sci.*, **2006**, 68, 185-89.

18. Dinesh, M. M. Evaluation of gum mastic (*Pistacia lentiscus)* as a microencapsulating and matrix forming material for sustained drug release. *Asian J. Pharm. Sci.,* **2017**, 12, 424-32.

19. Banker, A. U.; Banker, V. H.; Sunil, P.P. Formulation design and optimization of sustained released matrix tablets of Ambroxol hydrochloride. *Int. J. drug deliv.*, **2012**, 4, 375-85.

20. Ahmad, K.H.A.N.; Naqvi, B.S.; Shoaib, M.H.; Jallat, K.H.A.N.; Yousaf, R.I. Formulation Development and Optimization of Metoclopramide HCl Tablets for Future IVIVC Studies. *Lat. Am. J. Pharm.*, **2015**, *34*, 134-4

REVIEW ARTICLE

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Review: Polymers for Mucoadhesive Drug Delivery Systems

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ABSTRACT

In bio adhesive drug delivery, the word bio adhesion is used to define the connection between polymers (synthetic or natural) and gastro-intestinal mucosa or any soft tissue of the body. In cases when the bond is designed with the mucosa, the term mucoadhesion may be used synonymously with bio adhesion. For delivered the mucoadhesive drug by oral route following locations are used:-Oral cavities (lips, tongue, cheek or buccal, soft palate, hard palate and floor of the mouth. Importance of Antioxidant activity, it increase the dwelling time of the dosage time at the absorption site and it improves absorption and the therapeutic ability of the drug due to increase the residence time. Natural polysaccharides have an extensive variety of applications as a dietary supplement and pharmaceutical excipients with good biocompatibility, low cost, and easy accessibility. Polysaccharides are obtained from various sources like plants, marine, and animals.

Keywords: Polymers, Mucoadhesive drug delivery system, Antioxidant activity, Flaxseed mucilage, and Grafted Polysaccharides.

INTRODUCTION

Polysaccharides are biopolymers made up from different and lengthy chains of monosaccharides units which are joined together by a glycosidic link and on hydrolysis reactions yields oligo and monosaccharides which are known as polysaccharides. Polysaccharides are related to carbohydrates and 99% are founds in plants[1]. Polysaccharides are often quite homogenous, containing slight modifications of repeating units. Frequently, polysaccharides are not in pure form.

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They are associated with other polysaccharides, polyphenolics or proteins by covalent or non-covalent bonds. The structure of polysaccharides are frequently linear but may contain various degree of branching[2].

Polysaccharides are of mainly two types:

(A) Homo Polysaccharides: They are those polysaccharides which on hydrolysis yield a single type of monosaccharides. E.g., Cellulose, Dextrin, Inulin, Starch, Glycogen, etc.

(B) Hetero Polysaccharides: When polysaccharides are composed of different types of sugars are hetero polysaccharides. E.g., Proteoglycans, Hyaluronic acid, etc.

Natural polysaccharides have an extensive variety of applications as a dietary supplement and pharmaceutical excipients with good biocompatibility, low cost, and easy accessibility. Polysaccharides are obtained from various sources like plants, marine, and animals. Polysaccharides containing various polymers such as gums and mucilage for various uses in pharmaceutical formulations[3,4]. When natural polysaccharides are compared with synthetic polymers, natural polysaccharides are nontoxic, economic and biodegradable. Many polysaccharides such as chitosan, xanthan gum, sodium alginate, etc. are used in combination or either alone with their customized and indigenous form to evaluated controlled release of drugs, water treatment, improved oil recovery and flocculation of suspension[5]. Table 1. Shows the naturally occurring polysaccharides obtained from various sources:

Table 1. List of various polysaccharides and their sources

S.No.	Sources	Polysaccharides
1.	Plants	Starch, Pectin, Guar gum, Flaxseed gum, Pectin, inulin, Albizia gum
2.	Animals	Chitosan, Hyaluronic acid, Chitin, Chondroitin sulfate
3.	Seaweed	Alginates, Carrageenan, Agar
4.	Fungal	Scleroglucan
5.	Microbial	Xanthan gum, Gellan gum, Dextran, Glycan, Pullulan
6.	Tree exudates	Tragacanth, Gum acacia, Ghatti gum

Application of polysaccharides:

(A) Antitumor activity: Polysaccharides indicated important anti-tumor activity beside A375 and BGC823 cells and with vascular smooth muscles, cells show low cytotoxicity. Pectin a complex[6] polysaccharide, and a significant source of dietary fiber, it shows inhibitory action for several cancer lines. Polysaccharides anticancer activity is affected by the form of molecules, size of molecules, solubility in water and degree of branching. If water solubility of polysaccharide is higher and greater molecular weight then it shows greater antitumor activity.

(B) Immunoregulatory activity: Plant-derived polysaccharides have the best immunemodulatory constituents and have been displayed to be clinically therapeutic. Immune functions of macrophages, lymphocytes and natural killer cells directly active by plant polysaccharides.

(C) Antidiabetes activity: The polysaccharides which are obtained since natural resources (higher plants, algae, and mushrooms entirely have significant potential for the management of diabetes and also provide an annoying source for upcoming innovation and developed a new and unique compounds of therapeutic worth. Astragali Radix and Rahmanniae Radix exist two new compounds used for diabetes mellitus in China.

