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Address
İstanbul Medipol Üniversitesi
Kavacık Güney Kampüsü
Göztepe Mah. Atatürk Cad.
No: 40 34810 Beykoz/İSTANBUL
Tel: 0216 681 51 00

E-mail
editor@actapharmsci.com
secretary@actapharmsci.com

Web site
http://www.actapharmsci.com

Graphic Design
Murat Çakır
Levent Karabağlı

Printing Office
Forart Basımevi
Ziya Gökalp Mah.
Süleyman Demirel Bulvarı
Simpaş İş Modern Zemin Kat A 17-18
İkitelli - İstanbul
Tel: (0212) 501 82 20

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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 School of Medicine-Pharmacy Branch, Istanbul University, Turkey.

Starting from 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and became a journal for national and international manuscripts, in all fields of the 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 publishing only in English with the name, "Acta Pharmaceutica Sciencia" that represents internationally excepted high level scientific standards.

The journal has been publishing quarterly per year except an interval from 2002 to 2009 which released its issues trimestral in a year. Publication was discontinued from the end of 2011.

From 2016 October, Acta Pharmaceutica Sciencia is continuing publication with the reestablished Editorial Board and also with support of you as precious scientists.

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK

Editor

Brain power

Our brain team consists of a strong academic staff
who is specialized in neurosurgery (brain, spinal cord and nerve) and cooperates
with the new generation of intelligent technologies.

Third eye of the neurosurgeon: Technology


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CYBER KNIFE


O-ARM


STEALTHSTATION


INTRA-OPERATIVE MR


NAVIGATION

▸ ONKOLOJİK NÖROŞİRURJİ/NEUROSURGICAL ONCOLOGY ▸ SPİNAL NÖROŞİRURJİ/SPINAL NEUROSURGERY ▸ PEDİATRİK NÖROŞİRURJİ/PEDIATRIC NEUROSURGERY ▸ FONKSİYONEL NÖROŞİRURJİ/FUNCTIONAL NEUROSURGERY ▸ BEYİN VE OMURİLİĞİN DAMAR HASTALIKLARI/VASCULAR NEUROSURGERY ▸ PERİFERİK SİNİR CERRAHİSİ/PERIPHERAL NERVE SURGERY ▸ EPİLEPSİ CERRAHİSİ/EPILEPSY SURGERY ▸ NÖROENDOSKOPİ/NEUROENDOSCOPY ▸ HİDROSEFALİ/HYDROCEPHALUS

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Biosimilars: Current Scenario and Challenges in India

Anitha Nandagopal[1]*, Yusra Shakeel[1], Mamatha Tirunagari[2]

[1] Department of Pharmacology, Sultan-ul-Uloom College of Pharmacy, Road No: 3, Banjara Hills, Hyderabad, Telangana State, India.

[2] Department of Pharmaceutics, Sultan-ul-Uloom College of Pharmacy, Road No: 3, Banjara Hills, Hyderabad, Telangana State, India.

ABSTRACT

For a long period of time, biologics have been a major part of the pharmaceutical sector in providing treatment for most complex diseases like cancer, Alzheimer's disease, etc. With their patent expiration, a wide scope of opportunities has been unleashed for their replication. For the low molecular weight biologics, generics are preferable and safe option, whereas the large sized biologics being inaccessible for their price have led to the introduction of a new genre of medicine, Biosimilar. Biosimilar is designed with the intent to treat a patient in the same way as an existing biologic therapy. They are not the exact replicas of their innovator drugs. They are derived protein having immunogenic reaction and risk of adverse events. Despite the hurdles, biosimilar offer promising beneficial and cost effective option, if allowed to be understood by physicians and patients.

Keywords: Biosimilar, biologics, generics, replicas, cost effective.

INTRODUCTION

The field of medicine has been able to achieve a new class of medicine, especially with the advancement in biotechnology[1-2]. These types of medicine are large sized molecules that are made in the bodies of living organisms by addition of DNA to cells. The cells further copy or translate this piece of DNA into a protein and thus a biologic medicine is created. In comparison to chemical generics these are about 200-1000 times larger in size[3-5]. These products being highly complexed in comparison to chemical drugs, are in fact polypeptides or glycoproteins or nucleic acids[6-7]. Live cell lines are employed in the production of biologics which is why their reproduction is a difficulty. This is the reason biologics once manufactured are very hard to replicate exactly. The term biosimilar emphasize the difference

*Corresponding author: Prof. Anitha Nandagopal, e-mail: anirajan_76@yahoo.co.in
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in two products, similar to the innovator biologics but not their exact copy. In the past few years a number of biopharmaceutical patents have expired including granulocyte colony stimulating factors [G-CSFs], erythropoietin, interferons and human growth hormone. This has given way to the production of Biosimilar. When compared to their innovator biologics, few structural differences were found but however were clinically insignificant for consideration. Biosimilar, have the liberty to not undergo the same gruesome approval procedures as biologics which reflects in its cost, but in comparison to generic drugs they are still costlier[8-9].

Biologics-characteristics, significance and use

Biologics is a term to refer to the most developed and significant area of biotechnological products that include viruses, allergenic, somatic cells, blood products, tissues, gene therapy and recombinant therapy proteins. With biologics, some of the milestones in therapeutic medicine that were not thought to be achieved are now in existence that include production of proteins with a special set of functions (e.g., fligrastim) and monoclonal antibodies that can target specific molecules (e.g., adalimumab)[10].

The foundation of biologic medicines lies in recombinant DNA technology, which involves genetically engineering a cell that is an intricate, sensitive process and often specific to a particular medicine. The quality and efficacy of the biopharmaceutical at the final production stage depends on various factors such as, type of expression system, e.g. bacteria, yeast and mammalian cells, growth conditions offered, purification processes, storage and transport protocols undertaken. During cell synthesis, certain modifications are bound to occur such as, glycosylation, phosphorylation, sulphation, methylation, acetylation and hydroxylation which can alter the therapeutic potential of the drug. Such modifications need to be calculated as they can be the reason for production of a million variants of drugs[11].

Biologics are very sensitive to factors such as temperature and pH, which makes them hard to characterize and produce on large scale. Any minor modifications may lead to alteration in characteristics and change in structure, stability or other qualities of the final product. These have the possibility to affect the treatment safety, efficacy and shelf life and also increase the risk of immunogenic reactions.

In the present time, some of the most important medicines are biologics which include albumin, monoclonal antibodies, human insulin used for burns or liver failure, cancer and diabetes respectively.

Some other biologics include cloned proteins that occur naturally and erythropoietin in treatment of chronic anemia by stimulation of red blood cell production. In the treatment of some very critical conditions like cancer, rheumatoid

arthritis (RA), psoriasis and multiple sclerosis biologics have been observed to be most effective[12].

Biosimilar

Biosimilar can be said to be the latest biopharmaceutical agent that are 'similar' but in many aspects cannot be considered 'identical' to its reference product. They are synonymously also called Subsequent Entry Biologics (SEB) and Follow on Biologics.Post expiration of the patent(s) for these primarily approved biopharmaceuticals, other biotech and pharma companies acquire the chance to copy and market these biological drugs. These along with generic medicines can prove cost effective for patients and other sectors of society.

Biologic medicines exhibit both structural and molecular complexity. For the development and manufacture of high quality Biosimilar an intrinsic scientific knowledge and manufacturing expertise is required. Initially a cell line is chosen for the production of right clone. Thorough understanding of reference drug and its pharmacodynamics are the major criteria for Biosimilar development.

A Biosimilar is required to show similarity with reference biologic on the basis of:

1. Analytical (testing in a lab for structural/physical similarity),

2. Non-clinical (testing for function/activity/toxicity) and

3. Clinical data (testing in humans for safety and efficacy) in terms of structural characteristics, safety and efficacy.

How similar are biosimilar to biologics?

Biosimilar are not generic alternatives of innovator drugs nor can they be the exact duplicates of their innovator product. A Biosimilar and its reference cannot be entirely identical due to their differing manufacturing processes, but to qualify the approval process the protein sequence has to be same and structural alterations to a minor extent can be accepted[13-15]. It is critical to understand the differences and to demonstrate that they are not clinically meaningful and thus can be expected to be as safe and effective as the reference product for that approved condition of use.

The explicit details of the manufacturing processes employed in innovator drug production are a patented source and not always accessible. Thus, for Biosimilar to be exactly duplicated as their reference protein is impossible. Also the fact remains that detecting the protein characteristics require stringent analytical techniques, lack of which makes a thorough study of biopharmaceutical product difficult. Thus, regulatory guidelines have to be abided and pharmacovigilance is mandatory.

Although innovator biologic products and Biosimilar are both proteins by nature, they both tend to possess immunogenic potential. However Biosimilar can have more adverse drug reaction episodes of the immunogenicity type than reference products as they do not go through the exact same regulatory approval when compared to innovator drugs[16].

Biosimilar versus generics[17-18]

A generic drug is a chemically and therapeutically equivalent entity of a low molecular weight drug whose patent has expired. Biologics and Biosimilar are produced from live cells. On the other hand small molecule drugs and generics are produced by chemical / synthetic method. These discrepancies show that Biosimilar are not equal to generics. For a better understanding, the significant differences that exist between biologics and traditional small-molecule drugs in terms of basic chemical structure and manufacturing processes should be studied.

Chemical generics are small but well-defined molecules that can be generalized as chemical structures that are stable by nature. On the other hand, biopharmaceuticals are large peptides or proteins that have complex structural makeup. They are primarily made of amino acid sequence that should correspond to the traditional pharmaceutical. They have a three-dimensional structure which is highly sensitive to a lot of factors and need care while handling.

A finished generic drug can undergo chemical analysis to identify its various components whereas it is not just difficult but sometimes impossible to characterize a biologic and identify its components in a laboratory which explains its unique nature for many of its features may remain unknown.

Manufacturing differences between Biosimilar and generics[19-20]

Generic low molecular weight drugs are synthesized chemically by a combination of known reagents or ingredients in a series of controlled and predictable reactions. Biopharmaceuticals or Biosimilar are prepared in a living system that also secretes many other substances other than the required protein.

Therefore, generics can be replicated in an exact manner and they achieve an atomically identical structure to the reference drug. Biologics being complex will exhibit some physical and chemical differences. The variation in the production processes will also bring about some modifications (e.g. purification methods, post-traditional modification as glycosylation or sialylation, tertiary or quaternary structures) [21]. Apart from the above two major differences, there also exist some other characteristic differences that are listed below in Table 1.

Table 1. Characteristic differences between generics and biosimilar

Property	Generics	Biosimilar
Molecular weight	< 500-900 Daltons	> 140,000 Daltons
Manufacture	Synthesized in laboratory or extracted from natural sources	Manufactured from genetic material of living cell cultures or DNA technologies
Adverse immune reaction	Lower potential and predictable	Higher potential and unpredictable
Composition	Active ingredients that are identical to the innovator product	Active proteins or peptides that is not identical but similar to innovator drug to a questionable extent.
Approval requirements	Small clinical trials mostly in healthy volunteers	Phase 3 onwards extensive clinical trials in patients.

Development of Biosimilar

The development of Biosimilar is a highly skilled, multi-step process that requires extensive understanding of the reference drug product. The multi-step process involved in the assessment and creation of a Biosimilar are given as follows.

Creating and Producing a Biosimilar

Clinical studies

A first stage in healthy volunteers or patients to test that the body processes the biologic in the same way as the reference product and,

A second stage in patients to test that the Biosimilar works with similar level of efficacy and safety as a reference product.

Comparison between the reference product and biosimilar

Lab testing and clinical testing of drug on patients are performed for bringing out a comparative study of the Biosimilar produced to its reference product. However there still exists an insufficient analytical testing system that lacks the ability to predict certain activity or safety reactions in patients.

Challenges in biosimilar development

The complex and variable nature of biologics and biosimilar presents numerous challenges for manufacturers and also the regulatory bodies such as European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) and New India Guidelines that are responsible for approval process[22-24]. Several such issues include as follows.

Naming and labeling

Each Biosimilar medicine has given a unique and easily recognizable International Non-proprietary Name (INN). This naming program is recommended majorly by WHO with the intention to identify a drug by its active pharmaceutical ingredients. This name is a public property and can avoid confusion among prescribers, pharmacists or dispensers. Pharmacovigilance becomes easy by reporting the specific name of the medicine in case of an adverse event. Substitution should be avoided for both biologics and Biosimilar as this can lead to a delayed adverse event reporting because substitution leads to lack of traceability and tracking of the drug dispensed.

Safety and efficacy issues / Immunogenicity

The large molecular weight and size along with its complex nature, makes the immune system within a body recognize the biologic medicine, giving way to the possibility of a range of immunologic responses. This can result in consequences affecting the efficacy (neutralizing antibodies) and may give rise to anaphylactic or infusion reactions[25].Small changes in any of the production steps of biosimilar can be a reason for alternations in cell behavior and can eventually affect the structural detailing or quality of the product. These changes can in turn disturb the shelf life of the products by interfering with its safety and efficacy, also possibly causing an immune response. Differences in age, gender and subsequent medicines taken can also affect patient response and needs consideration[26].

Quality issues

The manufacturing methods of Biosimilar have concerns about the quality and standards. The manufacturing method for a biosimilar should have the same quality requirements as any new biologic manufacturers and show that their process can produce a high-quality product. The method must include the scientific knowledge by which Biosimilar manufacturer may have access to advanced processes in comparison to the ones used for older originator products.

Substitution issues

The rulings and purpose for substituting biosimilar is different than generics. Generic drugs chemically synthesized can be substituted with their original drugs because they are identical, have the same therapeutic effect and are cost effective. However, in the case of biosimilar such irrational substitution can lead to adverse events and even failure of the intended therapy. This information is significant for a pharmacist and prescriber because a pharmacist can substitute a prescribed reference drug with a biosimilar or vice versa. In case of an adverse event, lack of accuracy in the documentation of the product dispensed to the patient can prove a hassle in pharmacovigilance assessment.

Issues related to regulatory approval

Adequate data of safety and efficacy of post animal and human study, pharmacovigilance and risk management data have to be submitted by the innovator companies and biosimilar producers to the US FDA and EMA for their approval. According to FDA biosimilar guidance announced by US FDA on February 9th, 2012, there exist lists of factors that need to be considered to demonstrate similarity of a biosimilar product to its reference drug. These include an intrinsic understanding of the manufacturing processes, mechanism of action, pharmacodynamics as well as pharmacokinetic studies including the immunogenicity potential and comparison of structural features of the two. Biosimilar do not undergo very extensive clinical trials but have to submit relevant data to be considered equivalent to a reference drug in terms of safety and clinical efficacy.

Pharmacovigilance

All biologic medicines, including Biosimilar, present at least some risk of an unwanted immune response to the medicine, where the patient reacts against the proteins in the medicine. The timely reporting of this and other adverse events is important. The difference that exists in Biosimilar inherently could be the cause for adverse events that might get overlooked at the time of Biosimilar approval**.**

In the pre-marketing period only a fixed number of patients receive the product which is why a very profound or rather extensive pharmacovigilance program is

required for distinguishing immunogenic reaction episodes. Standard methods for detection of immunogenic reactions should be adapted. Lack of validated methods may not give information about the extent of adverse reaction and their management. The ADR monitoring should include complete details of the drug received, whether a reference or Biosimilar, proprietary name and International Non-proprietary Name (INN) along with the exact dosage administered[27].

Biosimilar in India

Biosimilar in India are defined as: "A biological product/drug produced by genetic engineering techniques and claimed to be 'similar' in terms of quality, safety, and efficacy to a reference innovator product, which has been granted a marketing authorization in India by a competent authority on the basis of a complete dossier, and with a history of safe use in India[28].

Here are few factors that facilitate the development and uptake of Biosimilar in India.

• Patent enforcements undertaken by manufacturing companies are guarded by poor laws that give opportunities for domestic biologic manufacturers and private sector partnerships to promote Biosimilar development.

• There are less strict regulatory requirements and low R&D costs, domestic biologics are priced much lower in India compared to originators.

• The low price of Biosimilar compared to originator brands is restricted by limited health insurance coverage and therefore there still exists poor access to biologic drugs.

• Issues regarding the quality and safety of some domestically manufactured Biosimilar and lack of patient education remain a concern among patients and physicians[29-30].

Indian regulations and guidelines on biosimilar production

In June 2012, the Department of Biotechnology (DBT) has announced The New India Guidelines, "Draft Guidelines on Similar Biologics: Regulatory Requirements for Marketing Authorization in India." These guidelines mention the regulatory pathway for a similar Biologic to declare to be similar to an already existing Reference Biologic.

The guidelines mention regarding manufacturing process, safety and efficacy and quality aspects for similar biologics. The guidelines also mention the premarket regulatory requirements along with comparability exercise for quality, preclinical and clinical studies and post market regulatory needs for Similar Biologics.

The Similar Biologics are regulated as per the Drugs and Cosmetics Act, 1940, the Drugs and Cosmetics Rules, 1945 (as amended from time to time) and Rules for the manufacture, use, import, export and storage of hazardous microorganisms / genetically engineered organisms or cells, 1989 (Rules, 1989) notified under the Environment (Protection) Act, 1986.

Various applicable guidelines are as follows:

• Recombinant DNA Safety Guidelines, 1990

• Guidelines for generating preclinical and clinical data for rDNA vaccines, diagnostics and other Biologicals, 1999

• CDSCO guidance for industry, 2008:

– Submission of Clinical Trial Application for Evaluating Safety and Efficacy

– Requirements for permission of New Drugs Approval

– Post approval changes in Biological products: Quality, Safety and Efficacy Documents

– Preparation of the Quality Information for Drug Submission for New Drug Approval: Biotechnological/Biological Products

• Guidelines and Handbook for Institutional Biosafety Committees (IBSCs), 2011

• Guidelines on Similar Biologics: Regulatory Requirements for Marketing authorization in India 2012

Competent Authorities in the Approval Process of Biosimilar

a) Institutional Biosafety Committee (IBSC)

A person in any research institution who is handling the hazardous microorganisms and / or genetically altered organisms need to constitute the IBSC. The main objective of IBSC is to ensure the biosafety and review the applications to be recommended to RCGM. It is also responsible to review and authorize firm for exchange of the above-mentioned organisms for the purpose of research.

b) Review Committee on Genetic Manipulation (RCGM)

The main role of RCGM is to authorize import / export for research and development, exchange of genetically modified organisms for the purpose of research and development and review of data up to preclinical screening.

c) Genetic Engineering Appraisal Committee (GEAC)

Under the Ministry of Environment and Forests (MOEF) the GEAC works as a statutory body to review the research proposals and approve the activities for the

final drug product which contains the genetically modified organisms / living modified organisms.

d) Central Drugs Standard Control Organization (CDSCO)

The main function of CDSCO is to grant the import / export license, clinical trial approval and giving permission for marketing and manufacturing. The State Food and Drug Administration works along with CDSCO for issuing the licenses to the manufacturer of similar biologic in India. Import of drugs for examination, test and analysis for research and development is done by zonal CDSCO.

Factors that favor and withhold biosimilar use in India

Various factors *contribute* in the development and uptake of Biosimilar in India. These include:

• Poor patent enforcement in India provides opportunities to obtain approval for domestically manufactured Biosimilar that are ruling the Indian Biologics market.

• Less stringentregular requirements, lack of monitoring of laws and low R&D costs promotes Biosimilar development. Phase 1-2 clinical trials are not required for copying biologics if pharmaco-equivalence is proved whereas phase 3 trials can be performed on equal to or less than 100 patients. Also Biosimilar development takes approximately 8 years in the Europe, it only takes 3–5 years in India for a copy biologic. For Indian Biosimilar industry cost is a major advantage. In India to manufacture a Biosimilar molecule it need of US $10 to 20 million when compared to developed countries which need US $ 50 to 100 million. It shows that in India there is nearly 40% reduction in cost which makes it attractive place for Biosimilar manufacture[31-32].

• Due to low development and manufacturing costs, domestic biologic manufacturers are able to sell their products at a heavy discount in the range 12- 74% in comparison to the original drug that is sold in Indian market. Such discounting forces the originators to reduce their price to retain their market share.

• Domestic and foreign companies owing to the commercial opportunity are trying to enter into the market of biosimilar. However, these companies face a major drawback for successfully developing, manufacturing and commercializing Biosimilar. This has drawn partnership as a solution. Such mutually beneficial collaborations help the source company with experience and resources whereas the partnering company benefits by establishing itself in the biologics market.

• However, if such drivers of development exist, on the other hand there are certain reasons that become *resistors* in the path of Biosimilar development. Some such are:

a) Low affordability of the comparatively expensive Biosimilar/biologics due to

lack of health insurance is a major drawback for obtaining therapeutic benefit. So majority of the patients fail to afford such expensive treatment options.

b) The physicians still hesitate to prescribe Biosimilar as an alternate to biologics. Moreover, there has been noticeable difference in the potency between several Indian Biosimilar compared to their reference products. Manufacturing processes being different introduces impurities that can pose a threat to the final product. In order to minimize the risk of immunogenicity prescribers often avoid any such substitution[33-34]. The Biosimilar which are produced in India after the amendment of "Similar Biologic guidelines" were shown in Table 2.

Table 2. Biosimilars produced in India after "Similar Biologics Guideline" which came into effect on 15 September 2012[29-31]

Product name	Active substance	Therapeutic area	Approval/ launch date in India	Company
AbcixiRel	Abciximab	Angina, Cardiac ischemia	Apr-13	Reliance Life Sciences
Actorise	Darbepoetin alfa	Anaemia, Cancer, Chronic kidney failure	Jan-14	Cipla/Hetero
Adfrar	Adalimumab	Ankylosing spondylitis, Plaque psoriasis, Psoriatic arthritis, Rheumatoid arthritis, Ulcerative colitis	Jan-16	Torrent Pharmaceuticals
Bevacirel	Bevacizumab	Colorectal cancer	Jun-16	Reliance Life Sciences (Lupin)
CanMab	Trastuzumab	Breast cancer	Oct-13	Biocon
Cizumab	Bevacizumab	Colorectal cancer	Jun-16	Hetero
Darbatitor	Darbepoetin alfa	Anaemia, Cancer, Chronic kidney failure	2014	Torrent Pharmaceuticals
Etacept	Etanercept	Ankylosing spondylitis, Rheumatoid arthritis, Psoriatic arthritis, Psoriasis, Juvenile rheumatoid arthritis	Apr-2013	Cipla
Exemptia	Adalimumab	Rheumatoid arthritis	Sep-14	Zydus Cadila
Filgrastim	Filgrastim	Neutropenia	Oct-13	Cadila Pharmaceutical
Filgrastim	Filgrastim	Neutropenia	Mar-13	Lupin

Product name	Active substance	Therapeutic area	Approval/ launch date in India	Company
Folisurge	Follitropin alfa (follicle stimulating hormone)	Female infertility, Spermatogenesis in men	May-13	Intas Pharmaceuticals
Infimab	Infliximab	Ankylosing spondylitis, Crohn's disease, Psoriasis, Psoriatic arthritis, Rheumatoid arthritis, Ulcerative colitis	Sep-14	Epirus Biopharmaceuticals
Intacept	Etanercept	Ankylosing spondylitis, Juvenile idiopathic arthritis Psoriasis, Psoriatic arthritis, Rheumatoid arthritis	Mar-15	Intas Pharmaceuticals
MabTas	Rituximab	Lymphoma, Non-Hodgkin's Lymphoma	Feb-13	Intas Pharmaceuticals
Molgramostim	Recombinant human granulocyte macrophage colony stimulating factor (molgramostim)	Neutropenia	May-13	Zenotech Laboratories
Peg-filgrastim	Pegfilgrastim	Cancer, Neutropenia	Sep-13	Lupin
Peg-interferon alfa 2b	Pegylated recombinant human interferon alfa 2b	Chronic hepatitis B, Chronic hepatitis C	Apr-13	Intas Pharmaceuticals
Rasburicase	Rasburicase	Malignancy associated hyperuricemia	Aug-12	Virchow Biotech
Rituximab	Rituximab	Non-Hodgkin's Lymphoma, Rheumatoid arthritis	Feb-15	Reliance Life Sciences
Teriparatide	Teriparatide (parathyroid hormone)	Post-menopausal women with osteoporosis who are at high risk for fracture	Aug-12	Cadila Healthcare
Wosulin	Human insulin	Diabetes mellitus	Aug-03	Wockhardt

CONCLUSION

The recent expiration of patents for some important biologics have given a ray of hope to the biosimilar manufacturers. As these products are proteins, their safety and efficacy will always be considered a concern that should require stringent analysis and pharmacovigilance. Awareness between biosimilar and innovator products in terms of safety, efficacy and immunogenicity is essential for effective prescription and safety of the patients. India the leading producer of biosimilar should frame effective guidelines, nomenclature regulations and proper pharmacovigilance program.