(D) Anticoagulant activity: After sulfate modification, anticoagulant activity of polysaccharides similar action as heparin but no anti-platelet activity and avoid the side effect which induced by heparin. Sulfated citrus pectin and sulfated glycol glucuronomannan have revealed antithrombotic and anticoagulant activity. Sulfated polysaccharides found in aquatic invertebrates and aquatic algae with anticoagulant activity.

(E) Antiviral activity: Astragalus polysaccharide displays antiviral activity beside infectious bursal disease viruses and also reduces mortality and morbidity of the IBD (Infectious bursal disease) virus-infected chicks. Polysaccharides existing in algae and their abstracts (alginate, fucoidan, and laminarin) can inhibit bacteriological as well as viral infections.

(F) Antioxidant activity: Natural polysaccharides is an extremely favorable source of anti-oxidants, exclusively those which have been capability to more unrestricted radicals, so it has definite antioxidant and antiaging activity. Algal polysaccharides play a significant part as free radical scavengers in-vitro for the anticipation of oxidative impairment inactive organisms.

Polymers used for mucoadhesive drug delivery system[7]

Before using any polymer for any mucoadhesive formulation, polymers have been examined to ensure if they fulfill all the requirements (high number of hydrogen bonding, swelling properties, suitable wetting and chain flexibility of polymer) to ensure diffusion of mucoadhesive polymer to the mucous membrane network. Hydration is an also important requirement for mucoadhesive polymer to create sufficient macromolecular mesh and release polymeric chains to increase elucidation between mucin and polymer. The use of mucoadhesive polymers increase day by day to modified the release of formulation dosage form and also increased their use for buccal and gastro retentive drug delivery system. The design of some bilayer polymeric mixture can provide new material

for biomedical application and increase adhesion time, prolonged dwelling time and greater surface contact with the mucosa.

A new advancement (Polymer blending) that can combine the advantage of many different polymers and usually faster and cheaper development as compared to new monomers and new polymerization routes. Because of this reason, especially thermo-responsive and mucoadhesive polymers are used.

Characteristics of ideal polymers

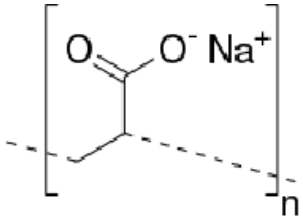
- Non-irritant to mucosal membrane
- Optimum molecular weight
- Good spreadability, wetting, swelling, and biodegradable properties.

Mucoadhesive polymers in drug delivery: Table 2 shows various types of polymers which are used in mucoadhesive drug delivery system[8,9].

Table 2. List of various polymers used in mucoadhesive drug delivery system

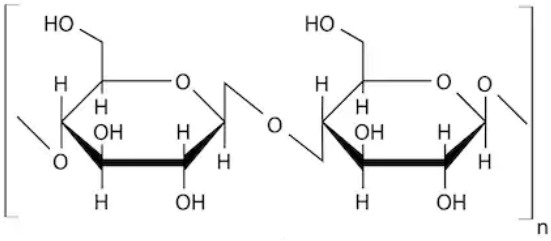
Source Dependent Polymers	Aqueous solubility	Charge	Potential Bioadhesive Force
Natural polymers E.g., Agarose, gelatin, chitosan Various gums (guar, xanthan, pectin, and sodium alginate) **Synthetic polymers** E.g., Cellulose derivatives: CMC, Thiolated CMC, sodium CMC, HPMC, HPC, methyl hydroxyethyl cellulose. Poly(acrylic acid)- based polymers E.g., Polyacrylates, PAA, PC, Poly(methyl vinyl ether-coethyl hexyl acrylates), polyoxyethylene, PVP, PVA, thiolated polymers	**Water-soluble** E.g., HEC, HPC, sodium CMC, Sodium alginate. HPMC flax seed **water-insoluble** chitosan (soluble in dilute aqueous acids), EC, PC	**Cationic charge** E.g., Amino dextran, Chitosan trimethylated chitosan, dimethyl aminoethyl- dextran **Anionic charge** E.g., chitosan, EDTA, CP, CMC, Pectin, PAA, PC, sodium alginate, Sodium CMC, Xanthan gum	**Covalent bonded polymers** E.g., hydroxyethyl starch, HPC, PVA, PVP **Hydrogen bond** E.g., Acrylates, poly (methacrylic acid), CP, PC, PVA Electrostatic interactions E.g., Chitosan

(A) Polyacrylates: There polymers are present in a broad variety of molecular weights, definitely modified systems of gel, safe besides non-toxic for oral uses. Polycarbophil and carbomers (PAA derivatives) are most preferred used as mucoadhesive polymer. Carbopol used as a polymer in oral preparation, suspension, and tablets. Polycarbophil is water-insoluble polymer but has a high swelling property that allowing high levels of enlargement within the mucus layer. The mucoadhesive properties of PAA increased by its conjugation with cysteine[10]. Sodium polyacrylate, also known as waterlock, is a sodium salt of polyacrylic acid with the chemical formula $[-CH_2-CH(CO_2Na)-]_n$.



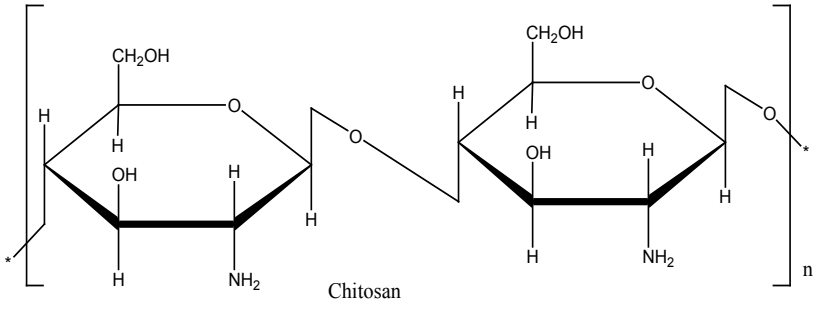
Chemical structure of Sodium polyacrylate

(B) Cellulose derivatives: Depending upon their type of substitution, cellulose derivatives exhibit different concerts. The most widely used cellulose derivatives as a mucoadhesive polymer are HPC (hydroxyl propyl cellulose), HPMC (hydroxyl propyl methyl cellulose), HEC (hydroxyethyl cellulose) and CMC (carboxymethylcellulose). As compared to other cellulose derivatives CMC shows greater mucoadhesive properties and also high hydrogen-bonding capacity[11].



Chemical structure of cellulose

(C) Chitosan: As compared to other cationic polymers, Chitosan is most broadly investigated polymer. Chitosan is a biocompatible, hydrophilic and biodegradable polymer with very low toxicity. Chitosan have moderate swelling and mucoadhesive properties. Used in the formulation of sustained release.

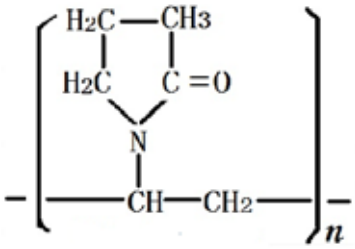


Chemical structure of chitosan

(D) Alginates: Alginates are naturally occurring polymers and extracted from seaweed, algae, and bacteria. Alginate is a negatively charged polymer and used for the preparation of microparticles because of their good mucoadhesive properties. Both low molecular weight alginate (too stiff) and high molecular weight (more flexible) are used as mucoadhesive polymer[12].

(E) Pectin: Pectin found in the cell wall of maximum plants and it is a water-soluble and heterogeneous polysaccharide. After mixing pectin and mucin in deionized water, the formation of large aggregates shown the relationship between pectin and mucin with hydrogen bonding.

(F) Polyvinyl Pyrrolidone: PVP is a non-ionic synthetic linear polymer having group (N-vinyl-2-pyrrolidone). PVP is non-toxic and due to this nature, it is used in non-parenteral exposure. PVP is soluble in various solvents (aqueous and organic) and these are chemically inert. It is mostly used in many formulations of pharmaceutical due to its mucoadhesive properties. The mucoadhesive properties of PVP at the pH of the intestine is 6.6.



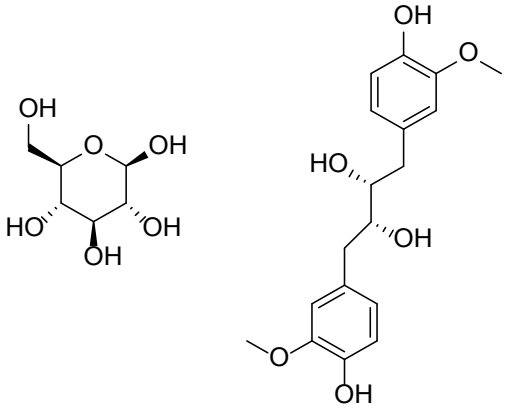
Chemical structure of Polyvinyl Pyrrolidone

Out of numerous polymers available in nature, Flaxseed mucilage is selected to study the mucoadhesive potential

Flaxseed: Flaxseed mucilage (Linum usitatissimum L.)

Family: Linaceae

Flaxseed mucilage (FSM) is a mucilage of polysaccharide extracted from fully developed dried seed of linseed or flax plant. Flax seed have two basic varieties: Golden or yellow (also known as golden linseed) and brown flax seed. The mucilage of flax seed contains two different polysaccharide types:- (1) acidic polysaccharides (rich in L- rhamnose, D-galactose) and (2) natural polysaccharides (rich in D-galacturonic acid, L-rhamnogalacturonan)[10].



Structure of chemical constituent's flaxseed mucilage

Flaxseed is well recognized for the content of chemical compounds with specific biological activity and functional properties: polyunsaturated fatty acids (PUFA), omega-3 family, lignans, soluble dietary fibers and carbohydrates[13]. The mucilaginous cells are present on the outer surface of the seed. The seed coat present on the outer surface of the flaxseed protect the flaxseed from mechanical damage and UV radiations[3].

The seed coat of flax seed have the following roles:

- Providing resistance against rain and wind
- Promoting water uptake
- Facilitating seed disposal by sticking to animals.

Flaxseed is rich in dietary fibers. Flax seed have many biologically active compounds such as linoleic acid, linoleic acid, lignans, polysaccharides, alkaloids, and cyanogenic glycosides. The production of flaxseed is for the production of oil, fiber, and food[13].

Properties

It displays a great thickness in liquid or aqueous media and embraces a virtuous water-holding and swelling capability. Moreover, it possesses exclusive nutritive significance as a dietary fiber and gelling agent. Nowadays, flax seed have been used also in pharmaceuticals.