CONFLICT OF INTEREST

There is no conflict of interest between the authors.

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Branding of Prescription and Non-prescription Drugs

Merve Memişoğlu[1*]

[1]Biruni University, Faculty of Pharmacy, Department of Pharmacy Management, 34010 Istanbul, Turkey

ABSTRACT

It is worthwhile to note that, while health related products are mostly function driven categories, health in itself is an emotive and personal subject. With this perspective, pharmaceutical companies need to focus on understanding the importance of branding in this rapidly changing social behaviour, competitive landscape, communication tools and techniques.

Although branding in the pharmaceutical industry has been changing for the past decade, major issues for industry stakeholders like healthcare professionals and patients are still ethical communication and marketing strategies. For pharmaceutical branding, companies should be engaged with related stakeholders in an effective and ongoing way while complying with regulations.

The purpose of this review is to evaluate the importance of branding for pharmaceuticals and underline the sentimental and functional benefits to consumers and/or patients. The focus of this review lies on branding and marketing communication process of pharmaceutical products and also providing insight for further pharmaceutical branding studies.

Keywords: OTC, over-the-counter drugs, prescription drugs, pharmaceutical marketing, pharmaceutical branding.

INTRODUCTION

What is Brand and Branding?

There are multiple definitions to this question, but according to the American Marketing Association (AMA), brand is defined as follows; "a brand is a name, term, design, symbol, or any other feature that identifies one seller's good or service as distinct from those of other sellers."[1]. In short, branding makes the product different and easily identifiable. A successful brand also survives the rest of time or "can be timeless" as quoted by Stephen King.

There are other definitions; Kotler et al. explained as "the brand creates value to the customers and can also become a competitive advantage for the company."[2].

*Corresponding author: Merve Memişoğlu, e-mail: mmerve.memisoglu@gmail.com, Tel: +904448276
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Schuiling and Moss also described a brand as "a name that will register the product in the consumers mind as a set of tangible and intangible benefits."[3].

Brand strategy is how, what, where, when and to whom they plan on communicating and delivering on the brand messages. In order to build a brand, companies must develop a brand strategy. Brand strategy building blocks include: brand positioning, brand personality, brand values, brand appears and perceived qualities which are based on product features such as reliability[4-5].

A brand can be divided into six different levels as attributes, benefits, values, culture, personality and user[2]. The value is recognised as one of the most vital part of branding, which in itself has three types: functional, expressive and central values. Normally, successful brands have a combination of value types[3,6-7].

Currently, people demand to be treated as whole human beings with minds, hearts, and spirits. Therefore brands should especially meet consumers deeper needs for social, economic, and environmental justice[8].

Kotler described brand meanings through different levels as listed in Table 1[9].

Table 1. Brand Meaning Levels[9]

Meaning Levels	Description
Attributes	Certain attributes that come to mind.
Benefits	Attributes must be translated into functional and emotional benefits.
Values	The brand says something about the manufacturer's value.
Culture	The brand may represent a certain culture.
Personality	The brand can project a certain personality.
User	The brand suggests the kind of customer who buys or uses the product.

Brand development journey often involves a combination of elements to co-exist like; a good name, recognisable logo, a slogan, a symbol, jingles, packaging, shape and colour. For example; a good slogan or jingle can help the brand to be kept in customers' mind. Together these elements contribute to the brand identity[10].

Possible benefits of branding are listed by Hoeffler and Keller[11-12] include the following attributes;

- improved perceptions of product performance;
- increased customer loyalty;
- less vulnerability to competitive marketing actions and marketing crises;
- larger margins;

- elastic customer response to price changing;
- greater trade or intermediary cooperation and support;
- increased marketing communication effectiveness;
- additional licensing and brand extension opportunities.

Branding is not only prominent for pharmaceutical products but also for other healthcare products such as probiotics and prebiotics[13].

What are the Differences Between Pharmaceutical and Fast-Moving Consumer Goods (FMCG) Industry?

The pharmaceutical industry is associated with a strong R & D as well as extensive sales force. Yet in time the pharmaceutical industry has been facing several challenges which include high cost of R & D, the patent expiration of products in parallel with the erosion of price and consolidating of the pharmaceutical companies[7].

A schematic perspective outlining the difference between the pharmaceutical industry and the FMCG industry would be as below;

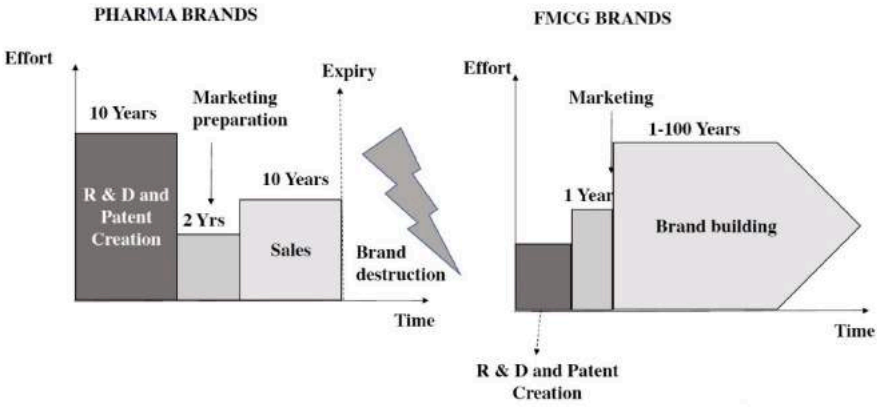

Figure 1. Differences between Pharma and FMCG brands[14]

The pharmaceutical industry has strict regulations. For this reason, a new product launch into the market is quite slow. Pharmaceutical products have relatively short-term patent protection. After patent expiration, pharmaceutical brands cannot be transferred in contrary to FMCG products. While brands of prescription drugs survive max 10 years, brands of non-prescription drugs can continue for a long time like FMCG products (Figure 1).

Given today's technology and sciences, when a new molecule discovery is made, which is more effective for a certain treatment, existing brands lose their importance[6]. Weston has suggested that 'super brands' are capable of surviving patent

expiration and can achieve "a bond with the consumer at a level transcending mere functional performance"[15].

Conversely, FMCG companies integrate brand development early in the product development cycle. The difference thereby is pharmaceutical companies focus on developing products rather than the brand. The latter have not been as efficient in using the power of brands. Yet, in certain markets like United States, certain OTC products have extensive advertising campaigns. This factor has led Moss and Schuiling to claim that branding in pharmaceuticals is ten years behind the FMCG industry[7].

Brand building is a long-term process and advertising is one of the key aspects. Within this concept, new strands have emerged in certain markets like that of direct to consumer advertising (DTCA). The advertising of prescription drugs is banned in a large extent of countries. The United States and New Zealand are the only countries that allow DTCA on television and other broadcasting media for prescription drugs where the consumer has little choice in drug selection[16]. Canada also does not allow advertisement which cover both the product and related indication[17-18].

In countries like Turkey where DTCA is not allowed, marketing activities of prescription drugs are based on functional attributes of products such as clinical and product related features. Promotion is directed primarily at healthcare professionals and includes several marketing activities such as personal sale, drug detailing, advertising in journals and medical administration software, sponsoring educational events, educational materials, promotional merchandise, public relations activities and disease awareness campaigns (DACs)[19].

Conversely, the regulatory restrictions are waived for OTC or non-prescription drugs as the consumer has direct access right to choose. Regarding OTC, products are allowed to be advertised to the public. OTC branding is similar to FMCG branding at many points and the aim of branding is to achieve brand recognition, brand preference and brand loyalty, increase market share accordingly.

End consumers are an important part of the purchase decision for OTC drugs making the role of brands very significant. Promotion is directed towards consumers/patients as well as healthcare professionals and includes several marketing activities such as **DTCA (including TV, radio, print ads, etc.),** detailing, sponsorship, events, direct mail, promotional merchandise, public relations activities, social media and viral marketing. For the OTC marketers, the most important question should be what the audience needs because brand association or brand image comes from customers' needs.

Table 2: Differences between Pharma and FMCG brand building[7,14]

FMCG	Pharmaceuticals
Focus on brand creation	Focus on product creation
Long time- decades	Short time-months, years
Lifecycle doesn't need to exist	Lifecycle management necessary
Marketing effort create the brand	R&D effort create the product
Investment maintains the brand	Patent expiry signals loss of resources
Brand destruction does not exist, portfolio rationalization does	Product cast out to provide revenue for new products
The brand is the asset	The product is the asset
Strategy and brand management	R & D and sales management
Brand name can be transferred	Brand name cannot be transferred to another molecule following patent expiry
The end users are simply the consumers	Doctor and pharmacists represent an additional layer between patient and company

Regarding the brand name strategy, FMCG sector adopts three basic brand name strategies. These names are descriptive brand, new brand and corporate brand.

Conversely, pharmaceutical brand name strategies utilise differing focus points. These range from the scientific name of the active substance (Indocin for Indomethacin), the therapy (Procardia), the indication (Glucophage), the class (Mevacor/Zocor) the corporate name (Novarapid-Novo Nordisk) and a new brand name (Zantac, Prozac).

Except the well known corporate and new brands, pharmaceutical brand name could be imitated and generics can threaten the brand name, image or positioning easily[7]. In some cases, corporate and product brand names may be closely associated which could impact each other and thus care must be taken in brand management. For example, the Merck corporate brand was negatively affected following the withdrawal of nonsteroidal anti-inflammatory drug Vioxx[7].

The benefits of branded pharmaceuticals towards different stakeholders such as prescriber, patient, payer and manufacturer are listed below according to Dogramatzis[20].

Table 3. The benefits of branded pharmaceuticals[20]

Prescriber	Patient	Payer	Manufacturer
Efficacy	Efficacy	Efficacy	Competitive advantage
Risk reduction	Risk reduction	Safety	Easier segmentation
Trust	Trust	Standardization	Global marketing
Patient retention	More information	Risk reduction	Customer loyalty
More information	Feeling good	Trust	Product bundling
Sponsorships	Convenience	Economies of sale	Easier line extensions
	Easier comparisons		Shorter time to market
	Company accountability		Image building
			Premium pricing
			Employee morale

Pharmaceutical Environment

To understand pharmaceutical branding, we should evaluate the environment of the pharmaceutical companies first.

Bendarik[21] mentioned in his article that the pharmaceutical industry is affected by several political, economic, social, technological, legal and environmental factors. It is believed that the most remarkable among them are social, technological and legal factors. Legal factors involve issues regarding patent protection and all regulation processes. It is a rather short period of time for companies to build the business of their product before the generic drugs capture the market. The market is highly regulated in every aspect.

Social factors cover the trends of aging population, self-medication, a desire to increase the life expectancy and most common chronic diseases such as diabetes or cardiac diseases. Therefore, pharmaceutical companies are building on the development of new drugs meant to target population and specific diseases[21].

With social and technological changes, it would appear that the consumers are becoming more educated and capable. With information everywhere, the consumer and patient is, able to drill deeper to seek; manufacturer details and product comments by others. To counter this information thirst and to create trust, manufacturers are disclosing more corporate aspects like sustainability, corporate responsibility and employee working conditions[22].

With change in corporate and consumer attitudes, the media sector has also changed dramatically. The new trend or communication medium is clearly the internet and social media as opposed to traditional channels like TV, radio, newspaper.

As the internet is becoming a much more powerful vehicle for branding and communications, the role of mass media is becoming less effective[12,23-24].

With the internet, interactivity between stakeholders, especially corporate and patient/consumer, are increasing. Within this framework, the use of social networks, mobile application platforms and internet user groups allow users to contribute with brand-related content, and share their experiences and opinions with other consumers anytime and anywhere [25].

New technologies (including devices and information platforms) like fast adaptation to smart devices is also educating the consumer to rely on self-medication and positively impacting the OTC market[26].

How Can a Successful Pharmaceutical Brand Be Built?

While branding creates awareness, brand positioning creates product differentiation. A strong brand creates high consumer loyalty and can influence the behaviour and attitude of patients and healthcare professionals directly[3,26].

In a survey conducted by Battistoni et all in 2014, it was seen that the benefit of OTC brand is most effective (60%) in determining consumer behaviour. Among the benefits, the communication strategy has the highest effectiveness. Within which the most prominent element was communication strategies. These strategies ranked advertising first (20.74%), media coverage second (9.71%), popular references (8.83%) and corporate social responsibility (7.32%)[27].

The fact remains that medical product communication and medical brand communication should not be confused with each other. Because the brand has a more flexible nature than the product, it can easily pass without being affected by environmental factors and barriers/limitations. In contrast, there are risks at various stages of product communication[28].

The power of branding has two assets. First, a powerful brand is borderless- can cross the borders and markets. Second, a powerful brand can also influence behaviour and attitudes. For example; Prozac has acquired almost iconic status with the term of 'Prozac generation' since introduction of Prozac to the market in the late 1980s.

From the perspective of the pharmaceutical industry, the following functional benefits are significant: 1. Efficacy 2. Safety (minimal adverse effects etc.) 3. Convenience (dosage form, pleasant taste/way related dosage administration etc.) 4. Cost-effectiveness[5].

Since, functional benefits are not the only way to encourage brand loyalty, companies must create the need for the consumer to be loyal by touching the consumers in an emotional manner. Next, companies must decide how to communicate these benefits in a consistent way to the target customers. This method

allows long term brand loyalty by establishing an emotional dialogue with the consumer via a sustained campaign[12,29-30].

Constitutively one of the most powerful emotional values in the pharmaceutical field is trust. Although trust in pharmaceutical brands is relatively low, once trust is gained, it can be contributed to the brand building process most successfully. A case in point is, the longevity of OTC products, is due to trust build up over the years[31].

For increasing brand loyalty, companies use several approaches which include education, logistic and reimbursement support. For example; physician-targeted continuing medical education (CME) schemes and patient-targeted disease awareness sites as education examples, such as "For My Bones" program (Novartis) as a logistic example and SHARE® program (Lundbeck) as an example of reimbursement support[32].

Another report by Roblek[33] et al show that consumer satisfaction with safety and product information had a significant effect on consumer trust. In addition to consumer trust, corporate social responsibility (CSR) had also a significant effect on virtual brand loyalty. Consumers can make brand changes according to their corporate social responsibility activities.

Successful brands are those who use a combination of any of the brand elements which include logos, slogans, packaging, names, characters and symbols. Furthermore, according to Pilling[34], both physicians and patients are more susceptible to pharmaceutical branding activities when they are supported by clinical evidence.

Positioning - The First Step to Branding

Positioning is the vital step of pharmaceutical branding. The message type does not impact positioning. Both the positive statements (features and benefits) and also negative statements (adverse events, overdose, contraindications and drug interactions) can be used for choosing, targeting and positioning strategies[20].

The proper positioning has been determined by Dogramatzis as follows[20];

- Identify competitive products (in both category and brand)
- Identify determinant attributes (Features, benefits, applications)
- Measure existing perceptions (recalls)
- Analyse competitive and relative position of alternatives (prescription, non-prescription)
- Determine preferred set of attributes (customer preference, survey)
- Define positioning (Competitive strengths of different brands)
- Devise repositioning (growth of segments, emerging attributes, new brands, new segments…etc.)

Within positioning, brand personality is an important differentiating factor. Within advertising the visual identity can include positioning as; masculine or feminine, young or old, radical or conservative, according to visual identity and advertisement[4].

Overall, the audience must see the combination of the brand name, packaging design and advertising. Brand personality is usually achieved through visual imagery and the use of celebrity endorsements[35].

Integrated Marketing Communication (IMC) - Speaking with One Voice

Successful branding ensures a consistent and continuous refined message and it can be possible by integrated marketing communication.

If one can bridge the gap within the audience perceptions hence the gap filler brand communication has achieved its objectives.

According to Kotler IMC is from reliance on one communication tool such as advertising to blending several tools to deliver a consistent brand image to customers at every brand contact[9].

IMC represents marketing tools, approaches (personalised, customer-oriented, technology–driven) and resources as a whole. All marketing communication must be integrated to create awareness of the brand, to link the brand image in consumers' memory and to facilitate a stronger bond.

Consumers meet the brand through advertising (such as: media advertising; direct response and interactive advertising; place advertising; point- of-purchase advertising), internet marketing, event marketing and sponsorship, direct marketing, database marketing, trade promotions, consumer promotions, publicity, public relations and personal selling[36].

On the other hand, the digital age has changed marketing channels. TV advertisement lost popularity among consumers especially young people. As time goes on, online and mobile advertising are of growing importance. Consumers can be informed about products and services without the limitations of time and place faced by traditional mass media. Social networks such as Facebook, Twitter, Instagram and LinkedIn have cultivated vast audiences. IMC is the exact key to success in the digital environment.

According to Mulhern, the principles of IMC are consumer insight, data-driven decision making and communications with multiple stakeholders represent an improved framework for managing communications in a digital world[37]. Regarding interactive marketing communication, it includes websites, search ads, dis-

play ads, videos, sponsorships, on-line communities, e-mail and mobile marketing[12].

IMC campaigns allow a celebrity speaking about a medical condition. For example; Pfizer first launched a massive advertising and public relations campaign with celebrities for branding Viagra[38].

There is an increasing trend in Australia, where marketers use "Un-named" product advertisements which were promoted to consumers without the product name and Disease State Awareness **(**DSA) campaigns which are implemented for brand building[39].

As it turns out, the disease is also branding by IMC to build public awareness and make the complexities of current and new diseases easier to understand.

Branding is widely used in several health awareness campaigns. For example "truth®" is a national tobacco prevention campaign for youth and young adults via branding techniques. Branded health campaigns differs from other branding activities mainly due to the fact that the brand object is voluntary. Successful health branding has the potential to decrease risky behaviors such as drug abuse, unsafe sex, unhealthy diet etc prominently[40].

The objectives of "The Heart Truth" as another branding campaign are increasing awareness among women about their risk of heart disease, encouraging women to talk to their doctors, finding out their risk and taking action to reduce them[41].

Signs and symptoms and their association with the disease play a key role in moving the patient to seek diagnosis and medical help. For example; a pink ribbon to mark breast cancer awareness.

Diseases often gain additional publicity with celebrities undergoing a case history. A recent case is of the Australian pop star Kylie Minogue who recovered after diagnosis with breast cancer or the cyclist Lance Armstrong who fought against testicle cancer[42-43].

As a Communication Tool, Storytelling

Storytelling is essential for successful branding. For pharmaceutical companies, storytelling is a relatively hot topic to communicate and share the key insight with different stakeholders. Pharmaceutical companies engage consumers through the art of storytelling. Marketers should provide truth via real patient stories. Marketers should also use social media to listen to patients and to leverage those stories to help motivate other patients and to enhance the image of their brands[44-45].

Fog et al. emphasized that today's strong brands are built on clear values and emotional connections with consumers; this is where storytelling comes to use to incorporate brand values into stories, develop emotional bonds with consumers and create consistent brand images[46].

Storytelling has positive effects on brand attitudes and purchase intentions. Compared to regular advertisements, storytelling can also make a brand more memorable in multiple ways (visually, factually and emotionally) and help to understand the benefits of the brand[44, 47-50].

Direct to Consumer Pharmaceutical Advertisement (DTCPA): The Good or The Bad?

With the information flow comes noise. Consumers are exposed to too many ads. Synchronously, consumers are paying less attention to the advertisement. The main reasons of this dramatic decrease are that they have lost faith in truthfulness of ad messages and they can access more information using the web among many channels[51].

There are currently three types of DTC pharmaceutical advertisements; 1) Reminder advertisements (limited information such as the name of drug, pricing and strength) 2) Help-seeking advertisements (only information about a disease or condition without mentioning a particular treatment) 3) Product-claim advertisements (the indication, efficacy and safety information)[52-55]. Pharmaceutical promotion should have both informative and persuasive elements[56].

DTCPA has several advantages like; educating, encouraging patients to contact a clinician, promotes patient dialogue with doctors, strengthens a patient's relationship with a clinician, encourages patient compliance, reduces underdiagnoses and under treatment of conditions and encourages product competition and lower prices[57-58].

On the other hand, DTCPA has also some disadvantages which include misinforming patients, overemphasizing drug benefits, encouraging drug overutilization, leading to inappropriate prescribing, wasting appointment time, nonrigid regulations and increasing costs[59-60].

For example; rofecoxib (Vioxx, Merck) was promoted but its side effects were neglected during advertising. After the discovery of serious side effects of Vioxx, it has been withdrawn from the market. Some heavily promoted drugs such as the diet pills Pondimin and Redux have been also withdrawn due to their potential harm[61-63].

Even though DTCA is a major factor in patient perception or awareness of cer-

tain medications, promotional activities directed at the medical community and particularly the promotion of free samples of medications are noted as the most effective means of influencing a physician's prescribing decisions[53,64].

In some studies, it is shown that consumers attend to and rely on advertising less and respond more to emotional ads than to information ads[65].

There has been an increase in the use of the Internet for prescription drug promotion which is an evolving area of interest for the pharmaceutical industry and regulators[66].

DTC marketing can be made in the US even for prescription drugs. The biggest danger here is that the consumer is more interested in attractive promotional messages and ignore the warnings in prescription drugs. Previous studies have shown that 33% of DTC advertising did not present accurate and balanced explanation of risks and benefits and only 12% of DTC presented useful information (drug interaction, overdose, etc.) for the consumer. For this reason, companies should focus on giving information to the customers rather than attracting them[67-68].

It is of course important that the public be informed about health, but the main problem is the content of the advertisement given as information.

On the other hand, unethical strategies such as the astroturfing are also applied by pharmaceutical companies in some countries where DTCA is banned[69].

As it turns out, potential ethical problems in pharmaceutical branding can be expected to arise in the future[70-71]. Finally, World Health Organisation's (WHO) ethical criteria for pharmaceutical promotion should be taken into consideration in this review. WHO states that all promotion-making claims concerning medicinal drugs should be reliable, accurate, truthful, informative, balanced, up-to-date, capable of substantiation and in good taste. They should not contain misleading or unverifiable statements or omissions likely to induce medically unjustifiable drug use or to give rise to undue risks[72].

CONCLUSION

It can be concluded that on the supply side, branding plays an increasingly important role for the pharmaceutical industry. The availability of the internet will create far greater public knowledge about pharmaceutical products and healthcare. On the demand side, patients have become strong advocates of their own healthcare as increasingly undertaking their research. In short, marketers need to fully understand the psychology of illness and consumers' health beliefs about therapeutic categories before creating an advertising campaign.

It should not be forgotten that side effects are a part of every drug. However, they

are never part of the brand.

For non-prescription drugs, pharmaceutical companies use the same branding techniques as FMCG marketers. On the other hand, branding of prescription drugs have a potential risk ahead. In branding process, marketers should ensure balance of message instead of mass promotion. The best way to achieve this balance is by taking responsibility to the public to act in an ethical manner.

Even if in this circumstance, marketers can build a successful pharmaceutical brand. They have new tools for the communication. In an increasingly networked environment and power of patients, companies should focus on fewer product advertisements and more educational events aimed at raising awareness of a disease.

Overall, this reviewed literature on pharmaceutical branding suggests that it will be possible to develop pharmaceutical brands in a way to promote trust in the product.

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Proposal for an "Althaeae flos (marshmallow flower)" Monograph set-up based on European Pharmacopoeia Template and Quality Evaluation of Commercial Samples

Esra Saçıcı[1*], Erdem Yeşilada[1]

[1]Yeditepe University, Faculty of Pharmacy, Department of Pharmacognosy, İstanbul, Turkey

ABSTRACT

Although marshmallow flowers are used more frequently than leaves and roots, only Althaeae folium and Althaeae radix monographs exist in European Pharmacopoeia. The aim of study was to set standards for marshmallow flowers which will be used for its quality assessment. Althaeae flos monograph was prepared based on the main principles described in European Pharmacopoeia for herbal drugs. After setting the limits, the specifications of 33 marshmallow samples purchased from herbal markets were studied to determine their suitability for the proposed monograph. Results have shown that all the commercial samples were obtained not from *Althaea*, but *Alcea* species, a close gender, based on the flowers size. Since main characteristics of both flowers were determined to be identical, this would not rise a problem. However, residual parts of insects or snails were found in 28 samples and thus they were evaluated unacceptable to be used in phytotherapy.

Keywords: Althaeae flos, marshmallow flower, Malvaceae, monograph, quality

INTRODUCTION

There are 4 species represent of *Althaea* L. genus (Malvaceae) in "The Flora of Turkey and the East Aegean Islands".[1] Among these species, *Althaea officinalis* L. (marshmallow) is indigenous to Turkish flora and it is native in Asia, Europe and China.[2,3] In Turkey, *A. officinalis* is commonly named as "hatmi, tıbbi hatmi, devegülü, fatma çiçeği, erkurtaran/ergurtaran çiçeği".[4-7]

The name of *Althaea* genus is derived from a word "althaino" that signifies "therapy, treatment" in Greek because of the healing potential of the plant and the species name "*officinalis"* signifies "to use in medicine".[8]

*Corresponding author: Esra Saçıcı, e-mail: esrascc@gmail.com
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According to the studies on chemical constituents, astragalin[9], 4-hydroxy-benzoic acid[10], ferulic acid[10], hypolaetin 8-o-gentiobioside[11], hypolaetin-4-o-methyl ether-8-o-β-D-glucoside[11], dihydrokaempferol 4-o-β-D-glucoside[11], dihydrokaempferol 4-o-glucoside[9], naringenin-4-o-β-D-glucoside[11], naringenin-4-o-glucoside[12], p-hydroxy-phenyl acetic acid[13,14], populnin[10], protocatechuic acid[14], isoquercitrin[10], salicyclic acid[13], scopoletin[14], sinapic acid[14], spiraeoside[12], syringic acid[13], tiliroside[15] and also vanillic acid[10] were isolated from the flower parts of *A. officinalis*.

In vitro antioxidant effect of the flower extract from *A. officinalis* was investigated and reported to possess strong antioxidant effect, i.e., significant metal chelating, superoxide anion radical scavenging, free radical scavenging and reducing power actions.[16] In another study, the total flavonoids content and the antioxidant properties of Althaeae flos were comparatively determined in terms of the petal colors, including white, pink and reddish pink. Although white petals possessed the highest flavonoid content, reddish-pink colored petals exhibited the highest antioxidant activity.[17]

In a study, possible role of the aqueous extract of Althaeae flos in platelet aggregation, lipemia, inflammation and gastric ulcer was examined in rats. It was reported that platelet aggregation was inhibited time-dependently. Serum HDL-cholesterol level was significantly increased, while no effects on stool cholesterol and triacylglycerol were observed. The aqueous extract also showed anti-inflammatory activity on acute and chronic inflammation models and also anti-ulcerogenic effect was reported without any apparent adverse effect.[18] In another study, hexane extract of Althaeae flos collected from northwestern Iran was found to contain fatty acid composed of alpha-linolenic acid, palmitic acid, heptacosane and nonacosane, and the extract displayed a good antimicrobial activity against *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae*.[19]

Roots and leaves of *Althaea* species have been used in traditional medicines worldwide for centuries as expectorant, demulcent, diuretic, and emollient for the treatment of cough, upper respiratory tract inflammations, gastritis, peptic ulceration, renal (i.e. cystitis) and uterine disorders.[20-23] However records for use of its flowers in traditional medicines are rare. Hot water extract prepared from the dried marshmallow flowers was utilized externally (in Peru)[24] or orally (in France)[25] as an emollient or as expectorant in India.[26] Additionally, decoctions prepared from the flowers and leaves were taken orally against asthmatic symptoms in Italy.[27] In Greece, root and flowers of *A. officinalis* have found a wide range of applications including coronary disorders, hypertension, consti-

pation, diarrhea, enteritis, gastritis, stomatitis, cystitis, kidney stones, nephritis, blood purification, laryngitis, pharyngitis, common cold, bronchitis, aphthae, gum wounds and pimples.[28] In the Iranian traditional documents, the flowers, leaves or roots of *A. officinalis* have all been suggested orally for gastrointestinal and respiratory system inflammations as well as externally for burns and insect stings.[29] Furthermore, as an evidence of safety of *A. officinalis*, it is listed by the Council of Europe in category N2 as a natural food as well as in the United States. [22,30,31]

In Turkey, infusions obtained from marshmallow flowers have been used internally for bronchial complaints, cough and cold due to its rich mucilage content.[5,32] Decoction of marshmallow root have been used internally for the treatment of gastritis, peptic, mouth and throat ulcerations as well as for respiratory tract irritations as gargle.[4] In East Anatolia, infusion obtained from *A. officinalis* aerial parts has been used as diuretic and antilithic in the treatment of kidney stone or sand.[33] It is reported that in Aydıncık district (Mersin), infusion prepared from *A. officinalis* herb has also been used orally as one teacup 3 times per day for 2-3 weeks or used as gargle for 3-5 successive days against stomachache, as well as asthma, cold and flu symptoms.[7] [Note: In both studies the plant part used as remedy was specified as "herb"]. Moreover, *Althaea* roots are externally applied on wounds to keep the skin soft and for wound-healing.[4]

Despite *A. officinalis* flowers have found a place in traditional medicinal practice, in the European Pharmacopoeia (Ph. Eur.), only the qualifications of its roots and leaves are described under two monographs; Marshmallow leaf (Althaeae folium)[34] and Marshmallow root (Althaeae radix)[35]. Although in French Pharmacopoeia the flowers were registered together with the roots and leaves as a herbal drug for the treatment of cough, but no specification was described except the common macroscopic and microscopic characteristics.[36] On the other hand, in Turkey only the dried flowers of the plant have been available in the herbal drug market, the official herbal drugs, i.e., roots or leaves of marshmallow, are not available. Eventually, there is no official data to describe the specifications of marshmallow flowers to determine its qualification. Therefore, the first aim of the present study was to define the macroscopical, microscopical and chemical characteristics of marshmallow flowers. Secondly, a draft monograph proposal for "Althaeae flos (marshmallow flower)" was suggested to set the standards for the herbal drug by predicating on the analysis criteria in the Ph. Eur. In compliance with the proposed template, quality evaluation was carried out on the marshmallow flower samples purchased from herbal shops.

MATERIALS AND METHODS

Plant materials and solvents

Thirty-three samples of marshmallow flowers were purchased from local herb markets in İstanbul and Tekirdağ (Turkey). The other herbal material *Alcea* sp. flowers collected from Karaman, Konya (Turkey) in order to be used in this work and they were dried in shade. In this study, specimens of marshmallow were encoded as A1-A33 throughout the study for reasons of confidentiality. All of these herbs were powdered by a laboratory mill. Ethanol and sulphuric acid were from Sigma-Aldrich (Steinheim, Germany); acetic acid, *n*-butanol and methanol were purchased from Riedel-de Haën (Seelze, Germany), Fluka (Steinheim, Germany) and Analar Normapur (Muarrie, Australia), respectively.

Methods

Macroscopic examination

The morphological characteristics of the whole materials were determined under the Stems DV4/DR Carl Zeiss binocular loop. Epicalyx, calyx, corolla, stamen, filament, anther, ovary, stylus and pedicel parts of these materials were evaluated. In addition, the petal lengths of marshmallow flowers and *Alcea* sp. flowers were measured by a ruler.

Microscopic examination

The microscopic properties of the powdered materials were determined under a Carl Zeiss AxioVision electronical microscope and a Nikon E200 microscope using chloral hydrate solution. Characteristic elements i.e. curved, stellate or glandular trichomes, calcium oxalate crystals, pollen grains and stomata were examined.

Detection of foreign matter

The amounts of foreign organs and foreign elements of the entire flower materials were calculated according to the technique for determination of foreign matter described under Methods in Pharmacognosy in the Ph. Eur. 50-100 g of the whole samples were weighed. Then, they were spread on a white paper and insects, snails, feathers, bristles and stones were separated by examining with unaided eye as well as a magnifying glass. The removed foreign materials were simply weighed and the percentages were calculated.

Loss on drying experiment

The quantities of losses on drying of all the samples were evaluated according to technique for loss on drying under Physical and Physicochemical Methods in

the Ph. Eur. 1 g of the whole material was weighed in capsules, and dried in the drying oven at 105 °C for 2 hours. Then, capsules were cooled, reweighed, and the percentages were calculated.

Total ash experiment

The ash amount of each material was determined according to the method described under Limit Tests in the Ph. Eur. for total ash. All the crucibles were heated for 30 minutes, cooled in a desiccator and tares of them were weighed. 1 g of the powdered substance was weighed in a crucible, and then was ignited in a furnace at 600 °C. After each burning, the crucible was removed from furnace, cooled in a desiccator and weighed when it was cool and then the total ash percentage was calculated.

Swelling index experiment

The swelling index of all samples were calculated according to the method described under Methods in Pharmacognosy in the Ph. Eur. 0.2 g of each of the powdered material was weighed and put in a 25 mL ground-glass stoppered special cylinder graduated in 0.5 mL divisions. The material was first humidified with 0.5 mL of ethanol, and 25 mL of distilled water was added. Then each glass cylinder was enclosed and shaken every ten minutes for one hour period, and left to stand for three hours. After 90 minutes from the start of the experiment, the cylinders were turned around their axes in order to mix large volumes of liquid retained in the layer of the drugs and fragments of these drugs floating on the top of the liquid. At the end of the test, the volume occupied by the swelling drug, which indicates mucilage content, was measured. Three individual experiments were performed for each material at the same time. The average of three trials was estimated as the swelling index.

High Performance Thin-Layer Chromatography (HPTLC) analysis

The analyses were carried out on alumina HPTLC plates covered with silica gel 60 F_{254} (Merck, Darmstadt, Germany). 1 g of each powdered material was weighed, and 10 mL of 60% ethanol was added. After stirring for 15 min, they were filtered to prepare test solutions. These solutions were applied on the plates as 9.0 mm bands by a mean of 100 µL syringe (Hamilton, Bonaduz, Switzerland) via Camag Linomat V sample applicator (Muttenz, Switzerland). The plates were developed in a twin through glass chamber saturated with the mobile phase. The same mobile system [acetic acid-water-butanol (15:30:60, v/v/v)] recorded in "Malvae sylvestris flos" monograph in Ph. Eur. was used. After developing the plates, they were dried. Chromatograms were displayed by exposing to UV-light at 366 nm using Camag TLC Visualizer. Then, the plates were sprayed with sul-

phuric acid 5% in methanol (H_2SO_4) reagent. The derivatized plates were heated at 105 °C and documented. All the devices were managed by WinCATS software.

RESULTS

The findings of thirty-three marshmallow samples and *Alcea* sp. materials were evaluated by using the data presented under "Malvae sylvestris flos" monograph in the Ph. Eur.[37] This monograph was used as a guiding reference because *Malva sylvestris* is a close member to *Althaea* sp. from the same family.

Macroscopic results

Macroscopic examinations of all commercial marshmallow materials demonstrated that they had similar morphological features to *Alcea* sp. On the other hand, petal lengths of all these marshmallow materials were not within the limits specified for *Althaea* L. in "The Flora of Turkey and the East Aegean Islands", but they were similar to that of the *Alcea* sp. (Table 1). The morphological findings obtained by macroscopic analyses are given in Table 2.

Table 1. Comparative macroscopic characteristics of *Althaea* L. and *Alcea* L.[38,39]

	***Alcea* L.**	***Althaea* L.**
Epicalyx	generally 6, rarely 7-9 partite connate	6-9 partite connate at the base
Petal	30 mm or longer	up to 16 mm
Anther	yellowish	brownish-purple
Carpel	sub-bilocular	unilocular

Table 2. Observed findings from the macroscopic analysis of commercial marshmallow flower samples

	Alcea sp.	A-3 & A-21	A-15 & A-33	A-1, A-7, A-8, A-9, A-13, A-19, A-23, A-24, A-25, A-26, A-27, A-28	
Epicalyx	6 partite stellate trichome	5 partite stellate trichome	5-6 partite stellate trichome	6 partite stellate trichome	
Calyx	stellate trichome 5 sepals	stellate trichome A-3: 5 sepals A-21: 6 sepals	stellate trichome 5 sepals	stellate trichome 5 sepals	
Corolla	16-32 mm white	25-44 mm A-3: pink A-21: purple	20-32 mm purple	21-49 mm A-1, 9, 24: longer than calyx A-7, 8, 28: purple A-9: pink, purple, red	A-13. purple, white A-19, 23, 24, 25: pink, purple A-26: light blue, light purple A-27: dark orange, purple
Stamen	numerous	numerous	numerous	numerous	
Filament	-	stellate trichome	stellate trichome	stellate trichome	
Anther	dorsifix	dorsifix A-21: generally spilled	dorsifix	dorsifix	
Flower	hermaphrodite	hermaphrodite	hermaphrodite	hermaphrodite	
Ovary	2-locular	2-locular	2-locular	2-locular	
Stylus	numerous	numerous	numerous	numerous	
Pedicel	stellate trichome	-	A-33: stellate trichome	-	

Table 2. Observed findings from the macroscopic analysis of commercial marshmallow flower samples (*continued*)

	A-4	**A-5, A-12, A-16, A-22, A-29, A-30**	**A-2, A-14, A31**	**A-6, A-10, A-17, A-18, A-20, A-32**	**A-11**
Epicalyx	6 partite stellate trichome	6 partite stellate trichome	6-7 partite stellate trichome	7 partite stellate trichome	7-8 partite stellate trichome
Calyx	stellate trichome 5 sepals	stellate trichome A-5: 6 sepals A-12, 16, 22, 29: 5 sepals A-30: 3-5 sepals	stellate trichome A-2 & A-31: 5 sepals A-14: 3 sepals	stellate trichome A-6, 17, 20, 32: 5 sepals A-10, 18: 6 sepals	stellate trichome 5 sepals
Corolla	29-31 mm dark pink	22-53 mm A-5: light & dark pink A-12, 22, 29: purple A-16: yellowish A-30: light & dark purple	17-43 mm A-14: pink, purple A-31: dark orange, purple	24-51 mm A-6: longer than calyx A-6, 10, 20: purple A-17: pink, purple A-18: dark pink, red, purple, pink A-32: light pink, dark purple	yellow, light pink, purple 32-45 mm
Stamen	numerous	numerous	numerous	numerous	numerous
Filament	stellate trichome	stellate trichome	stellate trichome	stellate trichome	stellate trichome
Anther	-	dorsifix	dorsifix	dorsifix	dorsifix
Flower	hermaphrodite	hermaphrodite	hermaphrodite	hermaphrodite	hermaphrodite
Ovary	2-locular	2-locular	2-locular	2-locular	2-locular
Stylus	numerous	numerous	numerous	numerous	numerous
Pedicel	-	stellate trichome	-	-	-

Results of Microscopic Analysis

The microscopic analysis of all commercial marshmallow samples exhibited that they had similar microscopic characteristics to *Alcea* sp. (Figure 1). Additionally, stomata of A-2, 5, 10, 22, 32 and 33 had 3 subsidiary cells as well as only A-3 had the Malvaceae type glandular trichome. The microscopic findings are presented in Table 3.

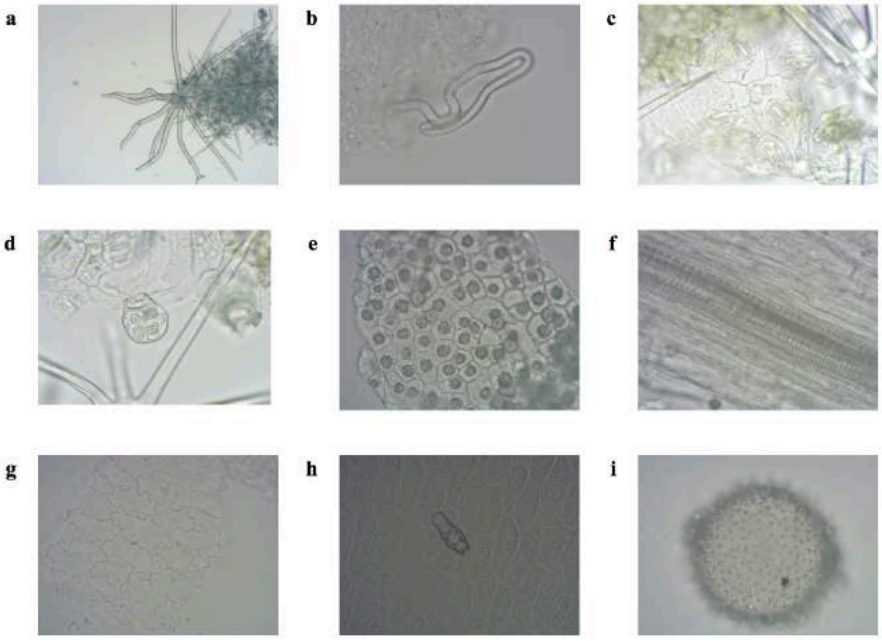

Figure 1. Microscopic illustrations of *Alcea* sp.: (a) stellate trichome (4x); (b) curved trichome (40x); (c) stomata (40x); (d) glandular trichome (40x); (e) cluster crystals of calcium oxalate (40x); (f) vessel (40x); (g) petal epidermis (40x); (h) glandular trichome in petal (40x); (i) pollen grain (40x).

Table 3. Comparative evaluation of the findings from microscopical analysis

	Alcea sp.	A-7	A-2, A-5, A-10, A-22, A-32, A-33	A-8, A-9, A-13, A-14, A-15	A-1, A-25	A-23, A-27, A-28, A-31
Stellate trichome	5-9 partite	3-8 partite	A-2: 3-9 partite A-5: 4-7 partite A-22, 32: 4-9 partite A-10: 5-7 partite A-33: 6-9 partite	3-9 partite	A-1: 4-8 partite A-25: 4-7 partite	4-8 partite
Curved trichome	+ numerous	+ numerous	+	+ numerous	+	+
Glandular trichome	+	-	-	-	-	-
Calcium oxalate crystal	+	+	A-2, 5, 10, 32, 33: + A-22: -	A-8, 9, 15: + A-13, 14: -	-	+
Pollen grain	+ numerous small & big	+ numerous small	+ numerous small & big	+ numerous small & big	+ numerous small & big	+ numerous small & big
Pollen grain	+ 3-5 subsidiary cells	-	+ 3 subsidiary cells	-	-	-

Table 3. Comparative evaluation of the findings from microscopical analysis (*continued*)

	A-4	**A-26**	**A-3, A-11, A-16, A-17, A-21, A-24**	**A-6, A-29**	**A-20**	**A-12, A-18, A-19**
Stellate trichome	4-9 partite	5-7 partite	5-8 partite	5-9 partite	6-8 partite	6-9 partite
Curved trichome	+ numerous	+ numerous	+	+	+	+
Glandular trichome	-	-	A-3: + A-11, 16, 17, 21, 24: -	-	-	-
Calcium oxalate crystal	-	+	A-3, 11, 17, 24: + A-16, 21: -	+	+	+
Pollen grain	+ numerous small & big	+ numerous small & big	+ numerous small & big	+ numerous small & big	+ numerous small & big	+ numerous small & big
Pollen grain	-	-	-	-	-	-

Foreign Matter

The ratio of foreign matter is set at maximum 2.0 per cent (w/w) as described at the general pharmacopoeia rules. Accordingly, 27 of 33 commercial marshmallow specimens were found to be lower than this ratio, while the percentages of the remaining six marshmallow materials [A-3, 16, 22, 27, 30, and 31] were over 2.0% (Figure 2). On the other hand, insects or insect parts and snails were detected in 26 among the 33 marshmallow samples, therefore they cannot be considered as safe herbal drugs. Among the remaining seven samples which did not contain insects and snails, two samples were previously eliminated due to high ratio of foreign matter. Consequently, only five samples [A-11, 12, 13, 18, and 19] were considered to be appropriate herbal drug. The percentages of foreign organs and foreign elements are given in Table 4.

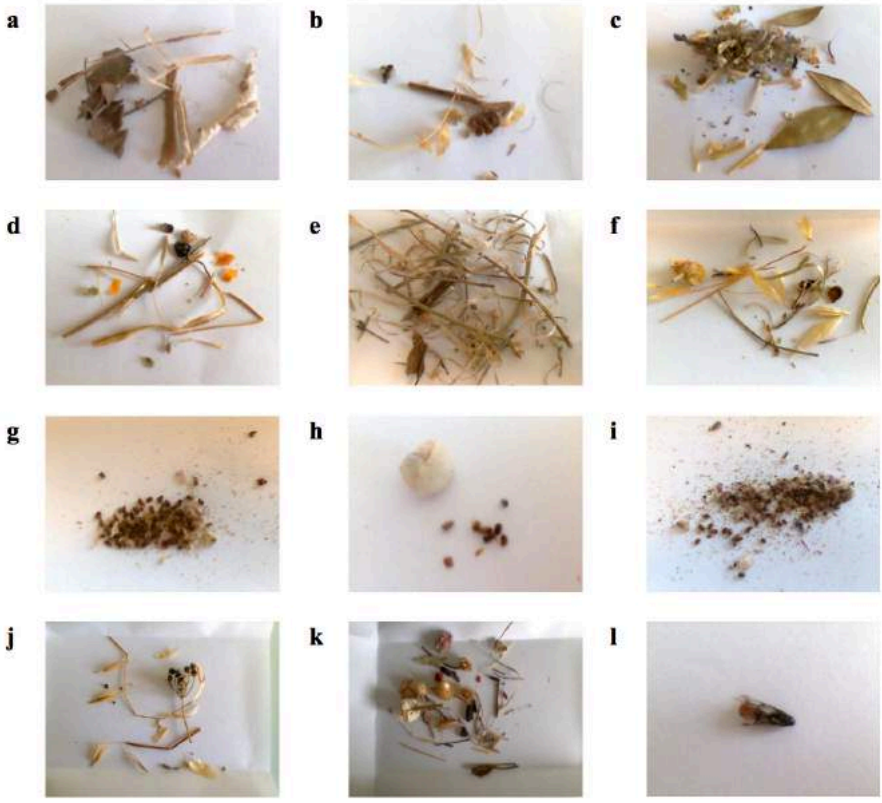

Figure 2. Foreign substances detected in marshmallow samples. (a) feather & different plant parts in A-3; (b) feather, bristle & different plant parts in A-7; (c) different plant parts in A-13; (d) stone & different plant parts in A-15; (e) different plant parts in A-16; (f) stone & different plant parts in A-20; (g) insects in A-21; (h) insects & snail in A-23; (i) insects in A-24; (j) different plant parts in A-29; (k) feather & different plant parts in A-30; (l) insect in A-32.

Table 4. Findings of foreign matter

Samples	Insects/Snails (%)	Feathers/Bristles/Stones/ Foreign plant parts (%)	Total foreign matter (%)
A-1	0.029	–	0.029
A-2	1.526	0.440	1.966
A-3	–	5.696	5.696
A-4	0.399	–	0.399
A-5	0.023	0.303	0.326
A-6	0.021	0.055	0.076
A-7	0.017	0.264	0.281
A-8	0.018	0.311	0.329
A-9	0.028	0.007	0.035
A-10	0.109	0.612	0.721
A-11	–	0.857	0.857
A-12	–	0.038	0.038
A-13	–	0.903	0.903
A-14	0.006	0.212	0.218
A-15	0.017	0.300	0.317
A-16	–	2.792	2.792
A-17	1.529	–	1.529
A-18	–	0.843	0.843
A-19	–	0.158	0.158
A-20	0.695	0.498	1.193
A-21	0.235	0.215	0.450
A-22	2.169	–	2.169
A-23	0.239	0.429	0.668
A-24	0.285	0.123	0.408
A-25	0.010	0.077	0.087
A-26	0.003	1.994	1.997
A-27	0.051	4.336	4.387
A-28	0.015	0.940	0.955
A-29	0.064	0.410	0.474
A-30	0.007	2.363	2.370
A-31	0.069	2.618	2.687
A-32	0.021	1.264	1.285
A-33	0.101	0.641	0.742

Loss on Drying

The limit of loss on drying was set at maximum 12.0 per cent (w/w) similarly to set value in "Malvae sylvestris flos" monograph [Ph. Eur.]. Accordingly, in 28 samples the rates were found to be lower [between 9.74 and 11.94 for A-1 to A-4, A-6 to A-16, A-18 to A-20, A-22, A-23, and A-26 to A-33], while in five samples [A-5, 17, 21, 24, 25] and reference *Alcea* sp. the ratio was higher than 12.0% [between 12.28 and 13.25].