Applications of flax seed

1. Pharmaceutical applications of flax seed

(a) Flaxseed mucilage and gums used information on mucoadhesive tablets be-

cause of their mucoadhesive nature. It is used as a disintegrant in tablets.
(b) Used as an emulsifying and suspending agent to stabilize the emulsion formulations.
(c) Used as sustaining material in dosage form
(d) Used as coating material
(e) Used as gelling agent[15]

2. Therapeutically applications of flax seed

(a) Used in cardiovascular disease (whole ground flaxseed, fiber, oil, and powder.
(b) Used for rheumatoid arthritis and inflammation of joints (flaxseed oil)
(c) Useful in hyper-cholesterolemia (flax seed powder and flaxseed oil)
(d) Used in the treatment of diabetes
(e) Useful to treat obesity
(f) Used in hypertension
(g) Used to treat tumor or cancer
(h) Used in kidney disease[16]

3. Dietary application of flaxseed: Flaxseed meal is gluten-free with a satisfying nutty (nuts like) flavor. The protein content also found in flax seed meals combined with binding properties of soluble fiber.

Flax seed works as a dietary supplements

(a) Fiber supplementation (bulk laxative with a demulcent action)
(b) Used as a component of protein powder blends.
(c) Many food ingredients (breads and other baked goods) are gluten-free products which are made by using flax seed.
(d) Healthy functional snack food (high protein energy bars)
(e) Oil of flax seed maintain the level of omega- 3 and omega-6 in the body
(f) Useful in the treatment of constipation and helps to maintain the overall bowel health.

Muco adhesion:

The term muco adhesion refers to any bond made between any two biological surfaces or a bond between a synthetic surface and mucosal surface (biological surface) and the constituents which are proficient for interaction with biological measurable and adhering them together for extended periods are known as mucoadhesive/ bioadhesives.

In bio adhesive drug delivery, the word bio adhesion is used to define the connection between polymers (synthetic or natural) and gastro-intestinal mucosa

or any soft tissue of the body. In cases when the bond is designed with the mucosa, the term mucoadhesion may be used synonymously with bio adhesion[17]

MECHANISM OF MUCOADHESION[17,18]

As stated, mucoadhesion is the connection of the drug and a suitable carrier to the mucous membrane. Mucoadhesion is a complex phenomenon which include:

STEP 1. Swelling and Wetting of polymer (contact stage)
STEP 2. Interpretation between the mucosal membrane and chain of polymers
STEP 3. Establishment of connections between the tangled chains (both known as consolidation stages). Figure 1. Shows the mechanism of mucoadhesion

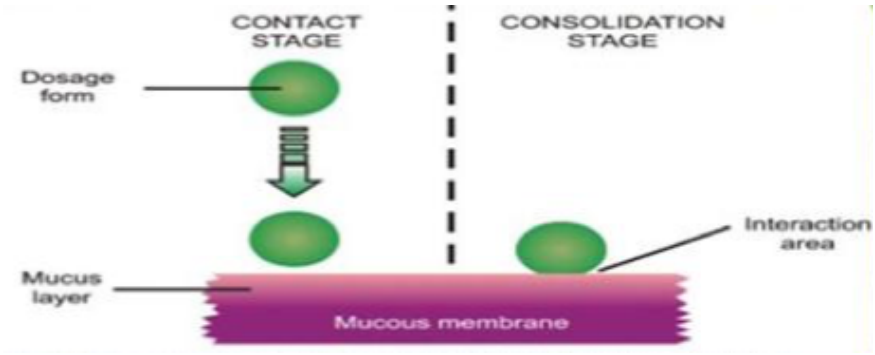


Figure 1. Mechanism of mucoadhesion

Theories of mucoadhesion[18,19]

1. Wettability theory

This theory is useful to low viscosity or liquid mucoadhesive organizations. Wettability theory defines the capability of a mucoadhesive polymer to the extent on biological membrane consequently it provides an interpretation of the "spreadability" of active drug delivery system. The process of wetting theory describes the energy essential to stabilize the surface rigidity at the interface amongst the two surfaces permitting for a virtuous mucoadhesive distribution and contact of the biological substrate. Figure 2. Shows the interaction angle between the device and the mucous membrane of mucoadhesion.