Total Ash

In the "Malvae sylvestris flos" monograph total ash limit was set at maximum 14.0 per cent (w/w). Accordingly, the total ash amount of all the commercial marshmallow samples [between 8.36 and 11.63 for A-1, A-2, A-4 to A-33] and *Alcea* sp. sample have remained below this limit. The ash content of only A-3 was somewhat closer to 14.0% [13.52%].

Swelling Index

Swelling index was determined following the "Malvae sylvestris flos" monograph [Ph. Eur.] and the limit therein was at least 15. In this regard, the swelling indices of all marshmallow specimens and *Alcea* sp. sample were far below the limit value. Besides, the greatest swelling indices were obtained from only A-24 and A-25. The swelling indices are presented in Table 5.

Table 5. Findings of swelling index

Samples	Trial-I	Trial-II	Trial-III	Mean
Alcea sp.	7.25	7.00	6.50	6.92
A-1	3.00	4.25	3.00	3.42
A-2	3.50	5.50	3.50	4.17
A-3	6.00	8.50	7.50	7.33
A-4	6.00	4.50	5.50	5.33
A-5	6.00	8.50	7.00	7.17
A-6	4.50	4.00	4.00	4.17
A-7	4.00	4.50	3.50	4.00
A-8	4.50	4.00	4.50	4.33
A-9	4.50	5.00	4.50	4.67
A-10	3.50	4.00	3.00	3.50
A-11	5.50	5.00	4.50	5.00
A-12	7.00	5.00	5.00	5.67
A-13	5.50	6.00	5.00	5.50
A-14	4.50	3.00	3.50	3.67
A-15	5.00	5.50	6.50	5.67
A-16	3.00	3.00	2.25	2.75
A-17	4.50	6.00	4.50	5.00
A-18	4.00	5.50	5.00	4.83
A-19	4.00	4.50	4.50	4.33
A-20	5.00	4.50	5.00	4.83
A-21	4.50	3.00	4.00	3.83
A-22	5.50	3.25	4.00	4.25
A-23	4.00	5.50	3.50	4.33
A-24	6.50	9.50	8.00	8.00
A-25	8.00	8.50	8.50	8.33
A-26	4.50	5.50	6.00	5.33
A-27	6.50	8.00	6.50	7.00
A-28	7.50	6.50	9.00	7.67
A-29	5.50	3.00	4.00	4.17
A-30	4.00	4.50	3.00	3.83
A-31	5.00	4.00	3.50	4.17
A-32	2.50	3.50	3.00	3.00
A-33	4.00	2.75	4.50	3.75

HPTLC Analysis

The fingerprints of the test solutions prepared from the flowers of all commercial marshmallow materials as well as the flower, corolla and calyx parts of reference *Alcea* sp. sample were compared (Figure 3, 4, 5, 6 and 7). Under 366 nm, A-4, 5, 9, 11, 12, 13, 17, 18, 19, 21, 23, 24, 25 and 32 showed two blue fluorescent zones and red fluorescent spot in the upper part of plate. Moreover, blue fluorescent zones at R_f 0.5 and 0.9 were seen in A-8, 10, 12, 14, 18, 20, 22, 28, 29, 30, 31 and 33. Furthermore, this blue zone was also detected in the flower, corolla and calyx parts of *Alcea* sp. Besides, after spraying with H_2SO_4 reagent, under white light, blackish yellow zone between R_f 0.2 and 0.4 was observed in A-4, 5, 9, 11, 12, 13, 17, 18, 19, 24, 25 and 32. In addition, they exhibited a dark yellow zone at R_f 0.8, so they were similar to each other.

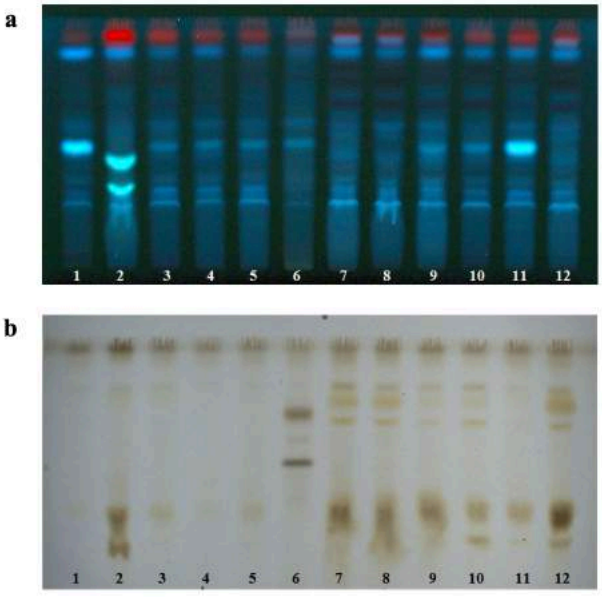

Figure 3. HPTLC chromatogram-1 visualized (a) at 366 nm; (b) under white light. Mobile phase: acetic acid-water-butanol (15:30:60, v/v/v); applied volumes: Mallow-7 µL, other extracts-9 µL, derivatization: H_2SO_4 reagent. Tracks: (1) *Alcea* sp. (reference); (2) mallow; (3) A-15; (4) A-1; (5) A-2; (6) A-3; (7) A-4; (8) A-5; (9) A-6; (10) A-7; (11) A-8; (12) A-9.

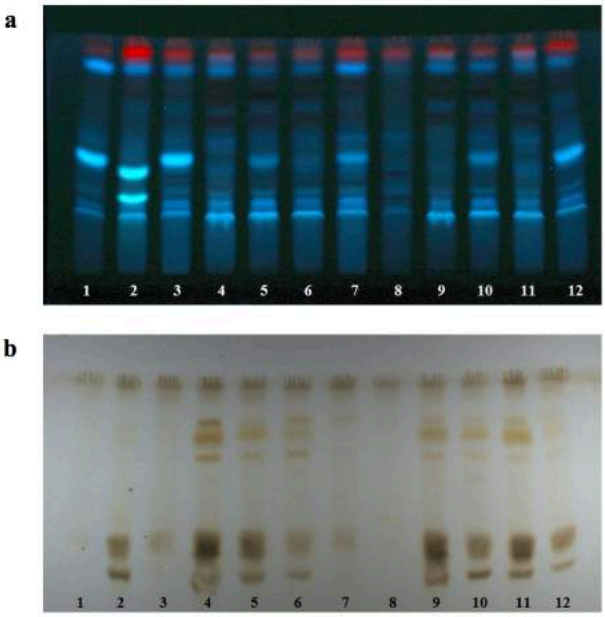

Figure 4. HPTLC chromatogram-2 visualized (a) at 366 nm; (b) under white light. Mobile phase: acetic acid-water-butanol (15:30:60, v/v/v), applied volumes: Mallow-7 µL, other extracts-9 µL, derivatization: H_2SO_4 reagent. Tracks: (1) *Alcea* sp. (reference); (2) mallow; (3) A-10; (4) A-11; (5) A-12; (6) A-13; (7) A-14; (8) A-16; (9) A-17; (10) A-18; (11) A-19; (12) A-20.

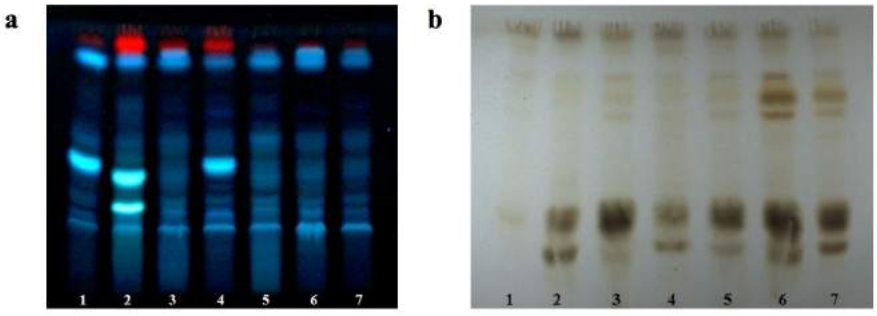

Figure 5. HPTLC chromatogram-3 visualized (a) at 366 nm; (b) under white light. Mobile phase: acetic acid-water-butanol (15:30:60, v/v/v), applied volumes: Mallow-7 µL, other extracts-9 µL, derivatization: H_2SO_4 reagent. Tracks: (1) *Alcea* sp. (reference); (2) mallow; (3) A-21; (4) A-22; (5) A-23; (6) A-24; (7) A-25.

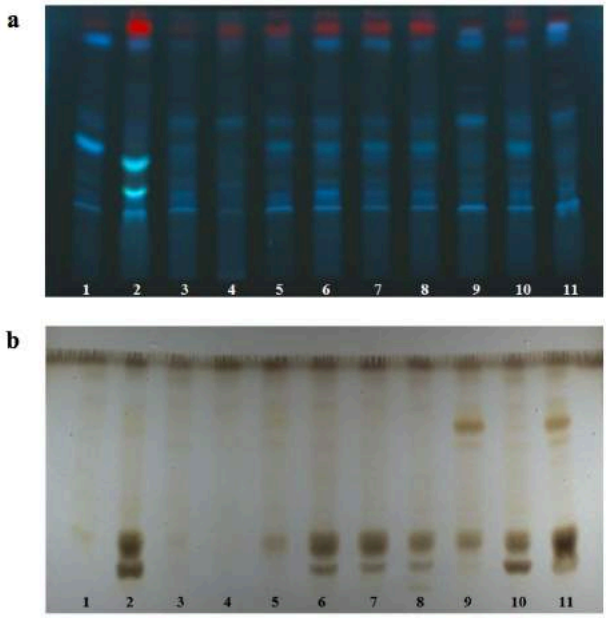

Figure 6. HPTLC chromatogram-4 visualized (a) at 366 nm; (b) under white light. Mobile phase: acetic acid-water-butanol (15:30:60, v/v/v), applied volumes: Mallow-7 µL, other extracts-9 µL, derivatization: H_2SO_4 reagent. Tracks: (1) *Alcea* sp. (reference); (2) Mallow; (3) A-26; (4) A-27; (5) A-28; (6) A-29; (7) A-30; (8) A-31; (9) A-32; (10) A-33; (11) A-5.

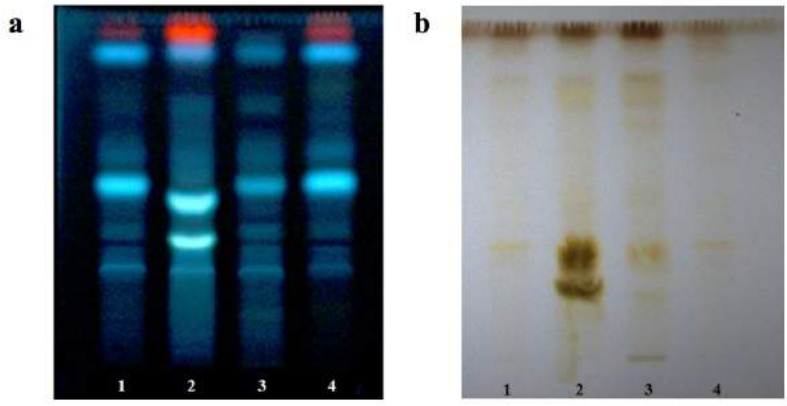

Figure 7. HPTLC chromatogram-5 visualized (a) at 366 nm; (b) under white light. Mobile phase: acetic acid-water-butanol (15:30:60, v/v/v), applied volumes: Mallow-7 µL, other extracts-9 µL, derivatization: H_2SO_4 reagent. Tracks: (1) *Alcea* sp. whole flower (reference); (2) Mallow flower; (3) *Alcea* sp. corolla; (4) *Alcea* sp. calyx.

DISCUSSION

In the present study, a monograph for "Althaeae flos" was proposed following the template for "Malvae sylvestris flos" in Ph. Eur. to be used in determination of the qualification of marshmallow flower samples and the developed template was applied for quality assessment of commercial marshmallow flower samples sold in herbal stores under the name of "hatmi çiçeği".

Macroscopic (Table 2) and microscopic (Table 3) examinations revealed that all commercial marshmallow samples had quite similar morphological and microscopic properties to those itemized under "Malvae sylvestris flos" monograph [Ph. Eur.]. In addition, it was observed that six samples had stomata with three subsidiary cells, and only one sample had Malvaceae type glandular trichome. On the other hand, it was found that petal lengths of all commercial marshmallow samples were longer than the specified values for *Althaea* species [9-16 mm][1], but were similar to those issued for *Alcea* species [30 mm or longer].[39] Besides, two-locular ovary was observed in all marshmallow materials. As a result, it was considered that all commercial marshmallow samples were originally obtained from *Alcea* sp. flowers, but not from the official species *Althaea officinalis*. This is possibly due to widespread distribution of *Alcea* species in Turkey comparing to *Althaea* species.

In foreign matter analysis, only five samples were considered to be acceptable since they were free from insects or snails residues as well as the ratio of foreign elements were found to be lower than the set value 2.0% (Table 4). On the other hand, 80% of the commercial marshmallow samples were found to be contaminated with insects, flies, snails and other animal wastes. The possible reason why these materials contain lots of such unacceptable matters is the closure of the petals during harvesting of the flowers, and the insects or flies collecting nectar from the flowers confined inside the closed flowers. Under these circumstances, it is not possible to prohibit such foreign substances without applying insecticide to the flowers, which would increase the health risk. For this reason, in contrast to *Althaea* or *Alcea* species, use of *Malva sylvestris* flowers does not have a risk for such contaminations and may be safely used.

In loss on drying test, it was detected that the rates of five commercial marshmallow samples and *Alcea* sp. were found to be more than 12.0%, but the rates in the remaining 28 marshmallow materials were below this ratio. In total ash analysis, the ratio of all commercial marshmallow materials and *Alcea* sp. were found to be lower than 14.0%. In swelling index test, it was determined that the results of all marshmallow materials and *Alcea* sp. were below the set value of 15 (Table 5), thus it was revealed that the mucilage content in marshmallow flowers was less than that in mallow flower.

In HPTLC analysis (Figure 3 to Figure 7), when the chromatographic profiles at 366 nm were compared, it was determined that fourteen marshmallow samples presented 2 blue fluorescent zones and a red fluorescent zone in the upper part. In addition, blue fluorescent zones at R_f 0.5 and 0.9 were found in the chromatogram of twelve samples. This blue zone was also detected in the flower, corolla and calyx parts of *Alcea* sp. Furthermore, after spraying with H_2SO_4 reagent, under white light, blackish yellow spot between R_f 0.2 and 0.4 as well as dark yellow zone at R_f 0.8 were observed in twelve specimens.

CONCLUSION

Although the roots (Althaeae radix) and leaves (Althaeae folium) of *Althaea officinalis* are the officially accepted herbal drugs in Ph. Eur., only its flowers are available in the herbal markets in Turkey. In this study, primarily a draft monograph for the flowers was prepared in order to set its specifications. Then the commercial flower samples which are sold under the name of "hatmi çiçeği" in the herbal markets were analyzed for their compliance to the specifications set. These results have revealed that all the available "hatmi" flowers samples were obtained from *Alcea* sp., but not from *Althaea*. On the other hand, due to insect and snails parts as well as high ratio of foreign matter, including feathers, bristles, stones and foreign plant parts, which were detected in the flower samples in 28 out of 33 samples obtained from herbal markets, they were rated out of official use.

In the light of the discussed above, a template for Althaeae flos monograph is prepared as follows:

The values in the experimental sections of the "Althaeae flos" monograph were the results obtained from the tests carried out in this study. The ratio of foreign matter was found to be between 0.029 and 5.696%. The mean calculated for 33 marshmallow samples was found to be 1.13. Maximum 2.0 per cent was approved as the amount of foreign matter by predicating on the general pharmacopeia rule.

In loss on drying test, the rates were between 9.74 and 13.25%. The average of 33 marshmallow materials was found to be 10.99. When "Malvae sylvestris flos" monograph [Ph. Eur.] value and the average of findings were considered, maximum 12.0% was accepted as the quantity of loss on drying in the "Althaeae flos" monograph.

In total ash experiment, the lowest rate was 8.36%, and the highest one was 13.52%. The mean of 33 marshmallow materials was found to be 10.24. When the results were taken into consideration, maximum 12.0% was approved as the content of total ash in the "Althaeae flos" monograph.

In swelling index trial, the lowest value was 2.75, and the highest value was 8.33.

The average of 33 marshmallow samples was 4.94. Regarding to the findings of trials, minimum 7 was accepted as the value of swelling index in the "Althaeae flos" monograph.

In TLC analyses, marshmallow specimens showed blue fluorescent spot and red fluorescent spot in the upper part of the chromatogram that was similar to Malvae sylvestris flos test solution [Ph. Eur.]. Considering the findings and TLC results stated in the "Malvae sylvestris flos" monograph, it is accepted that test solutions of marshmallow flowers exhibited a red spot in the upper part of the middle third and violet zone in the middle third.

According to the findings obtained from the Ph. Eur. analyses applied to all the samples and the evaluations of literature data, an "Althaeae flos" monograph prepared in Ph. Eur. template is as follows.

MARSHMALLOW FLOWER

Althaeae flos

DEFINITION

Whole or fragmented dried flower of *Althaea officinalis* L. and *Alcea* species.

IDENTIFICATION

A. Flowers borne in fascicles in the leaf axils. The flower involves an epicalyx with 6-9 lanceolate segments, connate at the base and located below the calyx. A calyx with 5 pubescent triangular lobes, gamosepalous at the base, not inflated. A corolla with 5 petals white to pinkish white, fused to the staminal tube at the base. Style much branched at the apex. *Althaea* petals 9-14 mm, *Alcea* petals 30 mm or more. Stamens numerous, the filaments of which fuse into a staminal tube covered by simple trichomes and small stellate trichomes visible using a lens. *Althaea* anthers brownish purple, *Alcea* anthers yellowish. Carpels numerous, glabrous, in one series around the base of the styles, 1-ovulate. *Althaea* carpels unilocular, *Alcea* carpels sub-bilocular. Fruit a schizocarp, splitting into numerous 1-seeded, indehiscent mericarps. Mericarps rugose, stellate-pilose.

B. Reduce to a powder (355) (*2.9.12*). The powder is pinkish white. Examine under a microscope using *chloral hydrate solution R*. The powder displays the following diagnostic characters (Figure 8): unicellular, thick-walled trichomes; small unicellular covering trichomes, somewhat curved [A] or in star-shaped groups of 2-9 [B]; capitate glandular trichomes with multicellular heads [C]; mesophyll fragments with cluster crystals of calcium oxalate [D]; veins of sepals with vessel [E]; fragments of petal epidermis [F] accompanied by glandular trichomes with multicellular heads [G]; numerous spherical pollen grains with a spiny exine, 140-160 μm in diameter [H].

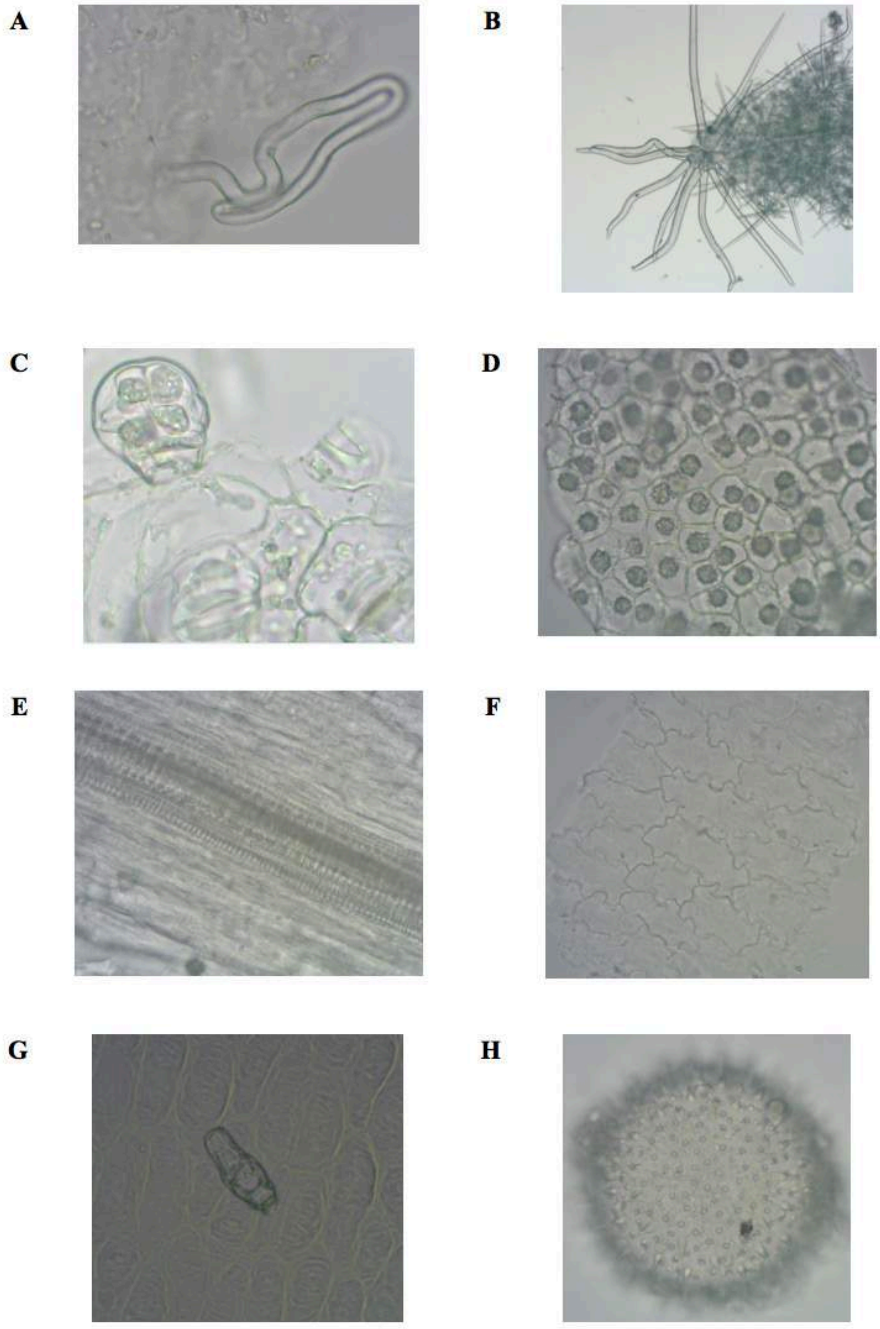

Figure 8. Illustration for identification test B of powdered herbal flower drug: (A) curved trichome; (B) stellate trichome; (C) glandular trichome; (D) mesophyll fragments with cluster crystals of calcium oxalate; (E) vessel; (F) petal epidermis; (G) petal epidermis accompanied by glandular trichome with multicellular heads; (E) pollen grain.

C. Thin-layer chromatography (*2.2.27*).

Test solution. To 1 g of the pulverized drug (355) (*2.9.12*) add 10 mL of *ethanol* (*60 per cent, v/v*) *R*. Stir for 15 min and filter.

Plate: *TLC silica gel plate R*.

Mobile phase: *acetic acid R, water R, butanol R* (15:30:60, v/v/v).

Application: 9 mL of test solution, as 9.0 mm bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in daylight, ultraviolet light at 366 nm, spray with *sulphuric acid solution* (5 per cent) and exsiccate in an oven at 105°C.

Results: chromatogram attained with test solution exhibits red zone in the upper part of the middle third and violet zone in the middle third.

TESTS

Foreign matter (*2.2.32*): maximum 2.0 per cent

Loss on drying (*2.4.16*): maximum 12.0 per cent, detected on 1.000 g of the pulverized herbal drug by desiccating in an oven at 105°C for 2 hours.