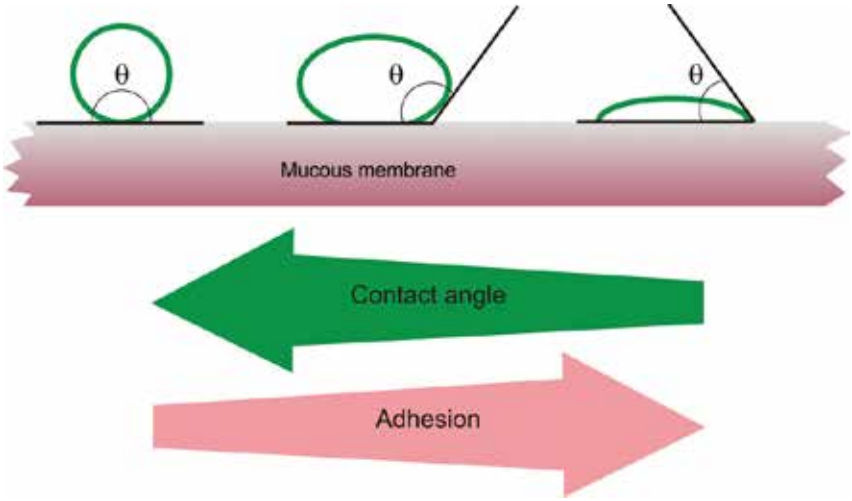


Figure 2. Contact angle between device and mucous membrane

2. Adsorption theory[20]:

Based on the adsorption theory, van der Waals' and hydrogen bond forces established for adhesive interactions. The mucoadhesive substance adhere because of superficial forces performing amongst the molecules of two surfaces, after preliminary contact angle between inter exteriors. According to the concept of chemisorption, collaboration through the interface follows as a consequence of compact covalent bonding.

3. Fracture theory

According to this concept, the strength which is required to discrete both the surfaces from each other is connected to the adhesive connections between the systems. This "fracture theory" transmits the strength for polymer impartiality from the mucus to the strength of their adhesive connection (Figure 3).

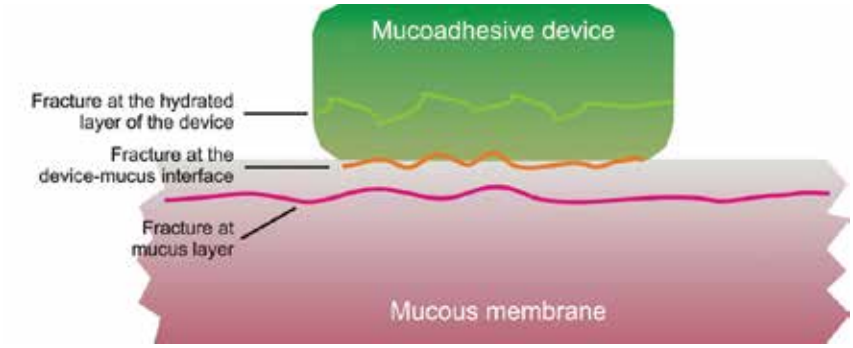


Figure 3. Picture of Secondary interaction resulting from inter-diffusion of polymer chain on bioadhesive device and bioadhesive membrane

4. Electronic theory

Each surfaces have their particular electronic structure and structural properties. This system is established on electronic modifications in arrangement or structures. It explains that bonding arises due to electron transmission between the arrangement of polymers and the mucous membrane epithelium. This results, double layer formed by electronic charges formed at mucoadhesive system interface and mucus. This is responsible for the development of attractive force between the two exteriors through the electronic double layer (Figure 4).

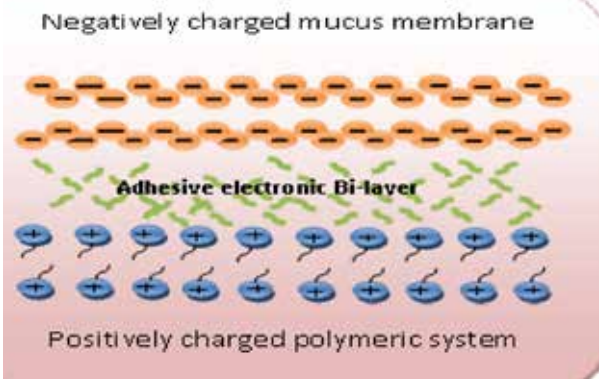


Figure 4. Electronic theory of mucoadhesion.

5. Diffusion theory

This theory explains the time-dependent mucoadhesive polymer chain diffusion into the glycoprotein chain network of the mucus stratum, shows in figure 5. This is a two-way diffusion method through permeation amount being based upon the diffusion coefficients of mutually relating polymers. While there are several factors elaborated in such procedures, the basic properties that considerably affect this diffusion are cross-linking density, molecular weight, and chain flexibility extension capacity of both networks and temperature (as major factor).

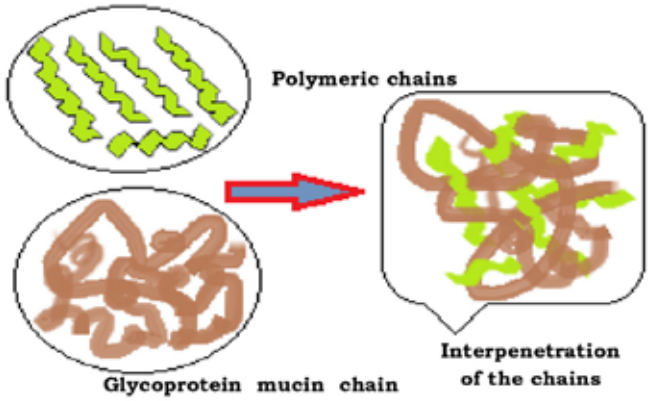


Figure 5. Picture of diffusion interlock of polymeric chains

Factors affecting mucoadhesion process

The mucoadhesion of the drug carrier system to the mucous membrane depends on the below-mentioned factors[18]. Different types of factors which affect the mucoadhesion process as following

- Concentration of polymer used
- Molecular weight of polymers
- Swelling factors & stereochemistry of polymer
- Mucin turnover rate
- Flexibility of polymers chain
- Polymer - substrate interaction pH [6,21]

Mucoadhesive drug delivery system

The use of MDDS is increased day by day because it can increase drug accretion on the mucosa by prolong the dwelling time. Hydrophobic interaction plays a vital role in case of mucoadhesion. In accumulation to electrostatic bonding and hydrophobic interaction, it has been shown that mucoadhesion can also occur through physical interlocking of polymer with mucosal membrane. The mucoadhesive formulations have been prepared as a medication to enhance the contact time of dosage form with the mucosal layer of various routes for drug delivery in body: Oral, Buccal, Oral, Nasal, Rectal, ocular, Sublingual routes[22,23].

For delivery of drug to the oral mucosa observe various areas:

For delivered the mucoadhesive drug by oral route following locations are used:-Oral cavities (lips, tongue, cheek or buccal, soft palate, hard palate and floor of the mouth[24].

Oral mucosa is a useful term to identify the lining of the oral cavity includes buccal, sublingual, gingival, palatal and labial mucosa.

Importance of mucoadhesive drug delivery[25]

1. It increase the dwelling time of the dosage time at the absorption site.
2. It improves absorption and the therapeutic ability of the drug due to increase the residence time.
3. Exceptional availability[21,26].
4. Better blood flow rates due to fast blood supply.
5. First, pass metabolism avoidance increase drug bioavailability.
6. Due to the acidic environment in the GIT drug is protected from degradation.
7. Enhanced patient Obedience: faster onset of action is achieved due to mucosal surface and easy to administer.

Limitation of mucoadhesives drug delivery system:

1. Long-time fixation at the site of application[26].
2. A new perspective is enzyme inhibition and penetration enhancement.
3. Drinking and eating are constrained at the time of mucoadhesive drug delivery.
4. Possibility of swallowing of formulation by patient.
5. Dosage form should not be disseminated after located at the site of absorption.

Mucoadhesive formulation:

(A) Tablet

Tablets are solid dosage form and small in size and use mucoadhesive coating. The coating on tablets used for mucoadhesive formulations for adherence of tablet to mucosa for both targeted local and systemic administration. Tablet have less patient compliance when given orally as compared to other route[27].

(B) Patches

Patches have three separate layers:

(1) Outer impermeable layer

(2) Reservoir layer (middle layer)

(3) Inner layer

Outer layer of patches control the direction of release of drug from the patches at the contact site and middle layer act as reservoir layer and hold the drug and help to provide specified amount of dose at the application site. The final inner layer allows the patch to adhere to the specified mucosa.

(C) Gels

Gels are liquid or semisolid dosage form. When a solid form of any formulation affects the comfort level of the patient then gels are used for treatment. Mucoadhesive formulations increase the viscosity of gels after applications so the drug in the gel form of drug effectively administer at the local sites and also maintaining the comfort of the patient.

(D) Solution

The solution form of mucoadhesive formulation is used when the drug is administered in the eye and nasal cavity. In mucoadhesive formulation, polymers which are mucoadhesive used to improve resistance of mucosal surface. The mucoadhesive system when used for eye then it called situ gelling system.

Advancement in mucoadhesive drug delivery system[26]

- Liposomes (used to convey vaccine drug, enzyme to cells and organs)
- Nanoparticles (used to improve ocular bioavailability and play a superior role to improve targeting at the site)
- Microsphere
- Nano suspension
- Mucosal vaccination

Modification of polysaccharides

Various approaches are used for chemical modifications of these natural polysaccharides are graft polymerization, etherification and cross-linking to overcome the problems of low microbiological stability, disagreeable odor, and uncontrolled hydration limit. Grafting by using vinyl monomers is a very useful application now a day to develop a new and better modified polysaccharides.

(A) Chemical modification

This technique of modification, can adapt the structure of polysaccharides by announcing substituent groups, and thus reinforce their parent bioactivities as well as generated new useful bioactivities. Some of the chemical modification techniques are:-

(a) Sulfation

This method is used for sulfation of neutral polysaccharides and sulfated polysaccharides.

(b) Carboxy methylation

Cellulose, pachymaran, and scleroglucan have poor water solubility so they can't induce bioactivities. This method is used to raise their biochemical events to stimulate water solubility[28].

(c) Phosphorylation

Glucose, Fructose, and some other monosaccharides which have no natural bioactivities, can be stimulated after phosphorylated modification.

(d) Acetylation

This method of modification used for the treatment of branches in polysaccharide molecules, which results in improvement in the solubility of polysaccharides.

(e) Alkylation

This method is frequently useful to modify chitosan to decrease their viscosity and improve solubility. Some polysaccharides need to modify by a methyl or ethyl group before analyzed the gas chromatograph is analyzed.

(f) Graft co-polymerization

The graft co-polymerization method involves a long sequence of one or more branches to another polysaccharide. The free radicals sites will create on the performed polymer with the help of external agents[2].

(g) Etherification

This method of chemical modification of polysaccharides involves the reaction of an alcohol (Saccharide alcohol) with alkylating agents (carboxylic acid used as alkylating agent).

(h) Oxidation

Polysaccharides may be oxidized in different ways to produce the structure of different types of polysaccharides. Chemical and biological modification have both seen used for oxidation of primary alcohol of polysaccharides. In oxidation reaction, chemical oxidation of primary alcohol to carboxylic acid and enzymatic oxidation of primary alcohol to aldehyde[2].