Total ash (*2.8.1*): maximum 12.0 per cent.

Swelling index (*2.8.4*): minimum 7, detected on 0.2 g of the pulverized herbal drug (710) (*2.9.12*) moistened with 0.5 mL of *ethanol R*.

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A Contraindication to Metfromin Therapy: Renal Impairment - Adherence to Prescribing Guidelines at a Hospital in Turkey

Neda Taner[1]*, Büşra Nur Çattık[1], Barkın Berk[1]

[1]Clinical Pharmacy, Istanbul Medipol University, Istanbul, Turkey

ABSTRACT

Objective: The aim of this study was to investigate whether renal functions of the patients were monitored and checked before and during metformin treatment as recommended in guidelines or whether they were disregarded and metformin was prescribed despite the contraindication of renal impairment in a hospital in Turkey.

Method: This retrospective cross-sectional study was conducted among the patients who were hospitalized at a university hospital, diagnosed with type 2 diabetes mellitus and had metformin included in their treatment between 2015-2016. The total number of patients with this diagnosis and treatment between these years was determined as 66 and all the patients were taken into the study. Renal functions of these patients were assessed by measuring serum creatinine levels and calculating GFR using the Cockcroft - Gault formula.

Results: During the duration of metformin treatment 10 patients (15%) were not monitored for their serum creatinine. The 56 patients who were monitored for their serum creatinine were at 1, 2, 3, 4, 5 (end-stage) renal failure stages with the rates of 30, 36, 30, 4, 0 % respectively.

Conclusion: Assessment of renal function, adjustment of drug doses accordingly and termination of the treatment when contraindicated, are essential strategies for metformin therapy to prevent medication errors. However, this study showed that adherence to these prescribing rules are low and in some patients renal function was not monitored, placing them in increased risk of lactic acidosis.

Keywords: Metformin, Renal Function, Lactic acidosis

INTRODUCTION

According to American Diabetes Association (ADA) guideline[1] and a position statement on the management of hyperglycemia in patients with type 2 diabetes published by ADA and European Association for the Study of Diabetes (EASD),

*Corresponding author: Neda Taner, e-mail: ntaner@medipol.edu.tr
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metformin is stated as the preferred first-line antidiabetic pharmacologic agent for the treatment of type 2 diabetes if it is tolerated and not contraindicated [2]. This information is also emphasized by the guideline of National Institute for Health and Clinical Excellence (NICE)[3]. Unlike some other antidiabetic agents such as sulfonylureas, thiazolidinediones and insulin, metformin is weight-neutral which makes it an attractive choice of drug for obese patients as well. It reduces the risks and rates of cardiovascular events and death. It also has low cost, proven safety record and effectiveness. During the management of type 2 diabetes mellitus (DM) hypoglycemia can occur and cause complications; however, metformin monotherapy rarely leads to hypoglycemic attacks when compared with insulin and sulfonylureas [1, 2, 4-7].

Metformin and phenformin, the two main biguanides, became available for DM treatment in the 1950s [8]. In the late 1970s all biguanides, except metformin, were withdrawn because of links to lactic acidosis and increased cardiac mortality. Ongoing research and its minimal clinical use fostered the approval of metformin by Food and Drug administration (FDA) in 1995. Especially The UK Prospective Diabetes Study (UKPDS) in 1998 set metformin to its current position [9].

Metformin is noticed as an antihyperglycemic agent because it lowers blood glucose concentrations in type 2 DM and it is also frequently described as an insulin-sensitizer, leading to reduction in insulin resistance by increasing insulin-stimulated glucose uptake of skeletal muscle and adipose tissue. Metformin exerts its antidiabetic effects mainly by reducing hepatic glucose production through inhibition of gluconeogenesis which is upregulated in type 2 DM. Reduction of hepatic glucose production by metformin is mediated by inhibition of mitochondrial respiratory chain resulting in a decrease in cellular ATP [7, 8, 10].

Metformin is a small molecule that is not bound to plasma proteins and it does not undergo relevant biotransformation in the liver or biliary excretion. It has low lipid solubility and high volume distribution. Excretion of unchanged drug in urine is the major mode of elimination of metformin. It is cleared by renal tubular secretion and glomerular filtration. As predicted, in case of impairment in renal function, clearance of metformin will reduce and accumulation may occur [11].

Although it has advantages metformin has some adverse effects and contraindications that narrow down the segment of the type 2 diabetic population that can benefit from this drug. The most frequent adverse effects, affecting approximately 30 % of the patients, result from gastro-intestinal (GI) disturbances including anorexia, metallic taste, nausea, abdominal discomfort and diarrhea. However, GI side effects are usually transient and can be minimized by slowly titrating the dose and administering the drug with or after food [10, 12].

Metformin and Lactic Acidosis

Lactic acidosis is a rare but serious adverse effect of metformin with a reported incidence of ≤10 cases per 100,000 patient-years which is estimated to be 20 times less than with phenformin, however it is life-threatening and associated with overall mortality of 25 to 50% [4, 13-16]. It is an anion-gap metabolic acidosis defined by plasma lactate level greater than 5 mmol/L and pH less than 7.35 [16, 17]. When severe, it is associated with multi-system organ dysfunction particularly neurologic (stupor, coma, seizures) and cardiovascular (hypotension, ventricular fibrillation) and carries a high mortality risk [17].

Predisposing factors of lactic acidosis are considered to be contraindications and precautions of metformin. For instance, in patients with kidney disease, liver function abnormalities, congestive heart failure, peripheral vascular disease, pulmonary disease, acute myocardial infarction, septicaemia, hypovolemia, shock, or other causes as these conditions may increase the risk of tissue anoxia and therefore the development of lactic acidosis [4, 16]. For the same reason it is recommended that metformin should be withdrawn in patients undergoing major surgery or requiring investigation using radiographic contrast media and should only be restarted once renal function has been evaluated and determined as within acceptable limits [11].

Metformin Treatment Preference in Renal Impairment

The prescribing information for metformin in the current label specifies the contraindication of renal disease or renal dysfunction as serum creatinine (SCr) levels≥1.5mg/dL (for males) and SCr levels≥1.4mg/dL (for women) [18]. According to FDA Revised Warning in April 2016, metformin can be used in patients with mild kidney impairment (Stage 2: 90>GFR≥60) and in some patients with moderate (Stage 3: 60>GFR≥30) impairment. It is also recommended to use estimated glomerular filtration rate (eGFR) instead of a single laboratory value such as a SCr to measure kidney functions in order to determine if patients can receive metformin [1, 19].

The updated Kidney Disease Outcomes Quality Initiative guidelines from the National Kidney Foundation are perfectly consistent with the label adding that a recent advice was adopted by the British National Formulary and the Japanese Society of Nephrology proposing that metformin use be reevaluated when GFR is 45 mL/min/1.73 m^2 and stopped when 30 mL/min/1.73 m^2 [20].

NICE guideline allows the use of metformin with eGFR less than 60 mL/min/1.73m^2, recommends to review the dose of metformin if the eGFR is below 45 mL/min/1.73m^2 in adults with type 2 diabetes, to stop metformin if the eGFR

is below 30 mL/min/1.73m², and to prescribe metformin with caution for those at risk of a sudden deterioration in kidney function and those at risk of eGFR falling below 45 mL/min/1.73m² [3].

Lastly Lipska et al[6] proposed recommendations for metformin use based on eGFR taking into consideration the eGFR thresholds of NICE guideline, Canadian Diabetes Association and the Australian Diabetes Society (Table 1).

Table 1. Proposed recommendations for metformin use based on eGFR [6]

Stage	Description	eGFR mL/ min/1.73m²	Action
1	Kidney damage with normal or high GFR	≥ 90	No renal contraindication to metformin Monitor renal function annually
2	Kidney damage with mild low GFR	<90 and ≥60	No renal contraindication to metformin Monitor renal function annually
3	Moderate decreased GFR	<60 and ≥45	Continue use Increase monitoring of renal function (every 3-6 months)
3	Moderate decreased GFR	<45 and ≥30	Prescribe metformin with caution Use lower dose Closely monitor renal function Do not start new patients on metformin
4	Severe decreased GFR	<30 and ≥15	Stop metformin
5	Kidney failure	< 15 (or dialysis)	Stop metformin

**Stages of chronic kidney failure by the National Kidney Foundation [21]; eGFR: estimated glomerular filtration rate.*

In view of these worldwide ambiguities about metformin use in presence of renal impairment, the aim of this study was to investigate whether renal functions of the patients were monitored and checked before or during metformin treatment as recommended in guidelines and if the treatments were adapted accordingly or whether they were disregarded and metformin was prescribed despite the contraindication of renal impairment in a hospital in Turkey.

MATERIALS AND METHODS

This retrospective cross-sectional study was conducted among the patients who were hospitalized at a university hospital, diagnosed with type 2 diabetes mellitus and had metformin included in their treatment between 2015-2016. The total number of patients with this diagnosis and treatment between these years was determined as 66 and all the patients were taken into the study. This study was

approved by the Ethics committee of the hospital. Relevant permissions were obtained to access patient data. Patient information was de-identified and just the investigators kept a confidential document revealing the identity of each patient. The data was collected by retrospective review of the hospital's electronic patient charts and included 1-year data. From the patients' chart, demographic data (age, gender, and diagnoses) and laboratory data (serum creatinine) were collected. Researchers used serum creatinine, when available on charts, and calculated CrCl using Cockcroft - Gault formula to estimate renal function of each subject. The number of patients who have not received a renal function assessment was identified. The number of cases of failure to de-prescribe metformin when contraindicated was also determined.

RESULTS

There were a total of 66 patients who met the inclusion criteria. When looked through the demographic characteristics of the patients enrolled in this study, 64 % were male. According to eGFR calculations, the 56 patients who were monitored for their serum creatinine were at 1, 2, 3, 4 and 5 (end stage renal failure) renal failure stages (Table 1) with the rates of 30, 36, 30, 4, 0 % respectively (Figure 1). It was determined that for 10 out of 66 patients (15%) who were on metformin treatment, a serum creatinine measurement and therefore renal function assessment was not performed. The study identified that according to eGFR calculations, 2 patients were contraindicated for having stage 4 kidney failure and according to SCr levels 2 more patients would be contraindicated for having a SCr level higher than 1.5 mg/dL although they were at stage 3 kidney failure and it is not accepted as a contraindication according to eGFR calculation.

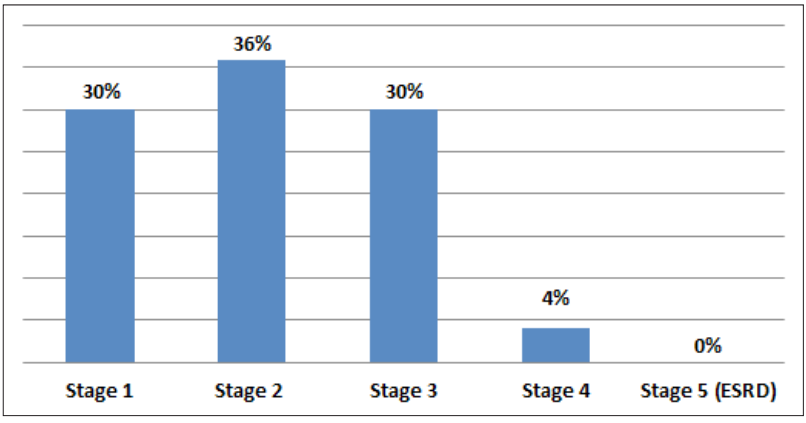

Figure 1. Renal Function Statutes of the patients (according to eGFR).

DISCUSSION AND CONCLUSION

The fear of lactic acidosis as an adverse effect originates from the experience with phenformin, although incidence of lactic acidosis is low with metformin. It has been interpreted that this experience led the physicians to be more cautious and lowered the incidence of metformin-associated lactic acidosis [14]. Studies reviewed by Inzucchi et al. [17] and Holstein et al [22] show that in real-life practice, recommendations to avoiding contraindications, including renal impairment, are not followed accordingly. The review also mentions that even though metformin clearance is reduced in the presence of chronic kidney disease, metformin levels remain within therapeutic range (0.47 – 2.5 mg/L) when eGFR is greater than 30 mL/min/1.73m^2. Richy et al [23] has noted that in their study the majority of metformin-treated patients (92.2%) had some level of renal impairment including severely reduced function. On the other hand, Eppenga et al [16] demonstrated in their retrospective cohort study that patients with all stages of renal impairment were treated with metformin, but they concluded mentioning the importance of current recommendations about renal function monitoring.

The approach towards the use of metformin in patients with renal impairment varies and the guidelines are not consistent in describing the contraindications as well [1,3,6,18-20]. It is shown that the recommendations of the guidelines are disregarded by the physicians in clinical practice. Studies conducted among patients on metformin treatment reveal that 25-28% of them had renal impairment as a contraindication [24-26]. Several reasons thought to cause this disregard, include 1) controversial ideas about the cause of MALA and the role of metformin [17, 26], 2) the low incidence of MALA, 3) high number of patients being deprived from the advantages of metformin treatment [6, 17, 22], 4) the thought that contraindications are unnecessarily strict because of the experience with phenformin [22], 5) antidiabetic drugs alternative to metformin might not be safer or better for these patients [17, 22]. This uncertainty shows the need to review the guidelines. Although the prescribing rules can be softened it must be acknowledged that lactic acidosis can lead to death and renal impairment is an inevitable complication of uncontrolled diabetes [27]. In the light of this study, the confusion worldwide seems to affect the physicians in Turkey as well. Examining different wards of departments in a hospital in Turkey revealed that the renal functions were not even monitored for 15 % of the patients. Although the guidelines aren't consistent about the approach to metformin use in the presence of renal impairment it is agreed by all that metformin must be stopped when eGFR is below 30 mL/min/1.73m^2. It is necessary to monitor the renal function initially to avoid this certain contraindication, besides there were also 2 patients with stage 4 renal failures. This might be explained as a lack of knowledge rather than a disregard.

Lack of clinical pharmacists serving in hospitals in Turkey presently, which is another factor increasing the risk of such oversights.

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Estimation of Abiraterone in Human Plasma by Liquid Chromatography Tandem Mass Spectrometry

Srinivasa Reddy[1], Licto Thomas[1], Lokesh Ramanna[1], Arindam Mukhopadhyay[1*], Saral Thangam[1]

[1]Norwich Clinical Services Pvt. Ltd., BA & MW, Bangalore, Karnataka, India

ABSTRACT

A LCMS/MS method for the determination of abiraterone in human plasma was described. After extraction of sample from plasma by LLE method it was dried and reconstituted in mobile phase. 20µL of sample was injected to a C_{18} column and eluted with a mobile phase (2 mM Ammonium formate, pH 3.5: Acetonitrile: 30:70, v/v) at a flow rate of 1.2 mL/min. MRM transitions were monitored as m/z 350.3 → 156.1 (abiraterone) and m/z 354.3 → 160.1 (abiraterone D4). Sample concentrations were calculated by linear regression analysis using the analyst software 1.5.1. An excellent linear response was obtained over the concentration ranges 0.20 ng/mL to 79.50 ng/mL. The intra-day and inter-day precision were within 14.4%. The assay accuracy was 91.35–105.05%. Mean recovery was 60.20% (2.84%) for abiraterone. The limit of detection was 0.052 ng/mL. The stability issue of abiraterone in plasma was also addressed. This method can be used for bioequivalence studies.

Keywords: abiraterone, prostate cancer, LLE, LCMS/MS, validation

INTRODUCTION

Prostate cancer is a slow growing, testosterone dependent cancer which affects approximately 1 out of 6 men in their lifetime. It causes significant morbidity and mortality in elderly males. Although it can be efficiently managed with hormonal therapy in the initial stages the cancer becomes resistant to conventional therapy as the duration increases and is termed as castration-resistant prostate cancer (CRPC)[1].

*Corresponding author: Dr. Arindam Mukhopadhyay, e-mail: arindam.mukhopadhyay@norwichclinical.com
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The backbone of first line treatment for metastatic prostate cancer is androgen deprivation therapy (ADT) which can be either medical or surgical[2]. Androgen receptor signaling is crucial in the progression from primary to metastatic prostate cancer[3]. The enzyme, CYP17A1 (17 alpha-hydroxylase/C17, 20 lyase), catalyzes the androgen biosynthesis and is expressed at higher amount in testicular, adrenal, and prostatic tumor tissue. Abiraterone inhibits CYP17A1 in a selective and irreversible manner via covalent binding mechanism. More specifically, it inhibits the conversion of 17-hydroxyprognenolone to dehydroepiandrosterone (DHEA) by the enzyme CYP17A1 to lower serum levels of testosterone and other androgens[4].

Abiraterone acetate is a pro-drug of its active metabolite, abiraterone. Abiraterone is poorly absorbed but abiraterone acetate is well and rapidly absorbed orally[5]. Abiraterone acetate is approved in the European Union and the US, in combination with prednisone or prednisolone, for the treatment of men with metastatic castration-resistant prostate cancer (CRPC)[3].

Several chromatographic methods have been reported for determination of abiraterone in plasma[6-11]. However, these methods had their own limitations in respect to the sample preparation, gradient elution, run time, etc. A liquid chromatographic method with fluorescence detection for estimation of abiraterone in plasma described by Tiphaine et al.[6] involves both protein precipitation performed with acetonitrile followed by liquid-liquid extraction using diethyl ether for sample preparation. Moreover, the run time was quite long (11-min). The LCMS/MS method described by Martins et al.[8] used SPE method for sample extraction and the linearity of this assay was in the range of 5 to 500 nM. Wani et al.[9] used protein precipitation during sample extraction and linearity in this LCMS/MS method was in the range of 0.1–50ng/mL. The linearity range was extended from 0.20 to 201ng/mL in another LCMS/MS method described by Gurav et al[10]. They used Phenacetin as an internal standard and protein precipitation method for sample extraction. In a recently published article, the abiraterone was estimated by LCMS/MS using a gradient method[11]. In this method, sample extraction was done by protein precipitation and deuterated abiraterone was used as an internal standard.

We developed a new, sensitive and relatively simple LC–MS/MS method for estimation of abiraterone in human plasma. In this method, the stability issue of abiraterone in human plasma has also been addressed. This method is validated as per FDA regulations[12] and can be used for pharmacokinetic study.

METHODOLOGY

Materials

Abiraterone (purity: 99.72%) was purchased from Vivan Life Sciences, India. Abiraterone D4 (purity: 99.83%) used as an internal standard was also from Vivan Life Sciences, India.

Methanol (HPLC-grade), acetonitrile, ammonium formate, potassium fluoride, oxalic acid dihydrate and formic acid of highest purity grade were purchased locally. In this study Milli Q purified water (Millipore, Milford, MA) was used.

Plasma lots collected in house were used for the experiments.

Preparation of Analyte and Internal Standard Solutions

Stock solution of abiraterone (200 µg/ml) was prepared in methanol. This concentration was then corrected by taking into account its potency and actual amount weighed. The stock solution of abiraterone was then diluted together with 50% methanol in water to concentration ranges of 9.98 ng/ml to 4000 ng/ml.

Similarly, stock solution of abiraterone D4 (1000 µg/ml) prepared in methanol was diluted to 100 ng/ml using 50% methanol in water. The concentration of the stock solution was corrected as mentioned in case of analyte before using for dilution.

Preparation of Stability Reagent

10g of potassium fluoride was dissolved in 100 mL 5% (w/v) oxalic acid dihydrate. 100 µl of 10% (w/v) potassium fluoride in 5% w/v oxalic acid dihydrate was added to 900 µl of pooled sodium fluoride + potassium oxalate plasma and stored at -70°C.

Preparation of Calibration Standards

To prepare calibration curve standards, 20 µl of the diluted samples of abiraterone was added to 980 µl of potassium fluoride stabilized sodium fluoride + potassium oxalate plasma to obtain a concentration range about 0.20 ng/ml to 80 ng/ml. All these bulk spiked samples were stored at about -70°C in aliquot of 300 µl.

Preparation of Quality Control Samples

Stock solution of abiraterone were diluted with 50% methanol in water to obtain the concentration ranges of 10.12 ng/ml to 3000 ng/ml. 20 µl of each diluted solution was added into 980 µl of potassium fluoride stabilized sodium fluoride + potassium oxalate plasma to obtain final concentration range about 0.20 ng/ml to 60 ng/ml for abiraterone.

Yellow monochromatic light was used throughout the study because of photosensitivity of abiraterone[13].

Sample Preparation

50 µl of internal standard mixture (abiraterone D4) was added to all RIA vials except blank. 200 µl of sample was then added to each labeled RIA vials. 100 µl of 0.1M Sodium dihydrogen orthophosphate dihydrate was added to respective RIA vials and mixed by vortex. 2 ml of TBME was then added to all vials, capped them and then placed on Vibramax at 2500 RPM for 10 mins. They were centrifuged at 4000 RPM for 5 mins at about 4°C. 1.6 ml of supernatant from each vial was transferred into fresh RIA vial and dried at 40°C in nitrogen evaporator. 0.3 ml of mobile phase was added and vortexed. The samples from each vial was transferred into a labeled HPLC vial and placed in the autosampler.

Chromatography

20 µL of sample was injected on a reversed phase column (BDS Hypersil C18, 100 × 4.6mm, 5µm). 2 mM Ammonium formate, pH 3.5 ± 0.2: Acetonitrile: 30:70, v/v was used as a mobile phase at a flow rate of 1.2 mL/min with splitter in Waters UPLC attached to API 4000 Mass spectrometer (Applied Biosystems, USA). The column was maintained at 40°C in the column oven. The run time was 4.0 minutes.

Mass Spectrometry

Electrospray ionization (ESI) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM). The operational conditions were optimized by infusing diluted stock solution of analyte and internal standard (Table 1).

Table 1. MS parameters optimized for analytes and internal standards

Analyte/IS	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)	Collision activated dissociation (CAD) (psi)	Dwell Time (ms)	Ion source voltage (V)	Curtain gas flow (CUR) (psi)
Abiraterone	90	10	65	15	8	400	5500	30
Abiraterone D4	90	10	65	15	8	400	5500	30

Source temperature was set at 500°C. Nebulizer gas (GS1) and auxiliary gas (GS2) flows were 45 and 55 psi, respectively. Quadrupoles Q1 and Q3 were set on unit resolution.

MRM transitions were monitored as m/z 350.3 → 156.1 (ABR) and m/z 354.3 → 160.1 (ABR D4).

Sample concentrations were calculated by linear regression analysis using the analyst software 1.5.1. Data was processed by peak area ratio. The concentration of unknown was calculated from the equation (Y= mX+ c) using regression analysis of spiked plasma calibration standards with reciprocal of the square of the drug concentration ($1/X^2$).

RESULTS AND DISCUSSION

It was reported earlier that abiraterone is unstable in blood/plasma in the absence of fluoride [14, 15]. During the study we also noticed a gradual decrease in abiraterone concentration in plasma with time during storage (data not shown). Hence to stabilize abiraterone in plasma we added a fluoride containing stability agent as mentioned under 'Materials and Methods'.

Method Development

Specific and effective sample clean-up procedures are required for sensitive and selective LC–MS/MS assays for determination of very low concentration levels of pharmaceutical targets present in biological samples. Three methods e.g. protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are generally used for preparing biological specimen. Protein precipitation method using organic solvent is the simplest one but the chances of matrix effect prevail. SPE technique for sample extraction is good but with an added cost. LLE was our method of choice for abiraterone since the extraction efficiency for highly non-polar analytes is more. This technique was shown to be robust, provided clean samples and gave good and reproducible recoveries of both analyte and IS. The extraction recovery of analyte was determined by comparing peak areas from plasma samples (n = 6) spiked before extraction with those from aqueous samples. The mean recoveries across QC levels (with precision) were 60.2% (2.84%) for ARB and 67.3% for ABR D4 (IS).

To make the method simpler we used isocratic mobile phase for eluting the analyte and IS. The total run time was only 4 minutes. A short run time is ideally required for being considered in high throughput analysis. The retention times for abiraterone and IS were 2.87 min and 2.83 min, respectively.

Method Validation

FDA Guidelines for specificity, linearity, intra- and inter-day precision & accuracy, and stability were followed to validate this method[13].