(i) Selenization

Selenium is an essential trace element for the human body and also an important component of glutathione peroxidase and superoxide dismutase. The combination of selenium and polysaccharide into an organic compound can promote the functions of new organic compounds.

(j) Acid/alkali degradation

This is a traditional method for Production of modified polysaccharides through acid/alkali degradation. To be specific, glycosidic bonds of polysaccharides are first cleaved by acidic/ alkaline solution, thus degradation of polysaccharides into lower molecular weight fragments with high solubility and improved biological activity, etc.

(B) Physical modification

This method is used to shorten the parent polysaccharide strength to develop lower molecular weight fragments. This technique is to confirm the maintenance of the basic arrangement of polysaccharides and the only reason for some conformational variations. The generally used techniques are microwave exposure, ultrasonic disruption, radiation-induced reaction, etc.

(C) Biological modifications

The biological modification of polysaccharides mostly denotes to enzyme modification, which is catalysis with enzymes results degradation of polysaccharides. When biological modification is associated with chemical modification then biological modification shows benefits of great efficiency, high specificity, and low toxic effects. The main aim of biological modification is to destroy the strength of polysaccharide before decreasing its viscosity and its molecular weight.

Grafted Polysaccharides

Polysaccharides such as vinyl monomers, Cellulose, gelatin, Starch, Eudragit, Chitosan, Carbopol, Agarose, and Acacia, etc. have been expansively graft modified to achieve macromolecular constituents better to their parent polysaccharides. The graft modified polysaccharide demonstrating better resistance to heat, antibacterial activity, greater oil/water repellant qualities, higher mechanical strength[29].

In natural form, polysaccharides have reduced shelf life because of their vulnerability to biodegradation but impartially unaffected to degradation below shear and perform as convenient flocculants and synthetic polymers suffer from poor shear dwelling properties but they can be easily tailored. By grafting technique, the synthetic polymers with any natural polysaccharides can be converted into identical customizable matrices with mixture properties appropriate for different uses. Chemical grafting is a useful and most effective method to obtain new material or polysaccharide by increasing the compatibility between natural and synthetic polymers and the new polysaccharide has the most useful and preferred hybrid characteristics. Grafted polysaccharide includes the connection of polymer chains, usually a monomer, to the strengthen polymer[30].

Grafting Techniques

The grafting technique is a multipurpose implementation for the copolymer production that may attain innovative property, a new and different arrangement of their parent molecule. Grafting technique carried out with the help of free radicals such as chemical inventors, U.V. Rays in the occurrence of photosensitizers, gamma rays, and microwave radiations. Grafting copolymerization is a new technique for the hybridization of natural polymer with any synthetic polymer for improvement in the functionality of natural polymer and modify a new and better polysaccharide. The most preferred process of graft copolymer production is the use of microwave radiation to produce the free radical sites on the strength polymer[29,30] .

The methods used for graft copolymerization has been classified into various types:

(1) Grafting initiated by free radicals (chemical method)[31]

(2) Grafting by enzymes (biological method)

(3) Grafting induced by plasma-radiation

(4) Radiation grafting (physical method:microwave radiation-induced grafting)

(5) Photochemical grafting

(6) Grafting by photochemical reactions

(7) Ionic grafting

(8) Grafting by chemical-free radical initiators etc.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

REFERENCES

1. Akin, A.; & Isiklan, N. Microwave assisted synthesis and characterization of sodium alginate-g-poly (N,N"- Dimethyl acrylamide). *Int. J. Biol. Macromol.*, **2016**, (82) 530-540.

2. Bhosale, R.R.; Osmani R.A.; & Moin A. Review on natural polysaccharide based particulate drug delivery systems: An inimitable tactic in novel drug delivery systems. *Int. J. Curr. Pharm. Rev. Res.,* **2014**, 5:138-155.

3. Tomada, M.; Shimada, K.; Satio, Y.; & Sugi, M. Plant mucilages: Isolation and structural features of Okra mucilage, from the immature fruits of Abelmoschus esculentus . *Chem. Pharm. Bull.*, **1980**, 28(10):2933-40.

4. Bemiller, J.N.; & Whistler, R.L. Industrial gums: Polysaccharides and their derivatives. *Academic Press*, **2012**.

5. Kulkarni, R.V.; Imamdar, S.Z.; Das, K.K.; & Biradar, M.S. Polysaccharides- based stimuli sensitive graft co-polymer for drug delivery. Advanced drug delivery review. *Woodhead publishing*, **2010**, 155-171.

6. Boddupalli, B.M.; Mohammed, Z.N.K.; Nath, R.K.; & Banji, D. Mucoadhesive drug delivery system: A review. *J. Adv. Pharm. Technol. Res.*, **2010**, Vol 1 (4): 381-387.

7. Nagaveni, P.; Sekhar, C. K.; & Reddy, J . Design and in-vitro evaluation of mucoadhesive drug delivery of nateglinide. *Int. J. Pharm. Sci. Res.*, **2017**, 8(4): 676-684.

8. Morales, J.O.; & McConville, J.T. Manufacture and characterization of mucoadhesion buccal films. *Eur. J. Pharm. Biopharm.*, **2011**, 77(2). 187-199

9. Singh, J.; & Deep, P. A Review Article on Mucoadhesive Buccal Drug Delivery System. *Int. J. Pharm. Sci. Res.*, **2013**, 4(3):916.