Selectivity

Selectivity of the method was evaluated in eight individual human plasma lots along with one lipemic and one hemolytic lot. No interference were observed at the retention times of analyte and internal standard when peak responses in blank lots were compared against the response of spiked LLOQ containing IS mixtures. Representative chromatograms in Figures 1 (blank plasma) and 2 (blank plasma spiked with analytes/IS) demonstrate the selectivity of the method. The minimum signal to noise ratio was 140.65 (more than 5 is acceptable).

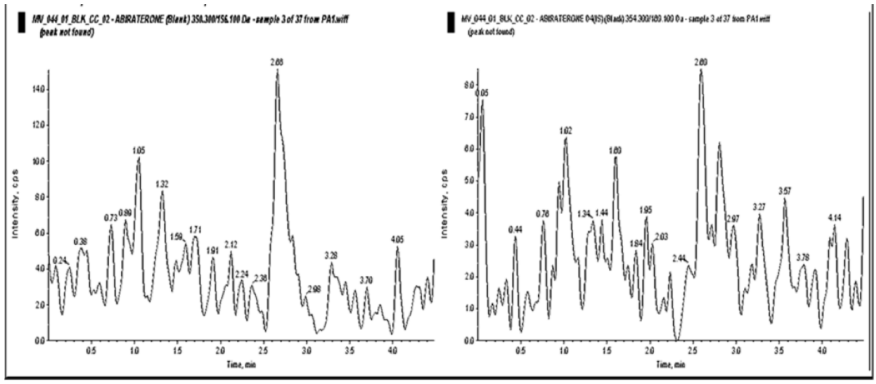

Figure 1. Chromatogram of extracted blank.

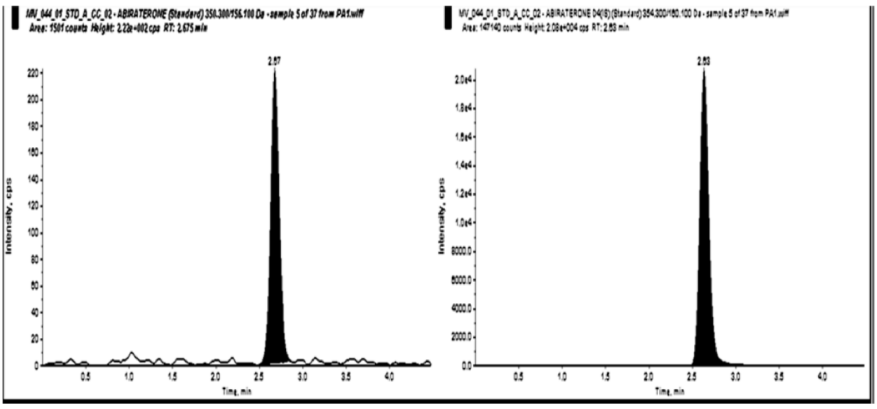

Figure 2. Chromatogram of an LLOQ calibration curve standard with IS.

Linearity and Sensitivity

Eight-point calibration curves were prepared with concentration ranging from 0.20 ng/mL to 79.5 ng/mL. The peak-area ratio (y) of analytes to internal standards was plotted against the nominal concentration ratio (x) of analyte to internal standard to determine the linearity of each calibration curve. Excellent linearity was achieved with correlation coefficients greater than 0.999 for all validation batches (Figure 3).

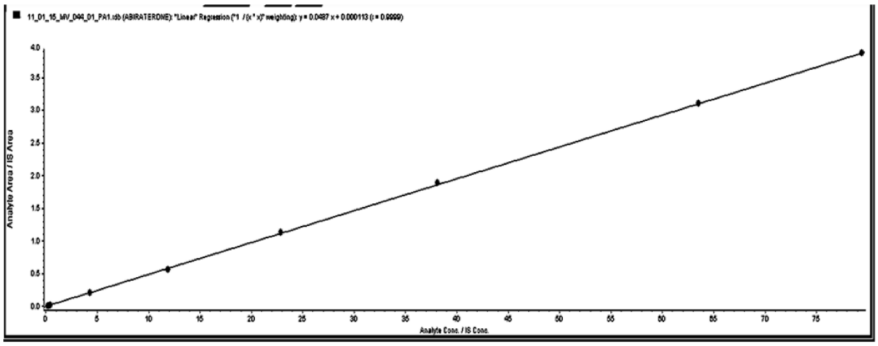

Figure 3. Calibration curve for Abiraterone.

The concentrations of calibration standards were back calculated to obtain the accuracy of each calibration point. The ranges of the calibration points' accuracy were 97.4–102%.

Six samples of LLOQ were processed and then injected along with a 'Precision and Accuracy' batch to assess the sensitivity of this method. Precision and accuracy for abiraterone at the LLOQs were 6.9% and 103.4% respectively. The LLOQ of the method is 0.20 ng/mL which is at par with the reported one [9]. Limit of detection was 0.05 ng/ml (signal to noise > 47.245). This indicates that this method is sensitive enough for a pharmacokinetic study. Moreover, a good signal-to-noise obtained at 0.05 ng/ml indicates that the LLOQ can be lowered further from 0.20 ng/mL or the volume of plasma can be decreased. This further widens the application of this method even to the pediatric patients where sample volume is always a challenge.

Precision and Accuracy

Precision and accuracy for intra- and inter-day batches for all analytes were determined by six replicate analyses of QC samples (n=6) at four different concentrations – Lower Limit Of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC). The respective concentrations for abiraterone were 0.20ng/mL, 0.55ng/mL, 25.09ng/mL and 62.74ng/mL for LLOQ, LQC, MQC and HQC. Results of precision and accuracy were presented in Table 2. The intra-day and inter-day precision were within 14.4% for all analytes. The assay accuracy was 91.35–105.05% of the nominal values. The accuracy of the assay was expressed by [(mean observed concentration) / (spiked concentration)] x 100% and precision was evaluated by relative standard deviation (RSD).

Table 2. Intra-day and inter-day accuracy and precision for the determination of abiraterone in human plasma

Sample ID	LOQQC (Nominal Conc. 0.208 ng/ml)			LQC (Nominal Conc 0.552 ng/ml)			MQC (Nominal Conc 25.096 ng/ml)			HQC (Nominal Conc 62.740 ng/ml)		
	Mean calculated Conc (ng/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (ng/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (ng/ml)	Mean accuracy (%)	% CV	Calculated Conc (ng/ml)	Mean accuracy (%)	% CV
PA - 1	0.20	98.5	5.3	0.52	94.4	3.8	24.14	96.2	1.1	60.57	96.5	1.1
PA - 2	0.19	92.4	5.9	0.50	91.5	2.6	24.72	98.5	2.0	60.79	96.9	1.6
PA - 3	0.19	91.3	6.9	0.51	92.1	6.2	25.12	100.1	0.9	61.49	98.0	1.8
PA - 4	0.21	100.9	5.3	0.53	95.7	2.3	24.79	98.8	0.4	59.80	95.3	0.7
PA - 5	0.22	105.0	14.4	0.53	95.4	1.7	24.92	99.3	1.4	61.16	97.5	1.0
Inter-day	0.20	97.6	9.5	0.52	93.8	3.8	24.74	98.6	1.8	60.76	96.8	1.5

Matrix Effect

Blank plasma from eight different sources was used for evaluation of matrix effect. One hemolytic and one lipemic plasma were included in these eight lots. 200 µL of blank plasma from each lot was processed as mentioned in sample preparation. Aqueous solution of analyte either at LQC or HQC level and known concentration of internal standard were added to each of the processed samples. These samples were considered as post extracted samples (presence of matrix).

Similarly, the aqueous solution of analyte either at LQC or HQC level containing same concentration of IS as above was prepared with the mobile phase solvent and was considered as aqueous samples (absence of matrix). Six replicates of each aqueous sample were injected along with post extracted samples of LQC or HQC.

Analyte and IS area responses of each post extracted sample were compared with the mean analyte area and mean IS area responses of the aqueous sample respectively. Calculation of the matrix effect was done using the formula: Matrix effect (%) = A_2/ A_1 x 100 (%), Where A_1= response of aqueous concentrations and A_2 is response of post-extracted concentrations.

Average (n=6) matrix factors were 105.19% with a CV of 5.49% at LQC level and 99.02% with a CV of 1.08% at HQC level which are within the accepted limit (% CV ≤15) (Table 3).

Table 3. Matrix effect of abiraterone in human plasma.

Matrix ID	LQC analyte area in absence of matrix	LQC analyte area in presence of matrix	LQC matrix factor for analyte	HQC analyte area in absence of matrix	HQC analyte area in presence of matrix	HQC matrix factor for analyte
PL-536	4993	5499	109.24	597558	577892	98.20
PL-537	5055	5368	106.63	586896	587961	99.91
PL-538	4925	5582	110.89	580961	586284	99.63
PL-539	5065	5116	101.63	580969	576825	98.02
PL-540	5070	4721	93.78	592048	576077	97.89
PL-541	5096	5162	102.54	592488	577367	98.11
LPL-499	-	5580	110.85	-	587693	99.87
HPL-504	-	5336	106.00	-	591694	100.55
Average	5034.00	5295.50	105.19	588486.66	582724.12	99.02
SD	63.41	290.67	5.77	6732.96	6282.38	1.07
%CV	1.26	5.49	5.49	1.14	1.08	1.08

Dilution Integrity

Dilution integrity was evaluated after spiking interference free human plasma with 2 times of HQC concentration of abiraterone (i.e. 2 x 80 = 160 ng/mL). These spiked plasmas was diluted either 2 fold (2T) or 4 fold (4T) with interference free human plasma. These samples (Six replicates of each dilution) were processed and then analyzed against a set of freshly spiked calibration standards. The mean accuracy and precision were 100.78% and 1.96% for 2T and 98.08% and 3.90% for 4T.

Carry – over Effect

To avoid any carry – over of injected sample in subsequent runs the cleaning ability of mobile phase used for rinsing the injection needle and port was evaluated. The order of placing samples was: LLOQ of analyte, blank plasma, upper limit of quantitation (ULOQ) of analyte and blank plasma. No carry – over was observed during the experiment. Benoist et al.[11] encountered with the carry – over in their recently published method which had been eliminated by using one more solvent as the mobile phase in their gradient method. However, our method is relatively simple where only isocratic elution was done without any carry – over problem.

Stability

Stability evaluations were performed in both aqueous and matrix based samples. For aqueous solution, both short-term and long-term stabilities were determined as follows:

a) Stability in aqueous solution

i) Short – Term stock solution stability (STSS)

Stock solutions of both analyte and IS were prepared separately and kept at 25°C for 26 h and named as stability stock. MQC concentration of analyte was prepared from the stability stock solution and stored at 25°C for 24h and marked as stability working solution. Just before injection, stock analyte solution and stock IS were diluted to MQC concentration of analyte and intended concentration of IS. Six replicate injections were given for MQC sample (both stock and working solutions) and diluted IS solutions. No significant differences were noticed when these results were compared with those obtained from the freshly prepared MQC solution indicating that analyte were stable at 25°C (Table 4). For IS, stability was 96.6% after 26h (data not shown). Accepted criteria for the ratio of mean response for stability samples should be between 90-110%.

ii) Long term stock solution stability (LTSS)

Aqueous MQC sample of analyte and solution of internal standard with known concentration were prepared by dilution from respective stock solutions and stored at 2-8 °C for 36 days. Mean area response of MQC of stored stock solution was then compared against MQC from freshly prepared stock solution. Similarly, mean area response for internal standard was also compared. Mean percent stabilities for abiraterone was 102.39 and 96.38 for abiraterone D4 (data not shown). These were well within accepted limit (90 – 110%). This indicated the stability of analyte and internal standard solutions for 36 days at 2-8 °C (Table 4).

Table 4. Short and long –term stability of Abiraterone aqueous solution

Short-term stability of stock solution at 25°C for 26h			Short-term stability of working solution at 25°C for 24h			Long-term stability of stock solution at 2-8°C for 36 days		
Average area of stock solution	Average area of fresh stock solution	% Stability	Average area of working solution	Average area of fresh working solution	% Stability	Average area of stock solution	Average area of fresh stock solution	% Stability
275952.3	284118.8	94.30	283617.2	284118.8	96.92	286243.8	278410.0	102.39

b) Stability in human plasma

i) Bench-top stability

Six aliquots of each analyte in human plasma (at LQC and HQC concentrations) from the -70°C were allowed to thaw unassisted at room temperature (25°C) for 6 h and processed along with a set of freshly prepared calibration standards as well as LQC and HQC samples. The stability for LQC and HQC samples were 104.74% and 100.96% respectively.

ii) Freeze thaw stability

After 4 freeze thaw cycles, the stability of abiraterone were 100.6% for LQC and 99.4% for HQC.

iii) In-injector stability

The stability for LQC and HQC samples kept in auto-sampler at 10°C for 46 h were 96.08 % and 101.05% respectively.

iv) Wet extract stability

The stability of abiraterone after 3 h at 25°C was 101.68% for LQC and 100.86% for HQC. As per FDA, accepted range for all the stability studies mentioned above is that the mean concentration for stability samples should be 85-115% of the mean concentration of freshly prepared samples. Thus, all the analytes were stable during the analysis process. Results of stability studies were provided in Table 5.

Table 5. Stability studies of Abiraterone in plasma

Parameters	Bench-top stability for 6h		Freeze-thaw stability after 4 cycles		In-injector stability for 46h		Wet extract stability for 3h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Nominal concentration (ng/ml)	0.55	62.74	0.55	62.74	0.55	62.74	0.55	62.74
Mean Calculated (ng/ml) gg(ngconcentration (ng/ml)	0.53	58.49	0.55	60.14	0.49	58.55	0.51	58.44
SD	0.02	0.30	0.02	0.58	0.01	0.91	0.01	0.84
%CV	4.75	0.52	3.26	0.96	2.24	1.56	2.46	1.43
% Stability	104.74	100.96	100.60	99.40	96.08	101.05	101.68	100.86

Extended precision and accuracy run

One set of CC and 50 sets of LQC and HQC as a batch (total 110 samples) were processed and then analyzed. Results of precision and accuracy were presented in Table 6. The precisions were 2.82% for LQC and 1.27% for HQC. The accuracies were 96.68% for LQC and 94.62% for HQC.

Table 6. Extended precision and accuracy of abiraterone

LQC				HQC			
Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL)	Accuracy (%)	% CV	Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL)	Accuracy (%)	% CV
0.55	0.53	96.68	2.82	62.74	59.36	94.62	1.27

This LC–MS/MS method for determination of abiraterone in human plasma is relatively simple, fast, sensitive and specific. It utilizes liquid-liquid extraction technique for this relatively non-polar molecule which offers consistent and reproducible recoveries with insignificant interference and matrix effect. Moreover, this method does not have any carry – over problem as reported earlier[11]. Stability issue of abiraterone in plasma has been resolved by using potassium fluoride[14, 15]. FDA guideline[12] mentions that internal standard should preferably be identical to the analyte and hence this method was developed using deuterated abiraterone. This method is also validated as per this guideline[12]. By using 200 µL plasma samples, the lower limits of quantification were achieved. It demonstrates that the method is reproducible, sensitive and suitable for high-throughput sample analysis. Moreover, as the sensitivity of this method is quite high this can be used even for analysis of pediatric samples where sample volume is always a challenge. This method has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

COMPETING INTERESTS

All authors hereby declare that no competing of interests is associated with the publication of this manuscript.

AUTHORS' CONTRIBUTIONS

All authors have equal contribution in this work.

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Investigation of the Anti-inflammatory, Hypoglycemic Activity and Median Lethal Dose (LD50) Level of Limonene in Mice and Rats

Betül Sever Yılmaz[1], Hanefi Özbek[2*]

[1]Ankara University, School of Pharmacy, Department of Pharmacognosy, Ankara, Turkey
[2]Istanbul Medipol University, School of Medicine, Department of Medical Pharmacology, Istanbul, Turkey

ABSTRACT

The aim of this study is to investigate the anti-inflammatory, hypoglycemic activity and median lethal lose (LD_{50}) level of limonene mice and rats.

Lethal dose levels were investigated using the probit analysis method. For the measurement of anti-inflammatory activity, seven separate work groups were established and limonene was administered in three different doses 0.15, 0.30, 0.60 mL/kg. Indomethacin and etodolac were used as reference anti-inflammatory agents.

For the evaluation of hypoglycemic activity, 6 separate work groups, consisting of healthy and diabetic mice were established and glibenclamide was used as the reference agent.

The LD_{50} level of limonene was determined to be 2.77 mL/kg. It is determined that all administered dosages of limonene possess anti-inflammatory activity; among these, 0.30 mL/kg was equivalent to indomethacin, and the remaining dosages were equivalent to etodolac. No hypoglycemic activity of limonene was observed in healthy and diabetic mice.

As a consequence, it is concluded that limonene did not show hypoglycemic activity, but possessed a strong anti-inflammatory activity.

Keywords: Limonene, anti-inflammatory activity, hypoglycemic activity, median lethal dose, rats, mice.

INTRODUCTION

Foeniculum vulgare Miller, (fennel) (Umbelliferae) is an annual, biennial or perennial aromatic herb, depending on the variety, and has been known since antiquity in Europe and Asia Minor. The leaves, stalks and seeds (fruits) of the plant are edible[1]. Extracts of the *Foeniculum vulgare* Miller (fennel) seeds are used in traditional Turkish medicine as an anti-inflammatory agent [2]. The anti-inflammtory, hypoglycemic and hepatoprotective effects of fennel were

*Corresponding author: Hanefi Özbek, e-mail: hozbek@medipol.edu.tr
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demonstrated by our previous scientific studies[3-7]. The volatile components of subsequent fennel seed extracts determined by chromatographic analysis contain *trans*-anethole, fenchone, methylchavicol, limonene, α-pinene, camphene, β-pinene, β-myrcene, α-phellandrene, 3-carene, camphor, and cisanethole[8]. The major components of essential oil samples are α-pinene, limonene, fenchone, methychavicol and *trans*-anethole [6].

Several studies related to limonene exist. Del-Toro Arreola *et. al.* reported that D-limonene modulates the immune response in BALB/c mice with lymphoma[9]. This study reported that limonene attenuates the gastric carcinogenesis enhanced by sodium chloride via increased apoptosis and decreased ODC activity in gastric cancers[10].

In our previous research we demonstrated that the volatile oil of fennel extract produced anti-inflammatory activity (0.050 and 0.200 mL/kg doses); additionally, volatile fennel oil showed hypoglycemic activity 4 hours after its application[3.] However, fixed fennel oil did not produce hypoglycemic activity[4-7]. In order to determine the compound responsible for these activities, we initiated a series of studies. In the present study we aimed to investigate the anti-inflammatory and hypoglycemic activities of limonene, one of the major compounds of volatile fennel oil extract and determined its lethal dose levels.

METHODOLOGY

Animals

Sprague-Dawley rats and Mus musculus Swiss albino mice were maintained in the animal laboratory. The animals were housed in standard cages with food and water *ad libitum*, at room temperature (22±2 ºC) with artificial light from 7.00 a.m. to 7.00 p.m., and provided with pelleted food. The ambient temperature was 22±2 ºC, ambient RH was % 55-60 and the rats were housed in groups. The approval of the Animal Ethics Committee was obtained.

Chemicals

(R)-(+)-Limonene was obtained from Aldrich (Steinheim-Germany), Lambda-carrageenan Type IV, indomethacin and alloxan were obtained from Sigma (Steinheim, Germany), etodolac was obtained from FAKO (İstanbul, Turkey) and Glibenclamide was obtained from Nobel, İstanbul, Turkey. Indomethacin and Etodolac were solved with ethyl alcohol.

Acute Toxicity

Swiss albino mice were randomly assigned to nine groups, with six animals in each group. The first group was treated with isotonic saline solution (ISS) (0.9%

NaCl) and employeded as a control group. The other eight groups were treated with limonene given intraperitoneally (ip) in increasing dosages of 0.20, 0.32, 0.40, 0.80, 1.60, 3.20, 4.80 and 6.40 mL/kg body weight. The mortality in each cage was assessed 72 h after administration of limonene. The percentage mortalities were converted to probits. Regression lines were fitted by the method of least squares and confidence limits for the LD_1, LD_{10}, LD_{50}, LD_{90} and LD_{99} values, and were calculated by the method of Litchfield & Wilcoxon and Kouadio et al. [11-12].

Anti-inflammatory Activity

The method of Winter *et. al.* with slight modification was employed[13]. Forty-two rats were divided into seven groups of six animals each. The rats were starved for 12 h and deprived of water only during the experiment. Deprivation of water was to ensure uniform hydration and to minimize variability in oedematous response. Inflammation of the right hind paw was induced by injecting 0.05 mL fresh lambda carrageenan (phlogistic agent) into the subplantar surface. Control Group I was given ISS and Control Group II was given ethyl alcohol. The third group (Reference Group I) received indomethacin (3 mg/kg, ip) and the fourth group (Reference Group II) received etodolac (50 mg/kg, ip) while the remaining three groups received the extract at doses of 0.15 mL/kg, 0.30 mL/kg and 0.60 mL/kg ip[14-15]. The doses utilized in the current study have been chosen according to the LD_1 value (LD_1 = 1.01147 mL/kg).

The measurement of foot volume was accomplished by a displacement technique using the plethysmometer (Ugo Basile 7140 plethysmometer, Italy), immediately before and three hours after the injection. The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated by the following formula[12]:

$$I\ \% = [(1-(dt/dc)] \times 100$$

where *dt* is the difference in paw volume in the drug-treated group and *dc* the difference in paw volume in the control group.

Preparation of Alloxan Diabetic Mice

Mice were starved for 18 h. and diabetes was induced by an ip injection of 150 mg/kg of alloxan monohydrate in ISS. This procedure were repeated three times[16]. 7 days after the last treatment, mice with blood glucose levels of 200 mL/dL and higher were taken into the study [17].

Hypoglycemic Activity in Diabetic Mice

Animals were randomly divided into three groups of six animals each. Group I

mice received 0.1 mL ISS ip. The animals of Group II were used as a standard, and treated orally with glibenclamide at a dose of 3.0 mg/kg. Group III received ip with 0.15 mL/kg body weight of limonene. Blood glucose levels were determined before treatment, 1, 2, 4 and 24 h after treatment by applying glucose oxidase peroxidase (Abbott, United Kingdom).

Hypoglycemic Activity in Normal (Healthy) Mice

The same protocol described above for normal mice was applied in mice made diabetic by administering 150 mg/kg i.p. of alloxan monohydrate. Also in this case, three groups of six animals each were used.

Statistical Analysis

Results were reported as mean ± standard error of mean (SEM). The total variation was analyzed by performing a one-way analysis of variance (ANOVA). An LSD (least significant difference) test test) was used for determining significance. Probability levels of less than 0.05 were considered significant[18]. Lethal dose levels were investigated by the probit analysis method. The medium effective dose (ED_{50}) value was calculated by non-linear regression analysis (SigmaPlot 2004 for Windows Version 9.01).

RESULTS AND DISCUSSION

Acute Toxicity

The results of lethal doses are shown in Table 1. The intraperitoneal medium lethal dose (LD_{50}) value for the total number of animals was found to be 2.77796 mL/kg.

Table 1. Lethal doses of limonene.

Lethal doses	Dose (mL/kg)	95 % confidence limits	
		Lower (mL/kg)	Upper (mL/kg)
LD_1	1.01147	0.16705	1.67328
LD_{10}	1.59225	0.49302	2.29631
LD_{50}	2.77796	1.68481	3.73663
LD_{90}	4.84663	3.61616	9.68089
LD_{99}	6.77774	4.74994	20.72301

Anti-inflammatory Activity

Table 2 illustrates the antioedema effect of intraperitoneally administered limonene on carrageenan paw oedema in rats. Limonene showed significant anti-inflammatory effects in all doses studied, peaking at a dose of 0.30 mL/kg (76.29

% inhibition), and with a lesser degree of inhibition at a dose of 0.15 mL/kg (51.729 %) and a dose of 0.60 mL/kg (51.732%).

Table 2. Anti-inflammatory effect of limonene.

Groups	Dose	Paw edema (% mL)	Inhibition (%)
Control-I (ISS)	0.1 mL	1.043 ± 0.127	-
Control-II (ethyl alcohol)	0.1 mL	0.988 ± 0.112	-
Indomethacin	3 mg/kg	[ab] 0.024 ± 0.061	87.44
Etodolac	50 mg/kg	[abc] 0.559 ± 0.040	43.42
Limonene	0.15 mL/kg	[abc] 0.504 ± 0.075	51.72
Limonene	0.30 mL/kg	[abde] 0.247 ± 0.074	76.29
Limonene	0.60 mL/kg	[abcf] 0.503 ± 0.106	51.73
F value		18.196	
p value		0.000	

Data presented as mean ± standard error of mean (n=6).

Post-hoc LSD test:

a : $p<0.05$ compared to control-I (ISS) group,

b : $p<0.05$ compared to control-II (ethyl alchohol) group,

c : $p<0.05$ compared to indomethacin group,

d : $p<0.05$ compared to etodolac group,

e : $p<0.05$ compared to limonene 0.15 mL/kg group,

f : $p<0.05$ compared to limonene 0.30 mL/kg group.

Compared to the controls, the greatest anti-inflammatory activity was observed in the first reference group receiving indomethacin with a 87.44% regression of inflammation. Etodolac, the second reference agent, showed significant but weaker anti-inflammatory activity with a 43.42% regression of oedema.

Limonene has a significantly lower anti-inflammatory effect compared to indomethacin at 0.15 mL/kg and 0.60 mL/kg doses, and a comparable effect at a 0.30 mL/kg dose. When compared to etodolac, the limonene had a statistically similar effect at 0.15 mL/kg and 0.60 mL/kg, and displayed higher activity at 0.30 mL/kg.

Limonene had significantly lower anti-inflammatory activity at 0.15 mL/kg and 0.60 mL/kg compared to 0.30 mL/kg, whereas the dose of 0.15 mL/kg showed no statistically meaningful difference with a 0.60 mL/kg dose.

The medium effective dose (ED_{50}) value of limonene was found to be 0.142 mL/kg.

Hypoglycemic Activity

The blood glucose levels of the alloxan diabetic mice are given in Table 3 and Figure 1. Table 4 and Figure 2 demonstrate theblood glucose levels of normal mice. Limonene did not have any hypoglycemic effect in alloxane-induced diabetic mice.

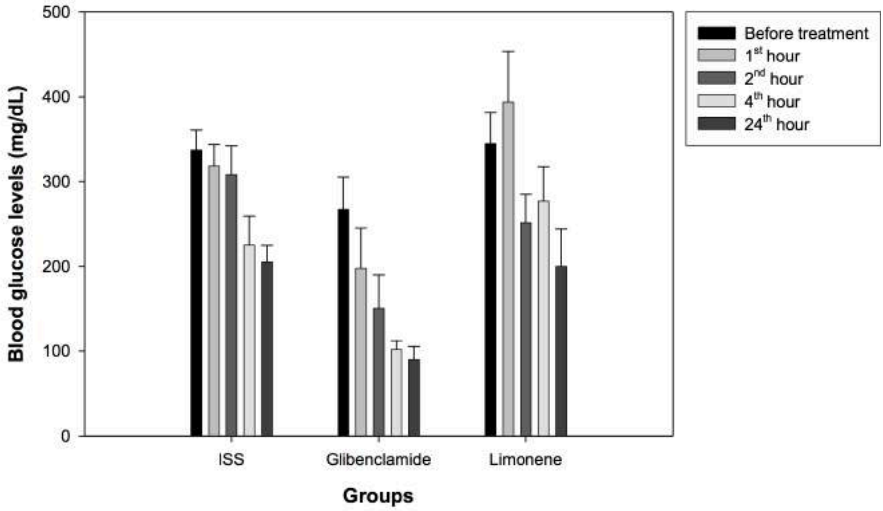

Figure 1. Blood glucose levels in glibenclamide, limonene and control groups of mice with alloxane-induced diabetes.

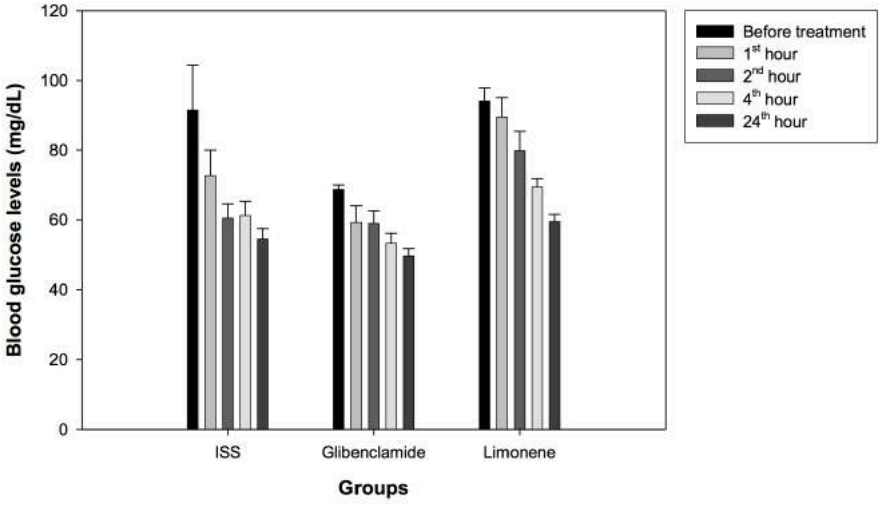

Figure 2. Blood glucose levels in glibenclamide, limonene and control groups of healthy mice.

Table 3. Blood glucose levels in glibenclamide, limonene and control groups of mice with alloxane-induced diabetes.

Groups	Fasting blood glucose (mg/dL)				
	Before treatment	1st hour	2nd hour	4th hour	24th hour
Control (ISS)	337.2±23.4	318.4±25.3	308.0±34.2	225.0±34.4	205.4±19.3
Glibenclamide	267.3±37.7	197.8±47.3	[a]150.5±39.7	[a]101.8±10.6	[a]90.1±15.4
Limonene	344.8±36.8	[b]393.8±59.9	251.7±33.3	[b]277.0±40.7	[b]200.0±43.8
F values	*1.509*	*4.053*	*4.559*	*7.901*	*4.753*
P values	*0.253*	*0.039*	*0.028*	*0.005*	*0.027*

Data were represented as mean ± standart error of mean.

Post-hoc LSD test:

a: $p<0.05$ compared to ISS group.

b: $p<0.05$ compared to glibenclamide group.

Table 4. Blood glucose levels in glibenclamide, limonene and control groups of healthy mice.

Groups	Fasting blood glucose (mg/dL)				
	Before treatment	1st hour	2nd hour	4th hour	24th hour
Control (ISS)	91.50±12.8	72.75±7.2	60.50±4.1	61.25±4.1	54.50±3.0
Glibenclamide	68.75±01.3	59.25±4.8	59.00±3.6	53.25±2.9	49.75±2.0
Limonene	94.1±03.8	[b]89.5±5.6	[ab]79.8±5.6	[b]69.5±2.4	[b]59.5±2.1
F values	*3.840*	*6.709*	*5.729*	*7.316*	*4.257*
P values	*0.054*	*0.012*	*0.020*	*0.010*	*0.043*

Data were represented as mean ± standart error of mean.

Post-hoc LSD test:

a: $p<0.05$ compared to ISS group.

b: $p<0.05$ compared to glibenclamide group.

It is observed that limonene group had significant levels of glucose in blood among healthy mice during the 1st, 2nd, 4th and 24th hours compared to glibenclamide group, but the ISS group had significant levels only during the 2nd hour.

In our previous research with volatile fennel oil, we demonstrated fennel's anti-inflammatory and hypoglycemic activities. In this part of the study - one of the studies of a series that we have initiated to determine the compound or compounds responsible - limonene, a major compound of the contents of volatile

fennel oil, anti-inflammatory and hypoglycemic activities have been examined. In addition, LD_{50} levels are also included.

In this work, an LD_{50} dose of limonene, a major component of the essential oil of *Foeniculum vulgare,* was determined to be 2.77796 mL/kg.

The current study clearly demonstrated the anti-inflammatory effect of limonene *in vivo*, equal to that of etodolac at 0.15 mL/kg and 0.60 mL/kg doses, and to that of indomethacin at a 0.30 mL/kg dose. The anti-inflammatory activity of an LD_{50} level of limonene is determined to be 0.142 mL/kg for rats.

Souza *et. al.* demonstrated the anti-inflammatory activity of limonene in the mouse model of pleurisy induced by zymosan (500 microg/cavity) and lipopolysaccharide (LPS) (250 ng/cavity), and this study supports our results[19]. In this study limonene was also effective in inhibiting the production of nitric oxide as well as a significant inhibition of gamma-interferon and IL-4 production by limonene. Yoon *et. al.* showed that limonene suppresses the lipopolysaccharide (LPS)-induced production of nitric oxide (NO), prostaglandin E2 (PGE_2), and pro-inflammatory cytokines in RAW 264.7 macrophages; detection of D-limonene reduced the expression of TNF-alpha, IL-1β and IL-6 in a dose-dependent manner[20]. Hirota *et. al.* showed that limonene may have a potential anti-inflammatory effect useful for the treatment of bronchial asthma by inhibiting cytokines, reactive oxygen species (ROS) production, and inactivating eosinophil migration [21]. Kummer *et. al.* showed that limonene isolated from *Citrus latifolia* Tanaka essential oil (CLEO) had potential anti-inflammatory effects, likely by inhibiting proinflammatory mediators present in inflammatory exudate and leukocyte chemotaxis[22]. In Rehman *et. al.* D-limonene also effectively decreased the doxorubicin induced overexpression of NF-κB, COX-2, iNOS and nitric oxide [23].

Determination of the activity of limonene to prevent the inflammation induced by carrageenan, may result from the aforementioned mechanisms. Further research is needed to reveal the mechanism of the anti-inflammatory activity.

Limonene did not show hypoglycemic activity in diabetic mice. It is found that the limonene group had significant levels of glucose in the blood of healthy mice during the 1^{st}, 2^{nd}, 4^{th} and 24^{th} hours compared to the Glibenclamide group, but the ISS group had significant levels during the 2^{nd} hour only. However, since these values were within normal limits related to the starved blood glucose level, these observed values are considered to have no clinical significance.

CONCLUSION

As a result, we can conclude that limonene can be totally or partially responsible

for the anti-inflammatory activity that volatile fennel oil extract produces, but is not responsible for the hypoglycemic activity. We think that by working on the limonene molecule, we can obtain a new drug that is as potent as indomethacin that has fewer and weaker side effects.

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Potential Mechanistic Profiling of an OTC Analgesic as a Cytotoxic Agent in the Treatment of Hepatocellular Carcinoma

Marwa E. Sayour[1*], Ibrahim Basheer[2], Rania Abd El Salam[3], Mohamed El Yamany[3], Amr Badr[4], Ahmed Farouk Al-Sadek[2], Raafat El-Awady[5]

[1] National Cancer Institute, Egypt, Central Pharmacy, Cairo, Egypt.

[2] Agricultural Research Center, Central Laboratory for Agricultural Expert Systems, Cairo, Egypt.

[3] Faculty of Pharmacy, Cairo University, Department of Pharmacology & Toxicology, Cairo, Egypt.

[4] Faculty of Computers and Information, Department of Computer Science, Cairo, Egypt.

[5] College of Pharmacy, University of Sharjah, Department of Pharmacy Practice and Pharmacotherapeutics, Sharjah, United Arab Emirates.

ABSTRACT

While being a safe over the counter drug, paracetamol has also proved to be a cytotoxic agent for cultured hepatocellular carcinoma cells (HepG2). In order to understand the biochemical mechanisms underlying its cytotoxic ability, molecular docking of paracetamol with cyclin dependent kinase 2 protein (CDK2) and breast cancer type 2 susceptibility protein (BRCA2) plus cyclooxygenase 1 (COX1) enzyme protein was undergone. Computational simulation was performed using Schrödinger software to describe the details of binding between atoms of the active sites and paracetamol. All COX1, CDK2 and BRCA2 proteins showed binding scores with paracetamol. Their G-scores were -5.32, -5.61 and -6.08 respectively leading to selective inhibition of these proteins and loss of their cell cycle related activity. The binding strength of COX1 and CDK2 with paracetamol was mainly dependent on the hydrophobic residues, while that of BRCA2 was contributed to charged residues. Binding is responsible for the subsequent loss of activity of these cell cycle related proteins and eventual cancer cell death via apoptosis.

Key words: Paracetamol; Cyclooxygenase 1; Cyclin Dependent kinase 2; Breast Cancer Type 2 Susceptibility Protein; Molecular Docking

INTRODUCTION

Paracetamol, chemically named as N-(4-hydroxyphenyl)acetamide, is a widely used safe non-opioid analgesic for pain management and antipyresis as an alternative to aspirin, NSAIDs and selective COX-2 inhibitors [1,2]. It is considered as one of the most famous over the counter drug used for pain relief in symptomatic treatment of slight and moderate pain. Moreover, it is the drug of choice for patients in whom application of non-steroidal anti-inflammatory drugs (NSAIDs) are contraindicated[3].

*Corresponding author: Marwa E. Sayour, e-mail: marwaezz@yahoo.com
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While being one of the oldest and most common OTC analgesics, the exact mechanism of action of paracetamol at the molecular level has not yet been elucidated [4]. It was found that the highly selective analgesic and antipyretic effects of paracetamol are due to its inhibitory effect on prostaglandin (PGs) synthesis via prostaglandin H synthase (COX) inhibition. PGs are lipid mediators derived from arachidonic acid that play central roles in the pathogenesis of inflammation, fever, pain and even cancer. PGs are generated by the oxygenation of arachidonic acid to the unstable intermediate prostaglandin H2 (PGH2) by prostaglandin H synthase (PGHS) of which there are two major isoforms; the constitutive PGSH-1 (COX 1) and the inducible PGSH-2 (COX 2).

In addition to its ability to treat pain and fever, paracetamol was found to induce apoptosis and necrosis in various types of cultured liver carcinoma cells specially HepG2 cell line [5]. Although considered safe at therapeutic doses, at higher doses, paracetamol produces hepatotoxic effect. The key event of paracetamol cytotoxicity is its biotransformation into the reactive metabolite N-acetyl-parabenzoquinonimine (NAPQI) by the P450 family [6-9] which can induce apoptosis or necrosis on different cell models [10,11]. *In vivo*, the balance among bioactivation, detoxification and defense/ repair mechanisms determines whether a compound will elicit a toxic effect or not. However, lack of correlation between *in vivo* hepatotoxicity and *in vitro* cytotoxicity may be a result of alteration in metabolic oxidation *in vitro*. Hepatoma cell lines constitute a simple, readily accessible and almost unlimited source of cells from a human liver. However, a major limitation is their reduced drug-metabolizing capability [12]. HepG2 cells, the most widely used for hepatotoxicity studies, showed substantially lower levels of most P450 enzymes than primary hepatocytes, which can contribute to the differences in cytotoxicity found in both cellular models [12,13]. Based on the two facts that paracetamol/NAPQI transfer ratio in HepG2 cells is still unveiled and the fact that drug-induced cytotoxicity can also be induced by parent drug molecule and/or its metabolite [14], two-part study will deal first with the interaction of paracetamol with some intracellular cell-cycle related proteins as part one (the current study). This in turn will elucidate the biochemical mechanisms underlying the established cytotoxic action of paracetamol on HepG2 cells.

Close interplay of the different disciplines is therefore of utmost importance to tackle the problem of metabolism prediction. Events that produce hepato-cellular death following the formation of paracetamol-protein adducts are poorly understood. Understanding metabolic processes at the molecular level is of fundamental importance for successful drug discovery and development. Knowing the metabolic properties of a molecule can help to optimize the stability and consequently the *in vivo* halflife and risk–benefit ratio of a drug [15].

Among such proteins which are strongly related to cell cycle, CDK2 and BRCA2 were selected due to their important role in cell cycle progression and DNA repair as attractive targets for the design of anti-proliferative drugs. The current study explored whether CDK2 and BRCA2 proteins have docking sites for paracetamol which can lead to inhibition of their functions and perturbation of cancer cell cycle. The loss of their activity or function may lead to cancer cell death via apoptosis.

Computational docking has become one of the most valuable tools to understand the behavior of the binding process between intracellular proteins and a certain ligand molecule. In laboratories, it's not possible to achieve that goal via microscopic examination which can only reach final results without revealing details. Computational simulation and its applications became indispensible as they are the only tool to describe the details of the binding affinity between atoms of the active sites of target proteins and their ligands. Free-energy simulation techniques have been developed for quantitative modeling of protein-ligand interactions and the prediction of binding affinity [16, 17].

Molecular docking could offer more insight into understanding the protein-inhibitor interactions and structural features of protein's active site. Glide [18-20] developed by Schrödinger, is one of the most popular docking methods. It includes preparation, docking and scoring the binding. Extra precision (XP) Glide was applied in docking to enhance sampling methods and scoring functions [2].

Depending on the results of previous studies which have already proved the cytotoxic ability of paracetamol on human liver carcinoma cells [22], the current study explores the possible molecular docking of paracetamol with some certain intracellular proteins. Computational docking software and algorithms were used as an *in-silico* experimental environment. Schrodinger's software suite [23, 24], including different utilities, was used to study and analyze the potential binding of paracetamol with COX1, CDK2 and BRCA2 proteins in order to recognize the mechanism underlying drug's cytotoxic effect. Deeper analysis of the resulting binding details and outcomes might be promising for future discovery of untraditional liver carcinoma treatment.

METHODOLOGY

Materials

In the current study, COX1, CDK2 and BRCA 2 were selected to undergo a dock trial with paracetamol to elucidate whether paracetamol has an inhibitory effect on them that stands for its cytotoxic action on HepG2 cells. Both CDK2 and BRCA2 were selected for this purpose due to their crucial role in cell cycle progression. In addition, COX1 was also selected for being the enzyme protein linked with paracetamol's mechanism of action.

Molecular Structures:

A. Structures of the ligand Paracetamol (PDB ID: TYL), (Figure 1A), in addition to the three studied cellular proteins, were retrieved from the Protein Data Bank (PDB)[25].

B. Cyclooxygenase enzyme 1 (COX-1), the structure of prostaglandin H2 synthase-1 was obtained complexed with P-(2'-IODO-5'-THENOYL) hydrotropic acid (IODOSUPROFEN), (PDB ID: 1PGE)[26]. The structure is shown in Figure (1B).

C. Cyclin-Dependant Kinase 2 (CDK2), the structure of CDK2 was obtained complexed with a disubstituted 4, 6-bis anilino pyrimidine CDK4 inhibitor, (PDB ID: 1H00)[27]. The structure is shown in Figure (1C).

D. Breast Cancer type 2 susceptibility protein (BRCA2), the structure of BRCA2-DSS1 complex was obtained (PDB ID: 1IYJ)[28]. The structure is shown in Figure (1D).

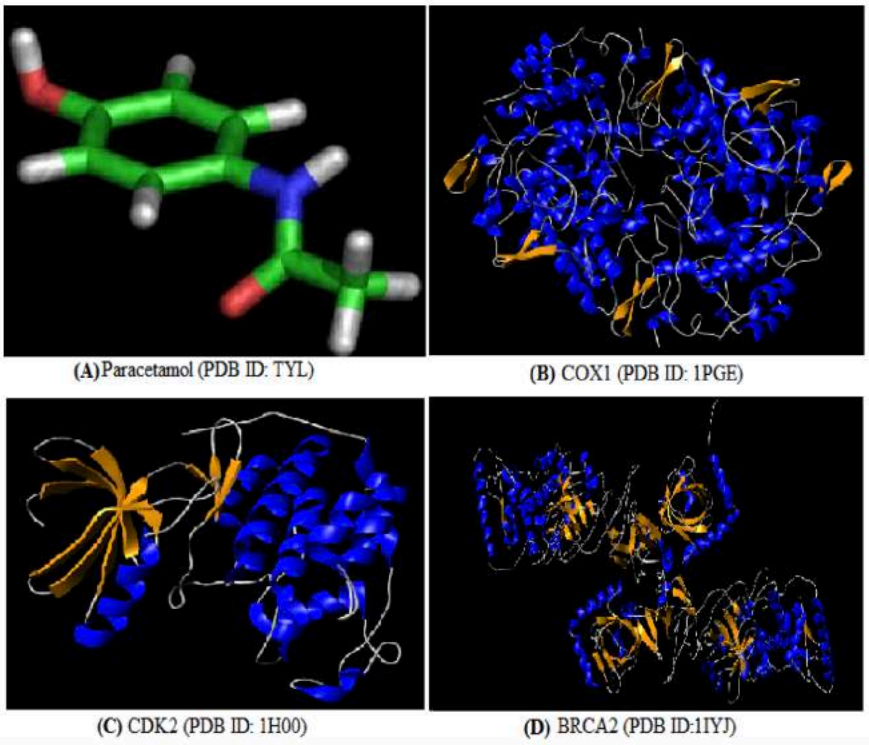


Figure 1. 3D Structures of the ligand paracetamol and the 3 tested proteins

Methodology

In the computational experiments, the methodology contains three main phases, which are preparation, binding site grid determination, and docking.

The overall flow of the docking process is shown in Figure 2. It describes the whole process and flow of steps to get the docking results as binding energy estimates.

Firstly, the preparation phase includes loading the file of the required molecule (either protein or ligand), then opening the corresponding preparation utility in Schrödinger Suite [23,24], setting the required parameters and running the preparation.

Secondly, the active site of each protein is determined by running the Grid Generation module in Schrödinger Suite. Different methods were followed for each protein according to the available structure which will be explained later.

Finally, after grid generation, docking was performed with Schrödinger Glide [18-20]. For each protein, the ligand was docked into the prepared structure active site determined by the generated receptor grid.

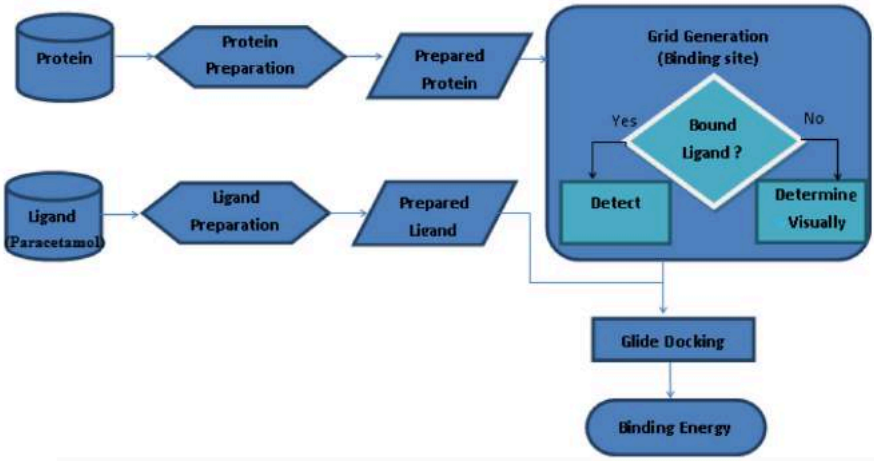
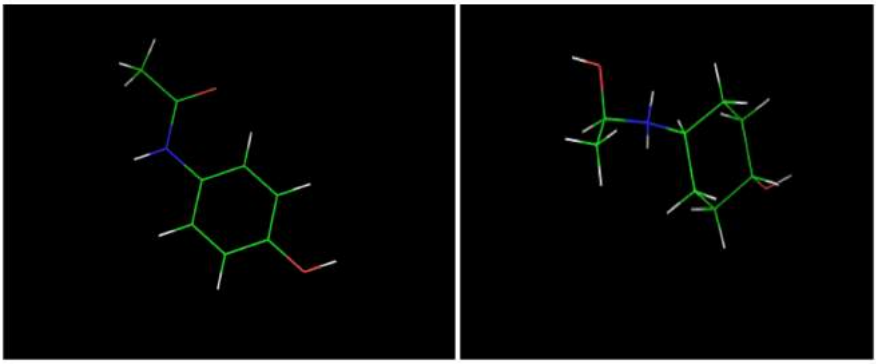


Figure 2. Docking process workflow

Preparing Molecules

The ligand, paracetamol, was loaded into Maestro 10.1 [23,24], Figure 3A, and prepared by the LigPrep utility[29]. Force field used was OPLS_2005, and states were generated at target pH 7.0. The prepared structure was saved to be used in docking experiments (Figure 3B).



carbon, oxygen, nitrogen and hydrogen are labeled green, red, blue and white respectively

Figure 3. (A) Paracetamol before preparation **(B)** Paracetamol after preparation

Protein structures were prepared using the Protein Preparation utility[30,31] in Schrödinger Suite. First, the structure of COX-1 protein complexed with hydrotropic acid (1PGE) was loaded into Maestro (Figure 4A). After preparation, the co-crystalized ligand [P-(2'-IODO-5'-THENOYL) hydrotropic acid] was exported as a single entry and saved in a separate file. This exported ligand was then docked back to the active site in the same way as it will be described later in the docking section. Glide uses full OPLS-AA force field at an intermediate docking stage and is claimed to be more sensitive to geometrical details compared to other docking algorithms. In the next step, water molecules were removed and H atoms were added to the structure, most likely positions of hydroxyl and thiol hydrogen atoms. Protonated states and tautomers of His residue and Chi 'flip' assignment for each Asn, Gln and His residue were selected by protein assignment script provided by Schrödinger. Minimization was performed until the average root mean square deviation of the nonhydrogen atoms reached 0.3 Å [18-21].