10. Russo, E.; Selmin, F.; Baldessari, S.; Caviglioli ,G.; Ciluzo, F.; Minghetti, P.; & Parodhi, B. A focus on mucoadhesive polymers and their applications in buccal dosage forms. *J. Drug Delivery Sci. Technol.*, **2016**, 32:113-125

11. Gopinath, V.; Saravanan, S.; Al-Malekin, A.R.; Ramesh, M.; & Vedivelu, J. Review of natural polysaccharides for drug delivery application. Biomed. Pharmacother., **2018**, (107), 96-108.

12. Barclay, T.G.; Day, C.M.; Petrovsky, N.; & Garg, S. Review of polysaccharide particle-based functional drug delivery. *Carbohydr. Polym.*, **2019**, (221) 94-112.

13. Shim, Y.Y.; Gui, B.; Arnison, P.G.; Wang, Y.; & Reaney, M.J. Flaxseed (Linum usitatissimum L.) bioactive compounds and peptide nomenclature: A review. *Trends in fo Trends Food Sci Technol*, **2014**, 38(1):5-20.

14. Rubilar, M.; Gutierrez, C.; Verdugo, M.; Shene, C.; & Sineiro, J. Flaxseed as a source of functional ingredients. *Soil Sci. Plant Nutr.*, **2010**, 10(3), 373-377.

15. Prajapati, V.D.; Jani, G.K.; Moradiya, N.G.; & Randeria, N.P. Pharmaceutical applications of various natural gums, mucilages and their modified forms. *Carbohydr. Polym.*, **2013**, 92(2):1685-99.

16. Goyal, A.; Sihag, M.K.; Patel, A.; & Tanwar, B. Therapeutic potential of flax seed. In therapeutic probiotic and unconventional foods. *Academic press*, **2018**, 256-267.

17. Tangri, P.; & Madhav, N.V.S. Oral mucoadhesive drug delivery system. *Int J Biopharmaceutics*, **2011**, 2(1): 36-46.

18. Mansuri, S.; Kesharwani, P.; Keerti, J.; Rakesh, K.T., & Jain. Mucoadhesion : A promising approach in drug delivery system. *React. Funct. Polym.,* **2016**, 100: 152-173.

19. Carvalho, C.F.; Braschi, L.M.; Evangelista, R.C.; Gremiao, M.F.D. Mucoadhesive drug delivery system. *Braz. J. Pharm. Sci.***2010**, vol. 46 (1), 1-17.

20. Ahuja, M.; Singh, S.; & Kumar, A. Evaluation of carboxymethyl gellan gum as a mucoadhesive. *Int. J. Biol. Macromol.*, **2013**, 53:114-121.

21. Salamat-Miller, N.; Chittchang, M.; & Johnston, T.P. The use of mucoadhesive polymers in buccal drug delivery. *Adv. Drug Delivery Rev.*, **2005**, 57(11):1666-91.

22. Ahuja, A.; Khar, R.K.; & Ali, J. Muco adhesive drug delivery system. *Drug Dev. Ind. Pharm.,* **1997**, 23(5) 489-515.

23. Pardeshi, C.V.; Rajput, P.V.; Belgamwar, V.S.; & Tekade, A.R. Formulation, optimization and evaluation of spray-dried mucoadhesive microspheres as intranasal carriers for Valsartan. *J. Microencapsulation,* **2012**, 29(2):103-14.

24. Silva, B.M.A.; Borges, A.F; Silva, C.; & Simoes, S. Muco adhesive oral films: The potential for unmet needs. *Int. J. Pharm.*,**2015**, 494 (1)537-551.

25. Kaur, A.; & Kaur, G. Mucoadhesive buccal patches based on interpolymer complexes of chitosan–pectin for delivery of carvedilol. *Saudi Pharm. J.*, **2012**, 20(1):21-7.

26. Babade, N.N.; Atran, S.C.; Wankhade, V.P.; Pande, S.D.; & Tabar, K.K. A review on buccal drug delivery system. *Int. J. Pharm. Pharm. Sci.*, **2013**, 3(1): 35-40.

27. Russo, E.; Selmin, F.; Baldessari, S.; Caviglioli, G.; Ciluzo, F.; Minghetti, P.; & Parodhi, B. A focus on mucoadhesive polymers and their applications in buccal dosage forms. *J. Drug Delivery Sci. Technol.*, **2016**, 32:113-125.

28. Liu, J.; Shen, J.; Shim, Y.; Reaney, M.J.T. Characterization and solution rheology of flax seed gum carboxy methylation derivatives. *Int. J. Food Sci. Technol.*, **2016**, 51(2), 530-541.

29. Singh, V.; Kumar, P.; & Sanghi, R. Use of microwave irradiation in the grafting modification of the polysaccharides-A review. *Prog. Polym. Sci.*, **2012**, 37(2): 340-364.

30. Mishra, S.; Mukul, A.; Sen, G.; & Jha, U. Microwave assisted synthesis of polyacrylamide grafted starch and its applicability as flocculant for water treatment. *Int. J. Biol. Macromol.,* **2011**, 48(1) 106-111.

31. Setia, A. Application of graft copolymerization: A revolutionary approach. *Biopolymer grafting*, **2018**, 1-44.