The 1PGE structure was noticed to have two chains, A and B. Chain B and the corresponding molecules were typical as chain A. It was also observed that the bound ligands resided away from the area between the two chains. This means that the binding site is away from this area, so, the decision was taken to remove chain B for simplification, and only chain A was considered for other preparation steps for docking (Figure 4B).

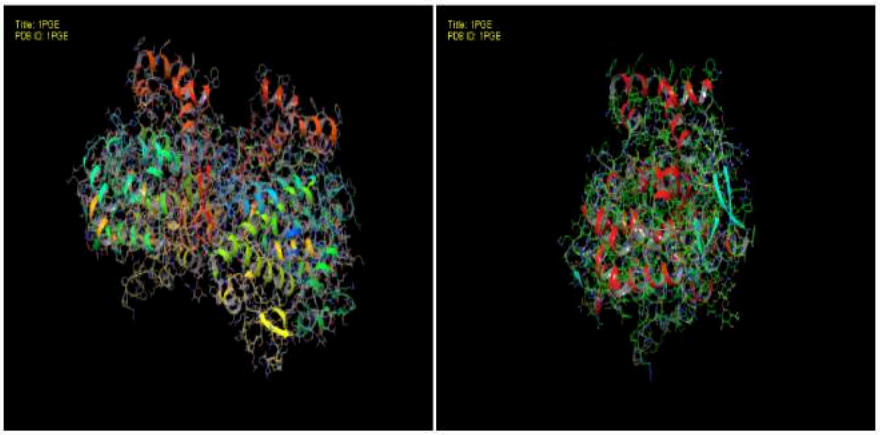


Figure 4. (A) COX1 (1PGE) before preparation **(B)** COX1 (1PGE) after preparation

In order to prepare the CDK2 protein, the structure of CDK2 protein complexed with a disubstituted 4, 6-bis anilino pyrimidine CDK4 inhibitor (1H00) was loaded. Since, this protein structure contained only one chain, no chain deletion was performed.

Regarding the BRCA2 protein, the structure of BRCA2-DSS1 protein complex (1IYJ) was loaded (Figure 5A). This protein structure contains four chains, A, B, C, and D. Chains A and B are typical as chains C and D. Thus, the decision was made to delete chains C and D (Figure 5B). Additionally, as no bound ligands exist, the protein was prepared to allow for the visual determination of the binding site later.

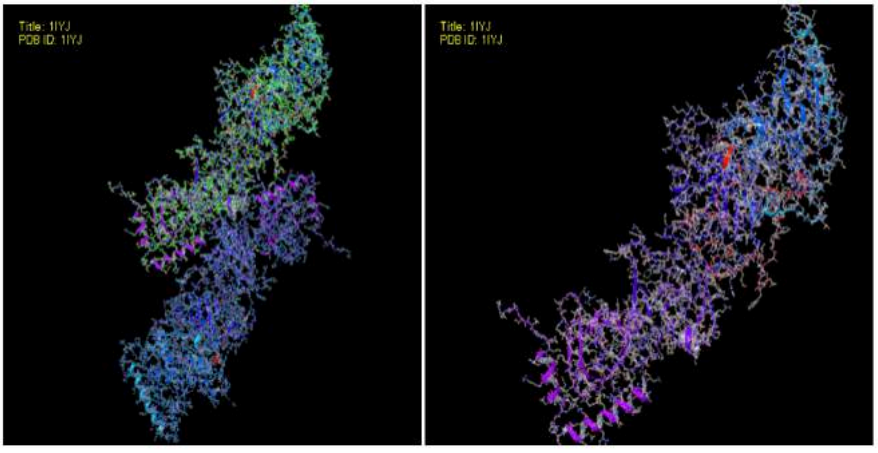


Figure 5. (A) BRCA2 (1IYJ) before preparation **(B)** BRCA2 (1IYJ) after preparation

After refining the structures and removing unnecessary chains or molecules, parameters were set and remaining steps were performed for each protein. pH was set to 7.0, protein structure was optimized, H-bond Assignment was applied, water removed, and Restrained Minimization was performed using OPLS_2005 force field.

Grid Determination

Receptor grid was generated for specifying the active site of the COX-1 protein. This was done using the prepared 1PGE complex structure which contains a ligand residing in the active site of the COX-1 protein. This complex structure was used to determine the active site of the protein to be used in the docking phase. The ligand was picked and the grid was determined around the picked ligand (Figure 6A).

For CDK2 protein, the receptor grid was generated for specifying the active site by using the prepared 1H00 complex structure, which contains a ligand residing in the active site of the CDK2 protein. The ligand was picked, and the grid was determined around the picked ligand, to be used in the docking phase (Figure 6B).

Finally, for the BRCA2 protein, the receptor grid for the active site was selected by visual inspection of the 1IYJ structure, as there was no bound ligand in the available structure. The potentially active areas of the protein were tested by setting the receptor grid manually for different times, then running the docking phase for each, and checking the results (selected grid in Figure 6C).

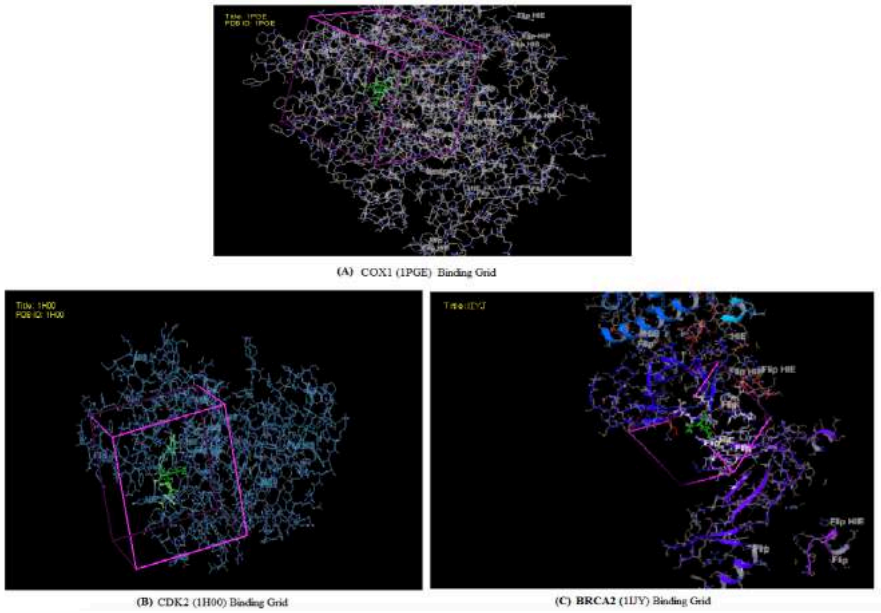


Figure 6. Binding grid for the 3 tested proteins

Docking

The prepared proteins and the ligand obtained from Maestro 10.1, Schrödinger software was used for docking studies. The active site of the selected protein was defined to include residues within 3.5 Å radius to the complexed ligand. For docking, the authors have used Maestro scoring function with grid resolution of 0.4 Å and a flexible mode of ligand docking. The docking score was calculated as the best ligand-pose energy (kcal/mole) and the score was assessed for correlation between paracetemol and COX1 CDK2 and BCRA1 binding affinity and selectivity. Docking was done using Glide by loading the prepared Paracetamol file and the receptor grid file for each protein separately. For COX-1, the generated grid was loaded into glide, with paracetamol ligand. Then, the glide docking was run in the XP mode, which represents extra precession docking and more output analysis options. The XP terms and the description of their visualizations is given in Table (1). In the visualizations, hydrogen atoms are not generally displayed, and the ligand carbon atoms are colored green.

The energy g score (17) is governed by the equations:

The XP Glide scoring function is presented in equation 1. The principal terms that favor binding are presented in equation 2, while those that hinder binding are presented in equation 3.

XP Glide Score= $E_{coul} + E_{vdW} + E_{bind} + E_{penalty}$-------Eq-1

$E_{bind} = E_{hyd\_enclosure} + E_{hb\_nn\_motif} + E_{hb\_cc\_motif} + E_{pt} + E_{hb\_pair} + E_{phobic\_pair}$—Eq-2

$E_{penalty} = E_{desolv} + E_{ligand\_strain}$------Eq-3

Where; G Score: Total Glide Score; sum of XP terms

Lipophilic EvdW: Lipophilic term derived from hydrophobic grid potential at the hydrophobic ligand atoms.

Phob En: Hydrophobic enclosure reward.

PhobEnHB: Reward for hydrophobically packed H-bond.

PhobEn Pair HB: Reward for hydrophobically packed correlated H-bonds.

H bond: Chem Score H-bond pair term.

Coul: Electrostatic rewards; includes Coulomb and metal terms.

Site Map: Site Map ligand-receptor non-H bonding polar-hydrophobic terms

Penaltiy: Polar atom burial and desolvation penalties, and penalty for intra-ligand contacts.

HB Penal: Penalty for ligands with large hydrophobic contacts and low H-bond scores.

RESULTS

Docking Analysis

The ligand paracetamol and the crystal structure of protein complex as a template were adopted to perform the validation steps. After following the steps in the methodology section, and getting the outputs of the docking phase of this experiment, the obtained results were analyzed visually and in terms of energy values. Visual analysis helps in understanding the number of bonds, bond types, and their position. Analysis of energy values describes the strength of different energies that bind the ligand to the target protein. For visual analysis, the total glide scores of COX-1, CDK2 and BCRA2 were -5.32, -5.61 and -6.08; respectively as shown in Table (1). The total glide scores are the sum of XP terms (G Score) due to the interactions between amino acid residues of proteins with paracetamol.

The resulting redocking pose and the bonds of the co-crystalized ligand (P-(2'-IODO-5'-THENOYL) hydrotropic acid) with COX-1 are the same as those used for docking paracetamol ligand as shown in (Figure 7 A, B). The glide score (G score) of the co-crystalized ligand with COX-1 was -12.644.

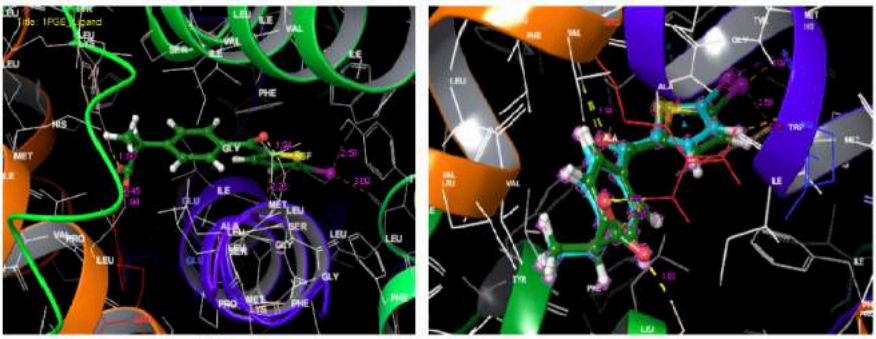


Figure 7. Confirmation of docking experiment

Table 1. Comparison of binding free energies due to interaction of paracetamol with COX1, CDK2 and BCRA2.

XP terms	COX1	CDK2	BCRA2
Total Glide Score= sum of XP terms (G Score)	-5.32	-5.61	-6.08
Dock Score	-2.78	-5.58	-6.05
Van der Waal lipophilic (EvdW)	-2.73	-2.72	-0.43
Hydrogen Bonding (HBond)	-1.79	-1.3	-2.94
Electrostatic reward (Electro)	-0.53	-0.36	-3.03
Polar interactions at the active site (Site map)	-0.29	-0.24	0
Expos penal	0	0	0.32

The acetyl oxygen (ketonic form) of paracetamol moiety showed H bond interaction (distance=2.03 Å) with the hydroxyl hydrogen of SER- COX1 protein amino residue. The phenolic oxygen of paracetomol moiety showed H-bond interaction (distance=2.03 Å) with the indolic hydrogen of TRP - COX1 protein amino residue. Moreover, the carboxylic oxygen (ketonic form) of LUE amino acid of the COX1 moiety showed H bond interaction (distance=2.35 Å) with the phenolic hydrogen of paracetamol moiety (Figure 8 A, B). The free energies of lipophilic term (LipophilicEvdW) score, ChemScore for H-bond pair term (HBond), electrostatic rewards and ligand/receptor non-Hydrogen bonding polar/hydrophobic and hydrophobic/hydrophilic complementarity terms (SiteMap) were found to be -2.73, -1.79, -0.53 and -0.29 respectively as seen in Table (1).

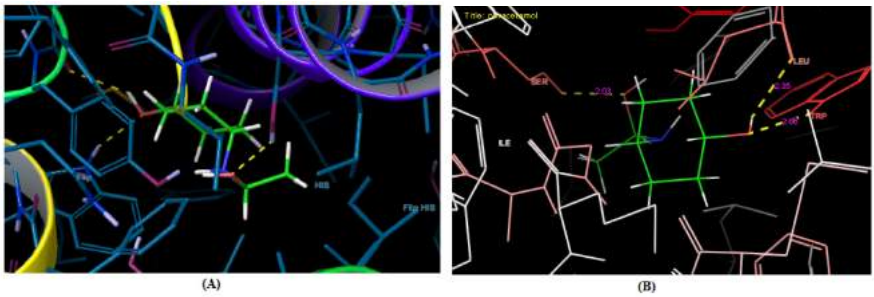


Figure 8. (A, B) Paracetamol Docked with COX1 (1PGE)

On the other hand, the detailed scores of the co-crystalized ligand [P-(2'-IODO-5'-THENOYL) hydrotropic acid] attached to COX-1 for the following bonds; lipophilic term (LipophilicEvdW) score, ChemScore for H-bond pair term (HBond), electrostatic rewards and ligand/receptor non-hydrogen bonding polar/hydrophobic and hydrophobic/hydrophilic complementarity terms (SiteMap) were

found to be -5.8, -1.96, -0.72 and -0.32 respectively. The resulting docking pose of [P-(2'-IODO-5'-THENOYL) hydrotropic acid] was compared with the original co-crystalized ligand (1PGE protein) using super position features in Maestro as seen in Figure 7 (B). The corresponding atoms in both poses of the ligand before co-crystalization and after redocking were selected in order and the calculated RMS for atom pairs was around 0.32. The objective for evaluating the redocking of the co-crystalised ligand was to indicate that the computational approach and the selected methodology proposed for docking was robust and could be used in the original ligand (paracetamol). The above result of RMS value (0.32) gives a level of confidence in the main docking experiment with paracetamol.

The acetyl hydrogen (enolic form) of paracetamol moiety showed H bond interaction (distance=1.91 Å) with the carboxylic oxygen (ketonic form) of ASP – CDK2 protein amino residue. The phenolic hydrogen of paracetomol moiety showed H-bond interaction (distance=2.16 Å) with the carboxylic oxygen (ketonic form) of Lue – CKD2 protein amino residue (Figure 8 A, B). The free energies of lipophilic term (LipophilicEvdW) score, ChemScore for H-bond pair term (HBond), electrostatic rewards and ligand/receptor non-Hydrogen bonding polar/hydrophobic and hydrophobic/hydrophilic complementarity terms (SiteMap) were found to be -2.72, -1.3, -0.36 and -0.24 respectively as seen in Table (1).

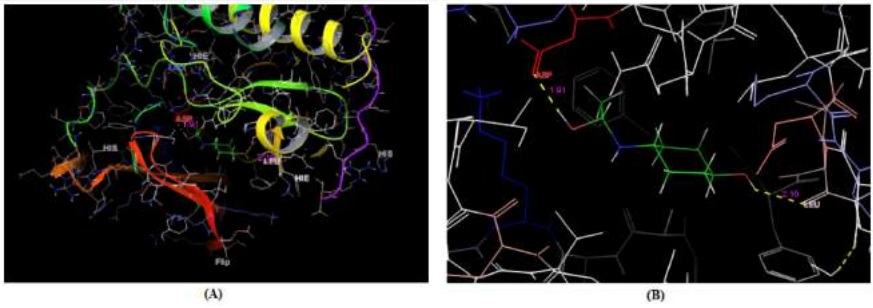


Figure 9. (A, B) Paracetamol Docked with CDK2 (1H00)

The amine hydrogens of paracetamol moiety showed H bond interaction (distance=1.61 and1.68 Å; respectively) with the carboxylic oxygen (enolic form) of GLU– BRCA2 protein amino residue. Another H-bond interaction with the same carboxylic oxygen (enolic form) of GLU– BRCA2 protein amino residue (distance=2.47 Å). The phenolic hydrogen of paracetomol moiety showed H-bond interaction (distance=1.87 Å) with the carboxylic oxygen (ketonic form) of LYS– BCRA2 protein amino residue. Finally, The phenolic oxygen of paracetamol moiety showed H bond interaction (distance=2.21 Å) with the guanidine hydrogen of ARG– BCRA2 protein amino residue (Figure 9 A, B).

The free energies showed different values in case of BCRA2 interactions where lipophilic term (LipophilicEvdW) score, ChemScore for H-bond pair term (HBond), electrostatic rewards and ligand/receptor non-hydrogen bonding polar/hydrophobic and penalty for solvent-exposed ligand groups that cancels vander Waals terms (Expos Penal) were found to be -0.43, -2.94, -3.03 and -0.24 respectively as seen in Table (1).

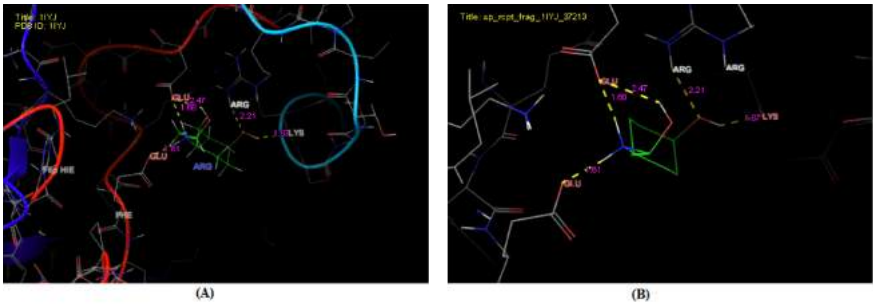


Figure 10. (A, B) Paracetamol Docked with BRCA2 (1IYJ)

The docking analysis for interactions of paracetamol and three studied proteins; COX1, CDK2 and BCRA2 are summarized in Table (2).

Table 2. Summary of overall interactions profile of paracetamol with COX1, CDK2 and BCRA2

	Paracetamol	Amino acid residue		Bond Length (Å)
COX1	Acetyl oxygen (ketonic form)	SER	Hydroxyl hydrogen	2.03
	Phenolic oxygen	TRP	Indole hydrogen	2.06
	Phenolic hydrogen	LUE	ketonic carboxylic oxygen	2.35
CDK2	Acetyl hydrogen (enolic form)	ASP	Carboxylic oxygen (ketonic form)	1.91
	Phenolic hydrogen	LUE	Carboxylic oxygen (ketonic form)	2.16
BCRA2	Amine hydrogen	GLU	Carboxylic oxygen (enolic form)	1.61
	Amine hydrogen			1.68
	Acetyl hydrogen (enol form)			2.47
	Phenolic hydrogen	LYS	Carboxylic oxygen (ketonic form)	1.87
	Phenolic oxygen	ARG	Guanidine hydrogen	2.21

DISCUSSION AND CONCLUSION

As one of the common OTC analgesics, paracetamol structurally constitute an acidic moiety (anilide moiety of carboxylic acid or enols) attached to a planar, aromatic group [32]. The acidic group in paracetamol serves a major binding group (ionic binding) with plasma proteins. Paracetamol conjugates with proteins through amino acid linkers, where the partial charge on the donor atom binds to the hydrogen. The structure of paracetamol is N-acetyl-para-aminophenol where both acetyl and phenolic moieties segregated into two subunits opposite ends clumped together by a phenyl ring molecule in the middle.

The binding pattern of paracetamol with COX-1, CDK2 and BCRA2 proteins with their best docking pose upon comparative assessment is being carried out between the established and the novel leads[33]. Schrödinger software suit counted the overall binding free energy and was more efficient for analyzing the binding of equal number of van der Waals interactions formed by both proteins with the ligand molecule. The non-bonded electrostatic energy swung towards the electrostatic contribution rather than the hydrophobic one to stabilize the complexes as seen with the binding of BRCA2 with paracetamol. In general, electrostatic interactions in proteins arise due to the presence of anionic amino acids, like aspartic acid, and cationic amino acids, like lysine, arginine and glutamine. When such residues are present in close vicinity of the ligand molecule, they exert a coulombic force at a shorter distance. On examination of the interactions made by paracetamol with COX-1, CDK-2 and BCRA2, it was noticed that while the latter formed multiple electrostatic interactions, the first and the second failed to contact any positive or negative residues[34,35].

Cyclin-dependent kinases (CDKs) play important roles in cell cycle progression and are attractive targets for the design of anti-proliferative drugs [36,37]. Many protein kinases have proved to harbor high affinity docking sites that may provide a potentially novel interface for the design of kinase inhibitors. Developing specific agents for CDK2 will assist in analyzing the role of CDK2 in cell cycle control. Moreover, BRCA2 protein is the protein product of BRCA2 gene which is responsible for DNA repairing. A Previous study reported that liver expresses BRCA2 gene which plays some role in malignancies other than breast cancer although the level of expression in the liver is much lower than in the mammary gland[38].

Depending on the results of previous studies[5,18] that proved the cytotoxic effect of paracetamol on HepG2 cells, the active sites of COX1, CDK2, and BRCA2 proteins were examined to figure out if there was any binding affinity between paracetamol and each of them. Results confirmed that both CDK2 and BRCA2 had

high affinity docking sites for paracetamol which presumed them as effective targets for inhibitor design.

The proposed mechanism of paracetamol cytotoxicity might be due to the binding of paracetamol with these cell cycle related proteins which result in subsequent loss of their activity or function and eventual cell death and lysis. This comes in line with the current study as paracetamol was the fair receptor for COX1, CDK2 and BRCA2. All of them were bound to paracetmol via hydrogen bonds recording negative glide score values confirming the stability of the formed adducts.

Conclusions

Computational docking showed that the binding strength of COX1 and CDK2 with paracetamol was mainly dependent on the hydrophobic residues while that of BRCA2 was contributed to charged residues. CDK2 and BRCA2 had high affinity docking sites for paracetamol which presumed them as effective targets for inhibitor design. The binding affinity between paracetamol and the studied cell cycle-related proteins was supposed to be responsible for the subsequent loss of their activity and eventual cancer cell cycle block and death via apoptosis. In addition to our findings, further investigations are recommended to prove that docking of paracetamol with cell cycle related proteins is a promising untraditional treatment strategy for liver carcinoma in the future. Docking of the metabolite NAPQI is also recommended as a complementary study has to be done since its formation is necessary for the incidence of cytotoxicity *in vivo* and to some extent *in vitro* due to the reduced levels of P450 enzymes in HepG2 cells.

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CONFLICT OF INTEREST

No conflict of interest is associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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